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Myc-Nick: A Cytoplasmic Cleavage Product of Myc that Promotes α-Tubulin Acetylation and Cell Differentiation

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SUMMARY

The Myc oncoprotein family comprises transcription factors that control multiple cellular functions and are widely involved in oncogenesis. Here we report the identification of Myc-nick, a cytoplasmic form of Myc generated by calpain-dependent proteolysis at lysine 298 of full-length Myc. Myc-nick retains conserved Myc box regions but lacks nuclear localization signals and the bHLHZ domain essential for heterodimerization with Max and DNA binding. Myc-nick induces α-tubulin acetylation and altered cell morphology by recruiting histone acetyltransferase GCN5 to microtubules. During muscle differentiation, while the levels of full-length Myc diminish, Myc-nick and acetylated α-tubulin levels are increased. Ectopic expression of Myc-nick accelerates myoblast fusion, triggers the expression of myogenic markers, and permits Myc-deficient fibroblasts to transdifferentiate in response to MyoD. We propose that the cleavage of Myc by calpain abrogates the transcriptional inhibition of differentiation by full-length Myc and generates Myc-nick, a driver of cytoplasmic reorganization and differentiation.

INTRODUCTION

The Myc family (c-Myc, N-Myc, and L-Myc) of basic-helix-loop-helix-zipper (bHLHZ) transcription factors controls the expression of a large number of target genes and noncoding RNA loci. These Myc targets mediate the physiological effects of Myc on cell proliferation, metabolism, apoptosis, growth, and differentiation (Eilers and Eisenman, 2008). To promote transcriptional activation at target genes, Myc forms heterodimers with its partner Max and recruits chromatin-modifying complexes to E-box-containing promoters. Myc is also involved in transcriptional repression through the inhibition of the transcriptional activator Miz1 (Kleine-Kohlbrecher et al., 2006). Aberrant elevation of Myc levels has been shown to contribute to the genesis of many types of human tumors (Hanahan and Weinberg, 2000).

Myc family proteins contain highly conserved regions termed Myc boxes (MB) that are essential for Myc's biological activities (see Figure 1E). A major determinant of Myc transcriptional function is MBII, which is the site of recruitment of coactivator complexes containing histone acetyltransferases (HATs) such as GCN5 (McMahon et al., 2000) and TIP60 (Frank et al., 2003). MBI functions as a phosphorylation-dependent binding site for the ubiquitin ligase Fbw7 (Welcker et al., 2004), whereas MBII is one of the binding sites for the ligase SKP2 (Kim et al., 2003; von der Lehr et al., 2003). Fbw7 and Skp2 both contribute to the rapid degradation of Myc protein ($t_{1/2}$ ~20 min). The C terminus of Myc harbors nuclear localization signals and the bHLHZ motif that mediates dimerization with Max and DNA binding.

Several variant forms of Myc protein have been previously identified. All of them are nuclear-localized, low-abundance proteins generated by alternative translation initiation. A weak CUG translational initiation site, upstream and in-frame of the predominant AUG codon, produces an N-terminally extended form of c-Myc called c-Myc1 (Hann et al., 1988). Another Myc protein variant is MycS, generated by internal translational initiations at two AUG codons located ~100 amino acids from the normal N terminus (Spotts et al., 1997). MycS lacks MBI but contains MBII and retains much of full-length Myc's biological activity (Xiao et al., 1998).

As expected, given their broad role as transcriptional regulators, Myc family proteins are predominantly localized to the cell nucleus during proliferation. Surprisingly, however, there have been multiple reports of cytoplasmically localized Myc, mostly in differentiated cells. For example, N-Myc localization was shown to change from nuclear to cytoplasmic in differentiating neurons of the neural crest, retinal ganglion cells, neurons of spinal ganglia (Wakamatsu et al., 1993, 1997), and Purkinje cells (Okano et al., 1999; Wakamatsu et al., 1993). Cytoplasmic Myc was also reported in tumors with diverse origins (Bai et al., 1994; Calcagno et al., 2009; Pietilainen et al., 1995). These studies relied on immunostaining protocols and the form of the Myc protein involved was not characterized.

Interestingly, association of Myc with several cytoplasmic proteins has been reported. The best characterized is the interaction of c-Myc with tubulins (Alexandrova et al., 1995) (Koch et al., 2007; Niklinski et al., 2000). Myc has also been reported to interact with other proteins that are predominantly

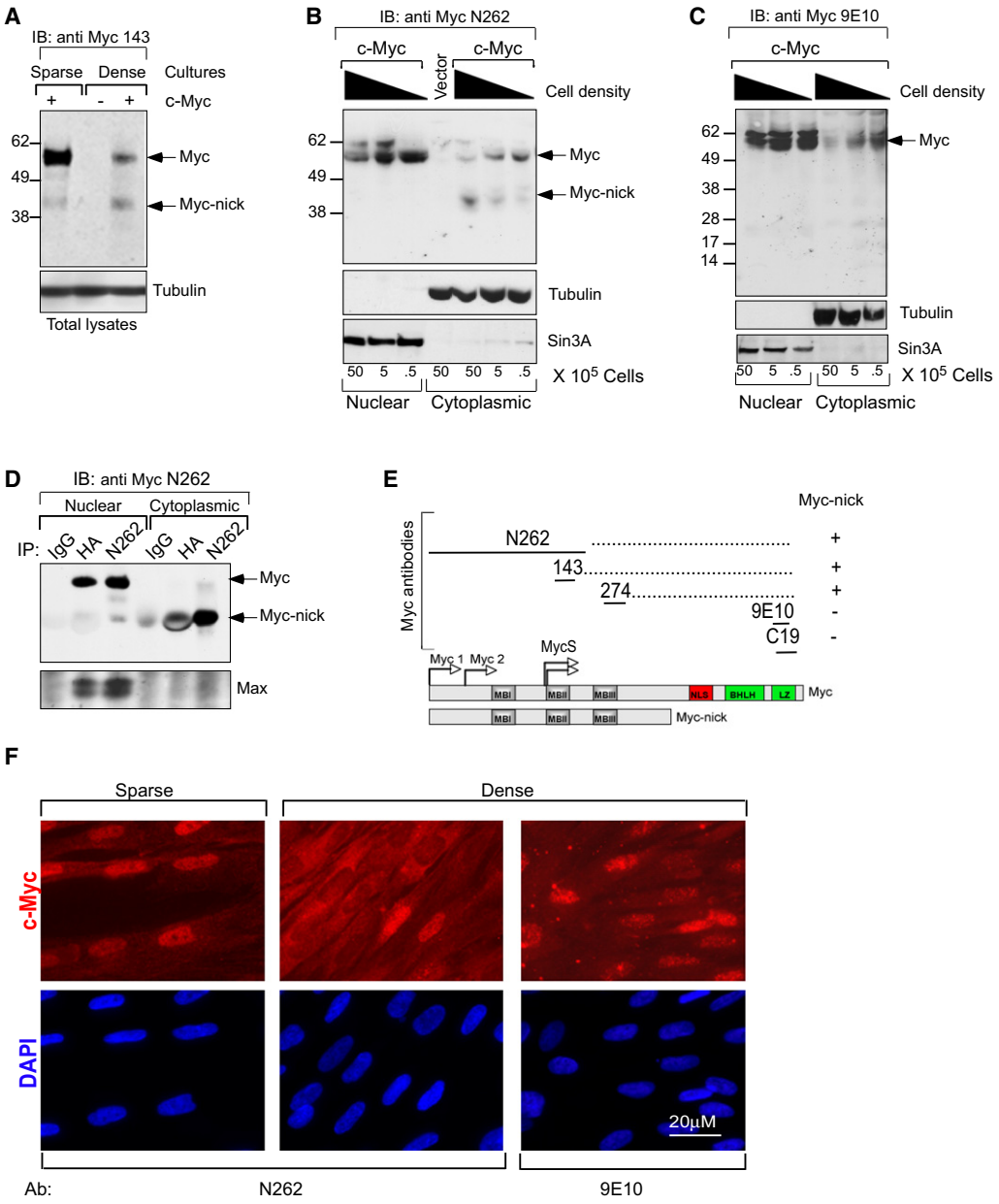

Figure 1. Identification of Myc-Nick in the Cytoplasm of Cells Grown at High Density

(A) Total cell lysates of Rat1 *myc* null fibroblasts infected with c-Myc or empty retroviral vectors were prepared for western blot by adding boiling sample buffer.

(B and C) Nuclear and cytoplasmic fractions of HFF cells expressing c-Myc were prepared 48 hr after plating at the indicated increasing densities.

(D) Immunoprecipitation of HA-c-Myc (N-terminal tag) with anti-N262, anti-HA, and normal IgG from nuclear and cytoplasmic fractions of HFFs. Note that Max is only coimmunoprecipitated along with nuclear c-Myc.

(E) Schematic representation of antibody mapping.

(F) HFF cells infected with c-*myc*-expressing retrovirus were cultured for 4 days after reaching confluency (middle and right panels) and compared with a subconfluent culture (left panel) by immunofluorescence using N262 and 9E10 antibodies.

See also Figure S1.

cytoplasmic, such as cdr2 (Okano et al., 1999) and AMY-1 (Taira et al., 1998). However the nature of the cytoplasmic Myc protein and its potential function remains an enigma. Here we report the identification of Myc-nick, a cytoplasmically localized cleavage product of Myc, and provide evidence for its role in cytoskeletal organization and cell differentiation.

RESULTS

Myc-Nick Is a Truncated Form of Myc Localized Predominantly in the Cytoplasm

While studying regulation of c-Myc degradation, we noticed an inverse correlation between the levels of full-length c-Myc and

a cytoplasmic 42 kDa protein in anti-Myc immunoblots derived from confluent fibroblast cultures (Figures 1A and 1B). As described below, this protein, which we have named Myc-nick, is a cytoplasmic cleavage product of full-length c-Myc generated at high cell density (Figure 1B). Myc-nick is recognized by three antibodies against the N-terminal two-thirds of c-Myc (anti-Myc N262, 274, 143; Figures 1A and 1B and Figure S1A available online) but not by anti- C-terminal antibodies (anti-Myc 9E10, C19; Figure 1C). Furthermore, an anti-HA antibody immunoprecipitates Myc-nick from cytoplasmic extracts of cells expressing N-terminally HA-tagged c-Myc (Figure 1D). In addition, cytoplasmic Myc bearing N-terminal but not C-terminal epitopes is detected by imunofluorescence in confluent cultures (see below, Figure 1F). Together, these results indicate that Myc-nick is a truncated protein lacking the C-terminal portion of c-Myc while preserving an intact N terminus comprising Myc boxes I–III (Figure 1E). This makes Myc-nick distinct from any other previously identified form of Myc (see Introduction).

We have detected endogenously expressed Myc-nick in the cytoplasm of a large number of cell lines including human foreskin fibroblasts (HFFs), Wi38, L cells, HCT116, SW480, HeLa (Figure S1B), C2C12 (Figure 7D), 293T, A431, Rat1, U2OS, ES cells, and mouse neurospheres (not shown), in addition to mouse tissues such as muscle, brain, and cerebellum (Figure 7A and Figure S7A). We also observed Myc cytoplasmic localization by immunofluorescence of confluent cultures of HFFs (Figure 1F and Figure S5A) and Rat1 *myc* null cells (not shown) expressing c-*myc*. Therefore a protein with the size and properties of Myc-nick is very widely expressed. In some settings we observe relatively low and variable amounts of Myc-nick in the nucleus (e.g., Figure 2A).

Myc-Nick Is Generated by Proteolytic Cleavage of c-Myc in the Cytoplasm

We considered the possibility that Myc-nick is generated in the cytoplasm because the nuclear export inhibitor Leptomycin B had no effect on the production or cytoplasmic localization of Myc-nick (Figure 2A). To determine whether a cytoplasmic activity could convert full-length c-Myc into Myc-nick, we incubated in vitro-translated [^{35}S]-methionine-labeled c-Myc or purified full-length recombinant c-Myc with nuclear or cytoplasmic extracts from Rat1 *myc* null cells. Only cytoplasmic extracts (CE) were capable of producing a protein (Figures 2B–2D) having the same apparent molecular weight as Myc-nick that was recognized by antibodies against the N terminus but not C terminus of c-Myc (Figure S2A). Increasing the incubation time with CE augmented Myc-nick production and decreased the amounts of full-length c-Myc input (Figure 2C). In agreement with our observations made in vivo, CE of dense cultures were more efficient in cleaving c-Myc than CE of sparse cultures (Figure S2B). In experiments designed to characterize the cytoplasmic activity responsible for formation of Myc-nick, we found that inhibitors of transcription or translation had no effect on Myc-nick formation (Figure 2E) whereas heating or adding protease inhibitors to the CE blocked Myc-nick, consistent with proteolysis (Figure 2F). Whereas MG132, an inhibitor of the proteasome (and other cysteine proteases), blocked Myc nick formation, specific proteasome inhibitors such as Epoxomycin and Lactacystein failed to block the formation of Myc-nick in vitro and in vivo (Figures 2G and 2H). In addition, inhibition of either the Fbw7 or Skp2 degradation pathways, known to be responsible for proteasomal Myc turnover, had no effect on Myc-nick formation (data not shown). Moreover, incubation of Myc with 20S and 26S proteasomes failed to produce Myc-nick (not shown). These results indicate that full-length c-Myc is converted into Myc-nick by a cytoplasmic protease that is independent of the proteasome.

Myc Is Cleaved by a Calcium-Activated Calpain to Produce Myc-Nick

In a systematic search for the proteases mediating cytoplasmic cleavage of Myc, we ruled out both caspases and lysosomal proteases on the basis of inhibitors and cleavage conditions (Figure S2C). However, we found that all calpain inhibitors tested including calpeptin, calpain inhibitor XII (Figure 3A), and calpain inhibitor VI (Figure 3B) blocked the formation of Myc-nick in vitro and in vivo. Moreover, MG132, although well known as a proteasome inhibitor, has also been shown to inhibit calpains (Mailhes et al., 2002). Calpains had been linked to Myc stability earlier, but the antibodies used in those studies would not have detected Myc-nick (Gonen et al., 1997; Small et al., 2002). Calpains comprise a large family of calcium-dependent cytoplasmic cysteine proteases that function at neutral pH and are primarily associated with partial protein cleavage rather than complete protein degradation. The most well studied members of this family are mcalpain and μcalpain. These ubiquitously expressed catalytic subunits form functional heterodimers with a calpain regulatory subunit (calpain r) to bind calcium. To determine whether Myc-cleavage is regulated by calcium-activated calpains, we employed siRNA to knock down calpain r in cells expressing c-Myc either endogenously or under control of a retroviral vector. In both settings a partial silencing of calpain r correlated with decreased Myc-nick and increased full-length c-Myc (Figure 3C). Knockdown of m or μcalpains alone did not block the formation of Myc-nick, most likely due to the presence of other redundant calpains (not shown). Because calpain activity is calcium dependent we next examined the effects on Myc-nick of modulating calcium levels. Treatment with the calcium chelators Bapta or EGTA reduced the ability of CE to cleave c-Myc (Figures 3D and 3E). Incubation of either IVT c-Myc (Figure 3F) or recombinant c-Myc (Figure 3G) with purified m or μcalpain (together with calpain r) produced Myc-nick. These results demonstrate that Myc-nick is directly generated by calcium-dependant calpain cleavage of full-length c-Myc.

Lysine 298 Is the Primary Calpain Cleavage Site in c-Myc

To map the calpain cleavage region on c-Myc, we used a series of internal deletion mutants lacking 60 residue segments (c-Myc ΔA-ΔG) (Tworkowski et al., 2002) (Figure 3H) and determined whether any failed to generate a shorter c-Myc protein. Only the deletion of residues 252–315 (ΔE) resulted in loss of a Myc-nick-like product (Figure 3I). This region contains a high scoring PEST domain, often present in unstable proteins and frequently associated with calpain cleavage sites. We further narrowed our search to residues 270–315, based on the ability of antibody 274 to detect Myc-nick (Figure S1A and Figure 3H). Although there is

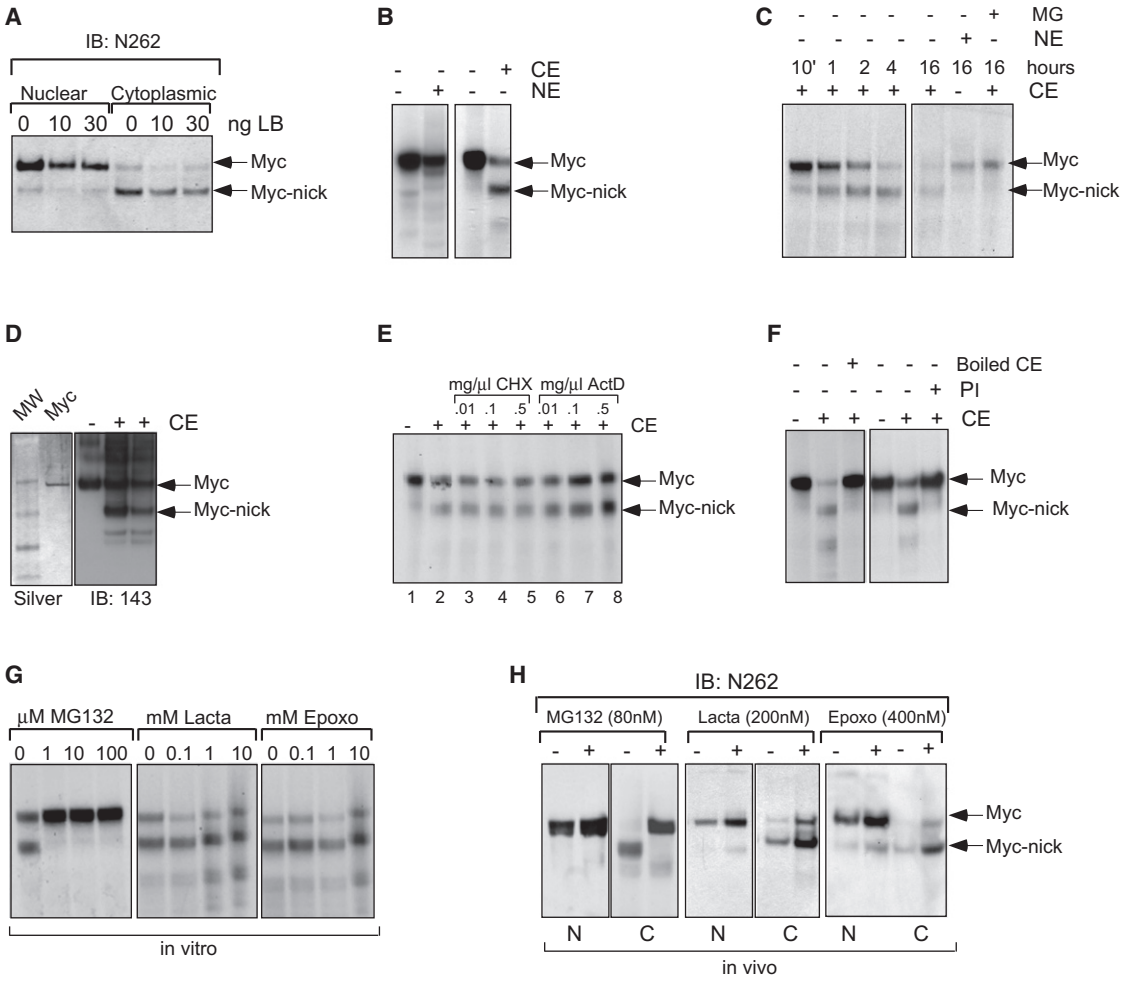

Figure 2. Myc-Nick Is a Product of c-Myc Cytoplasmic Cleavage Independent of the Proteasome

(A) CRM1-dependant nuclear export is not involved in the formation or localization of Myc-nick. Rat1 *myc* null cells infected with c-Myc were treated with leptomycin B (LB) for 4 hr before harvesting.

(B) c-Myc gives rise to Myc-nick in vitro when incubated with cytoplasmic extracts. Radiolabeled IVT c-Myc was incubated for 2 hr with cytoplasmic (CE) or nuclear extracts (NE) from Rat1 c-*myc* null cells.

(C) Timecourse of in vitro cleavage of c-Myc.

(D) Recombinant c-Myc is cleaved in the presence of CE. One microgram of recombinant c-Myc was incubated with 30 µg of CE and processed for western blot.

(E) IVT c-Myc was incubated with CE for 4 hr in the presence of of Actinomycin D (ActD) or cyclohexamide (CHX).

(F) The cleavage of c-Myc is inhibited by protease inhibitors and by heat inactivation. CE was boiled prior to incubation with IVT c-Myc and the protease inhibitor (PI) was added to the incubation mixture.

(G and H) The cleavage of c-Myc is inhibited by MG132, but not by Lactacystein or Epoxomycin. (G) IVT c-Myc was incubated with CE, in the presence of increasing amounts of MG132, Lactacystein, and Epoxomycin for 1 hr. (H) Rat1 *myc* null cells expressing c-Myc were incubated with MG132, Lactacystein, or Epoxomycin for 2 hr prior to harvesting. Nuclear (N) and cytoplasmic (C) fractions are shown.

See also Figure S2.

no universal consensus for a calpain cleavage site, a comparison of 106 sites present in 49 known calpain substrates indicated a preference for calpain cleavage after K or R, and to lesser extent Y, especially when these amino acids are flanked by P, V, and L (Figure 3J) (Tompa et al., 2004). We noted a region localized C-terminal to the PEST domain containing the sequence PLVLKRC (Figure 3H, marked in red). This region is evolutionarily conserved in c-Myc and N-Myc but not L-Myc (Figure S3A), consistent with the fact that c-Myc and N-Myc, but not L-Myc, are cleaved (Figures S3B and S3C). To determine whether this region functions as a calpain cleavage site, we synthesized a 10 amino acid peptide corresponding to the putative c-Myc calpain cleavage region (291–300) and to another nearby region of c-Myc (236–245) (Figure 3H, in red and blue, respectively) and asked whether they could act as competitive inhibitors of Myc-nick formation in vitro. Addition of increasing amounts of the peptide containing the putative calpain cleavage site blocked the formation of Myc-nick in vitro whereas the control peptide

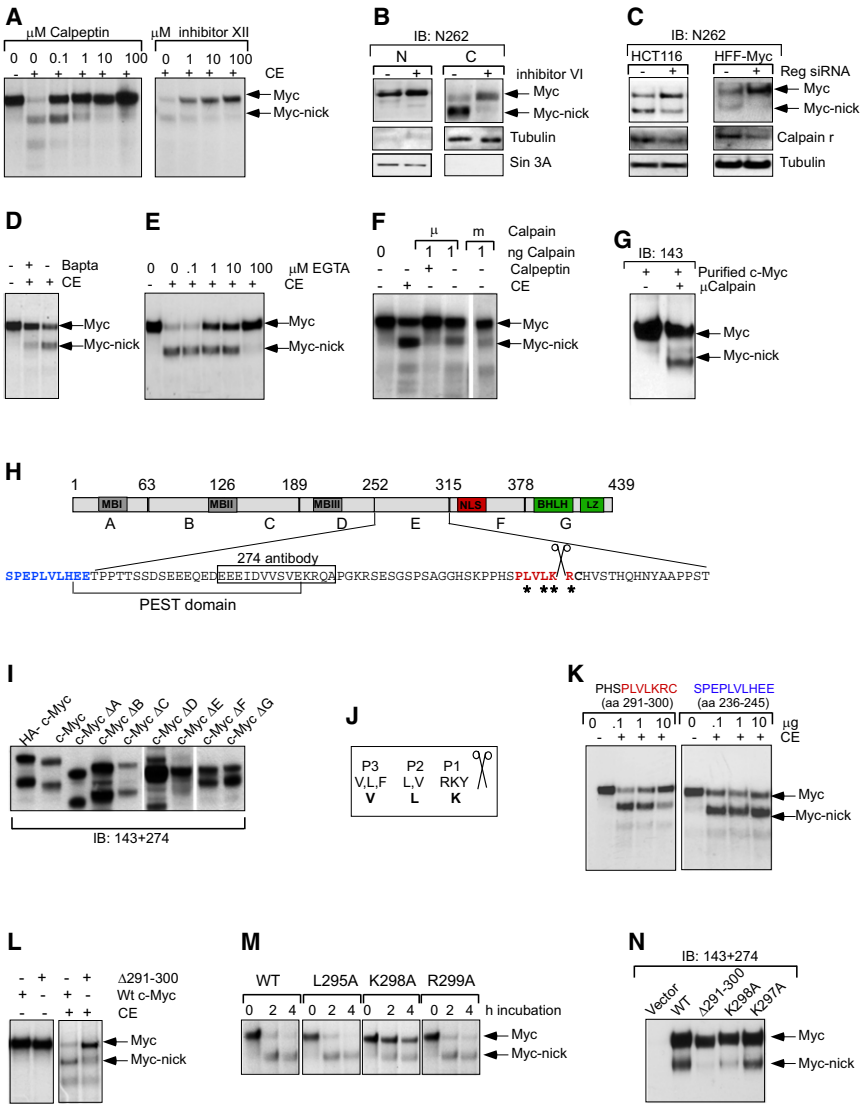

Figure 3. Myc-Nick Is Generated by Calpain Cleavage of Full-Length Myc

(A) IVT c-Myc was incubated with CE for 1 hr in the presence of the calpain inhibitors calpeptin and calpain inhibitor XII.

(B) Dense cultures of HFF cells infected with a c-Myc retroviral vector were incubated with calpain inhibitor VI for 2 hr prior to harvesting.

(C) siRNA for calpain regulatory subunit (Reg) reduces the formation of Myc-nick in HCT116 and HFF-Myc cells.

(D) Rat1 *myc* null cells were incubated for 2 hr in the presence of the calcium chelant Bapta, and then cytoplasmic extracts were prepared and incubated with IVT c-Myc (as in 2B).

(E) IVT c-Myc was incubated with CE for 1 hr in the presence of increasing amounts of EGTA.

(F) IVT c-Myc was incubated with purified recombinant μcalpain or mcalpain and r subunit for 1 hr on ice.

(G) One hundred nanograms of purified μcalpain and r subunit was incubated with 2 μg of recombinant c-Myc for 30 min on ice.

(H) Schematic representation of c-Myc protein indicating A-G deletion regions (deletion endpoints indicated above), putative calpain cleavage region in red.

(I) c-Myc and the deletion mutants ΔA-ΔG were expressed in 293T cells, and 48 hr later the presence of a Myc-nick-like protein in cytoplasmic extracts was determined.

(J) Amino acid preference for calpain cleavage region according to Tompa et al. (2004). P1–P3 indicate the position of preferred residues in relation to the cleavage site, bold letters indicate the c-Myc calpain cleavage site.

(K) IVT c-Myc was incubated with CE in the presence of a peptide that contains the potential calpain cleavage site (amino acids 291–300 in red) or a nearby sequence (amino acids 236–245 in blue).

(L) WT and Δ291–300 IVT c-Myc were incubated with CE for 1 hr (as in 2B).

(M) IVT WT, L295A, K298A, and K299A c-Myc were incubated with CE for the indicated time points.

(N) Cleavage products produced from the indicated c-Myc mutants in 293T cells.

See also Figure S3.

had no effect (Figure 3K). Next, we generated a deletion mutant of Myc lacking residues 291–300 and found it to be resistant to cleavage by CE (Figure 3L). When this mutant was ectopically expressed in 293T cells the cleavage was also reduced in comparison to the wild-type (WT) c-Myc (Figure 3N), indicating that this is the major calpain cleavage site within c-Myc. Next, we made point mutations in the calpain cleavage region (labeled with asterisks in Figure 3H) and assessed their cleavage. We found that the K298A mutation reduced the cleavage of c-Myc in vitro (Figure 3M) and in vivo when ectopically expressed in 293T cells (Figure 3N). Although K298 appears to be the major calpain cleavage site, a K298A mutant was still cleaved in vivo most likely because when this residue is mutated the cleavage is shifted to R299 (and perhaps L297, V296). We also identified weaker calpain cleavage sites localized in the C terminus of Myc that become more pronounced in the cleavage-deficient mutants.

Expression of Myc-Nick Alters Cell Morphology and Increases Acetylation of α-Tubulin

To study Myc-nick function, we generated a truncated form of Myc containing amino acids 1–298 (referred to as Myc-nick*). We found that ectopically expressed Myc-nick* is localized predominantly to the cytoplasm and migrates on SDS-PAGE with the same apparent molecular weight as Myc-nick generated by cleavage of full-length c-Myc (Figure 4A). Myc-nick is degraded at a comparable rate to full-length c-Myc with a half-life of about 30 min (data not shown). Because Myc-nick contains the MBI phosphodegron, with its GSK3β phosphorylation and Fbw7-binding sites, we would expect Myc-nick to be

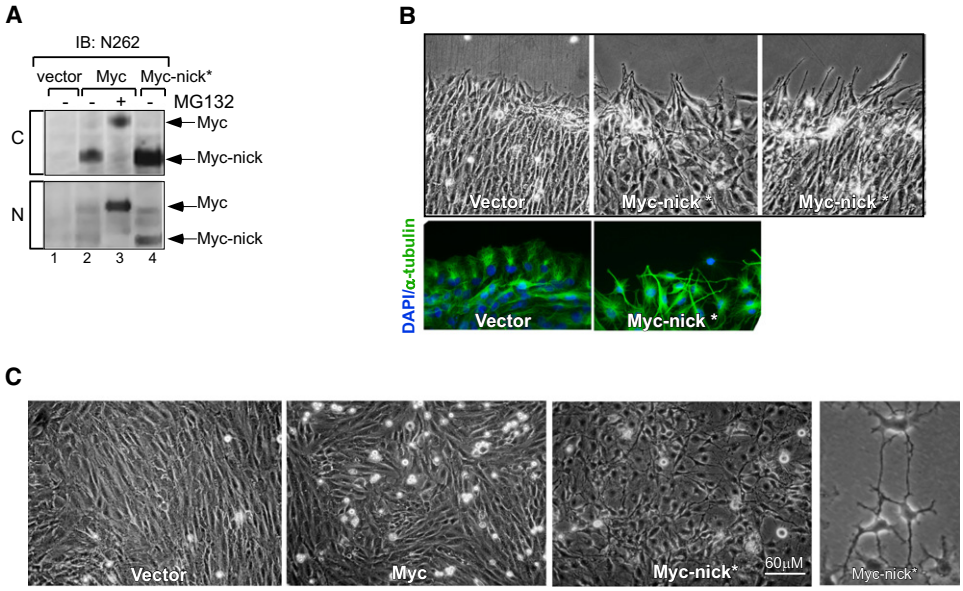

Figure 4. The Expression of Myc-Nick* Promotes Changes in Cell Morphology

(A) Myc-nick* (1–298) is cytoplasmic and has the same apparent molecular weight as Myc-nick derived from full-length c-Myc (compare lanes 2 and 4, upper panel). Rat1 *myc* null cells expressing empty vector, full-length c-Myc, and Myc-nick were fractionated into nuclear (N) and cytoplasmic (C) fractionations.

(B) Myc-nick*-expressing cells extend protrusions at the wound edge. A confluent monolayer of Rat1 *myc* null cells expressing either vector or Myc-nick was scratched using a 100 μl tip and phase contrast images were taken at 12 hr.

(C) Rat1 *myc* null cells expressing empty vector, c-Myc, and Myc-nick at 14 days after selection.

See also Figure S4.

degraded through similar proteasomal pathways as full-length Myc. Indeed, blocking proteasome activity by pharmacological inhibitors or by silencing E3 ligases and components of the proteasome induced the stabilization of both full-length Myc and Myc-nick (data not shown). Calpain inhibitors are incapable of inducing accumulation of Myc-nick, indicating that calpains are unlikely to play a major role in Myc-nick turnover in vivo.

Whereas overexpression of full-length c-Myc in Rat1a *myc* null fibroblasts is associated with increased proliferation and apoptosis, the ectopic expression of Myc-nick* had no detectable effect on either cell doubling time or survival (data not shown). However, Myc-nick*-expressing cells displayed dramatic morphological changes—they appeared spindle-like with long protrusions that occasionally formed intercellular contacts (Figure 4C, Figure S4, Figure S5C). This morphology was specific for Myc-nick* expression and could not be produced by expressing the C-terminal 100 residues of c-Myc (not shown). Introducing a scratch wound across a confluent cell monolayer showed that whereas control cells migrated into the wound by extending lamellipodia, Myc-nick*-expressing cells aligned parallel to each other and extended long protrusions into the wound (Figure 4B).

The morphological changes induced by Myc-nick* are suggestive of altered cell-cell contacts and/or major cytoskeletal reorganization. The elongated cellular protrusions present in Myc-nick-expressing cells resemble specialized structures formed by stable microtubules. Because stable microtubules display increased acetylation of α-tubulin on lysine 40 (Hubbert et al., 2002), we stained Myc-nick*-expressing cells with antibodies against α-tubulin and acetylated α-tubulin. Figure 5A shows that although acetylated α-tubulin immunostaining in vector and c-Myc-expressing cells is low, Myc-nick* cells display intense staining of elongated structures (Figures 5A and 5F). This was confirmed by immunoblotting for acetylated α-tubulin in Rat1 *myc* null or 293T cells expressing Myc-nick* (Figure 5C, Figure S6B). Importantly neither the levels of total cellular acetylated lysine (Figure S6A) nor the levels of tyrosinated tubulin (not shown) were affected. To ask whether Myc-nick* expression increased microtubule stability, we treated cells expressing Myc-nick*, c-Myc, or vector with nocodozole for 15 min. This treatment disrupted unstable microtubules with a half-life of about 10 min but not stable microtubules with a half-life >2 hr. Only Myc-nick*-expressing cells possessed microtubules that survived nocodozole treatment and these stained strongly with antiacetylated α-tubulin (Figure 5B).

Myc-Nick Interacts with α- and β-Tubulins

We observed partial cytoplasmic colocalization between Myc and α-tubulin in several cell types expressing c-Myc or Myc-nick (Figure 5E; Figures S5A–S5C). Using 293T cells expressing GFP-tubulin and Myc-nick we performed immunoprecipitation with anti-GFP and immunobloted with anti-Myc. Myc-nick was coimmunoprecipitated with GFP-tubulin but not GFP-EB1, a microtubule-binding protein (Figure S5D). In addition, immunoprecipitation of either c-Myc or endogenous α-tubulin from cytoplasmic extracts indicated that Myc-nick interacts with α-tubulin in vivo (Figure 5D). To examine in vitro interactions we incubated ^{35}[S]-methionine-labeled IVT c-Myc with purified brain tubulins

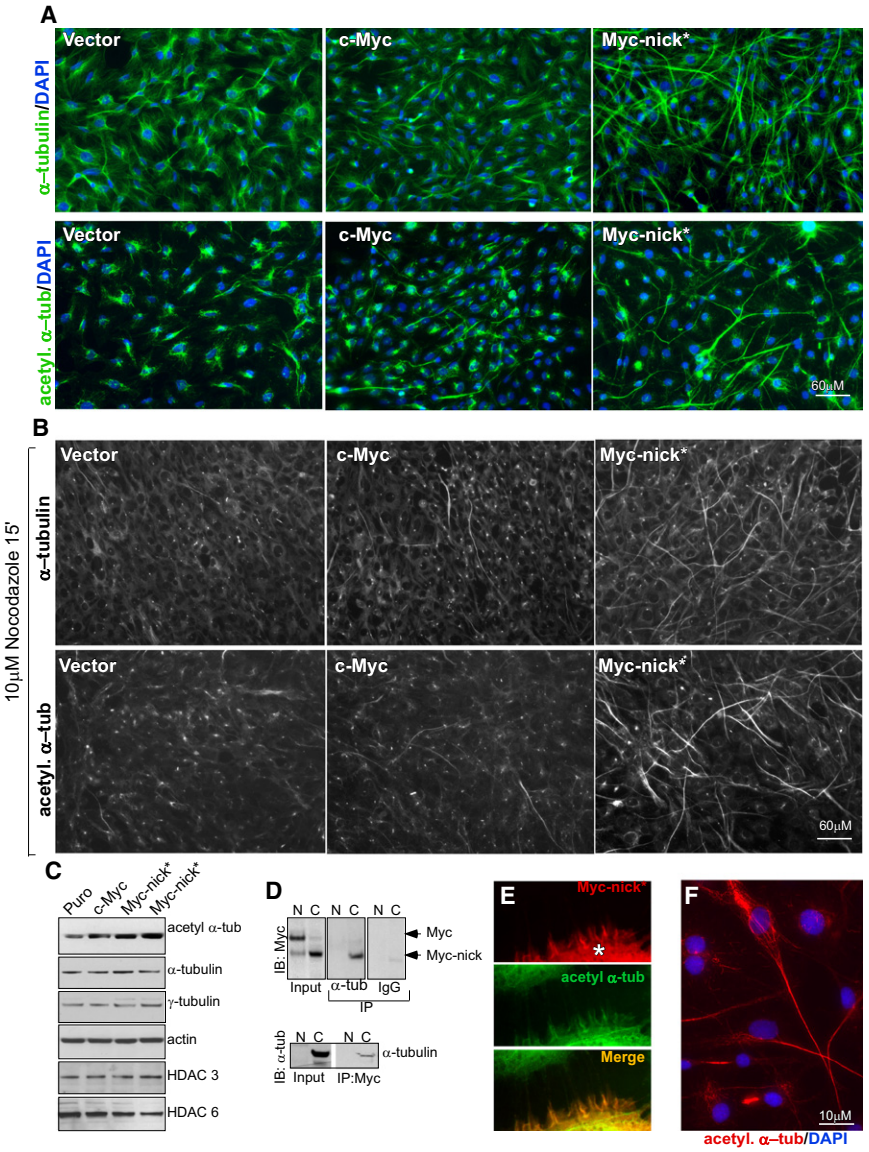

Figure 5. Myc-Nick* Cells Display Increased Levels of Acetylated α-Tubulin and Microtubule Stabilization

(A) Immunofluorescence for α-tubulin (upper panels) and acetylated α-tubulin (lower panels) of Rat1 *myc* null cells infected with empty vector, c-Myc, or Myc-nick*.

(B) As for (A) but incubated in the presence of nocodazole for 15 min prior to fixation.

(C) Immunoblotting of Rat1 cell extracts using antibodies against the indicated proteins.

(D) NE or CE of Rat1 *myc* null cells expressing c-Myc were immunoprecipitated with anti α-tubulin or normal mouse IgG and immunoblotted for Myc (top panel), or immunoprecipitated with anti-c-Myc N262 antibody, and immunoblotted with anti-α-tubulin.

(E) Immunofluorescence for Myc and acetylated α-tubulin in A431 lung epithelial cells cell transfected with Myc-nick.

(F) Detail of Myc-nick-expressing cell stained for acetylated α-tubulin.

See also Figure S5.

for 1 hr, then immunoprecipitated α, β, γ, βIII and acetylated α-tubulin and exposed the gel to detect radioactive Myc. Full-length c-Myc was coimmunoprecipitated mostly with β-tubulin (Figure S5E, middle panel). We also incubated IVT c-Myc with CE for 1 hr to produce Myc-nick and then performed immunoprecipitation for tubulins as above. The results showed that Myc-nick interacts with α- and β-tubulin (Figure S5E, upper panel). The binding of Myc-nick to tubulin is consistent with the previous report of an N-terminal region of Myc capable of associating with tubulin (Alexandrova et al., 1995).

Myc-Nick Directly Regulates α-Tubulin Acetylation through GCN5

Because Myc-nick lacks the Myc C-terminal dimerization and DNA-binding domains, we surmised that the increase in acetylated α-tubulin induced by Myc-nick* expression is independent of Myc transcriptional activity. One possibility is that Myc-nick recruits an acetyltransferase to microtubules. We observed marked acetylation of α-tubulin in cytoplasmic extracts incubated in the presence of either recombinant c-Myc (Figure 6A) or IVT c-Myc (Figure 6B and Figure S6C). Importantly, Myc proteins are known to associate with the acetyltransferases GCN5 and TIP60 (Frank et al., 2003; Sterner and Berger, 2000) via TRRAP that interacts with MBII (residues 106–143) (McMahon et al., 1998). We therefore tested a Myc-nick deletion mutant lacking Myc box II (ΔMBII) and found that its ability to promote α-tubulin acetylation was reduced (Figures 6C and 6D). In addition, the ΔMBII Myc-nick* mutant failed to induce the cell morphological changes that we had detected with WT Myc-nick* whereas a comparably sized deletion in a region adjacent to MBII was similar to WT (Figure 6E).

The dependence on MBII for tubulin acetylation and altered cell morphology suggests involvement of the acetyltranferases known to bind this region. We detected substantial amounts of GCN5 and TRRAP in the cytoplasm (Figure S6D), whereas Tip60 was predominantly nuclear (not shown). To test GCN5′s involvement in α-tubulin acetylation we performed siRNA-mediated knockdown of TRRAP and GCN5 in Myc-nick*-expressing cells and found that decreasing either one of these proteins reduced the levels of acetylated α-tubulin (Figure 6F) and reverted the changes in cell morphology induced by Myc-nick to that of vector-infected cells (Figure 6G). Moreover, GCN5 coimmunoprecipated with α-tubulin and Myc in cytoplasmic extracts of 293T cells transfected with GCN5 (Figure 6H).

Both ectopic expression of GCN5 in 293T cells (Figures 6D and 6I) and addition of full-length recombinant GCN5 to

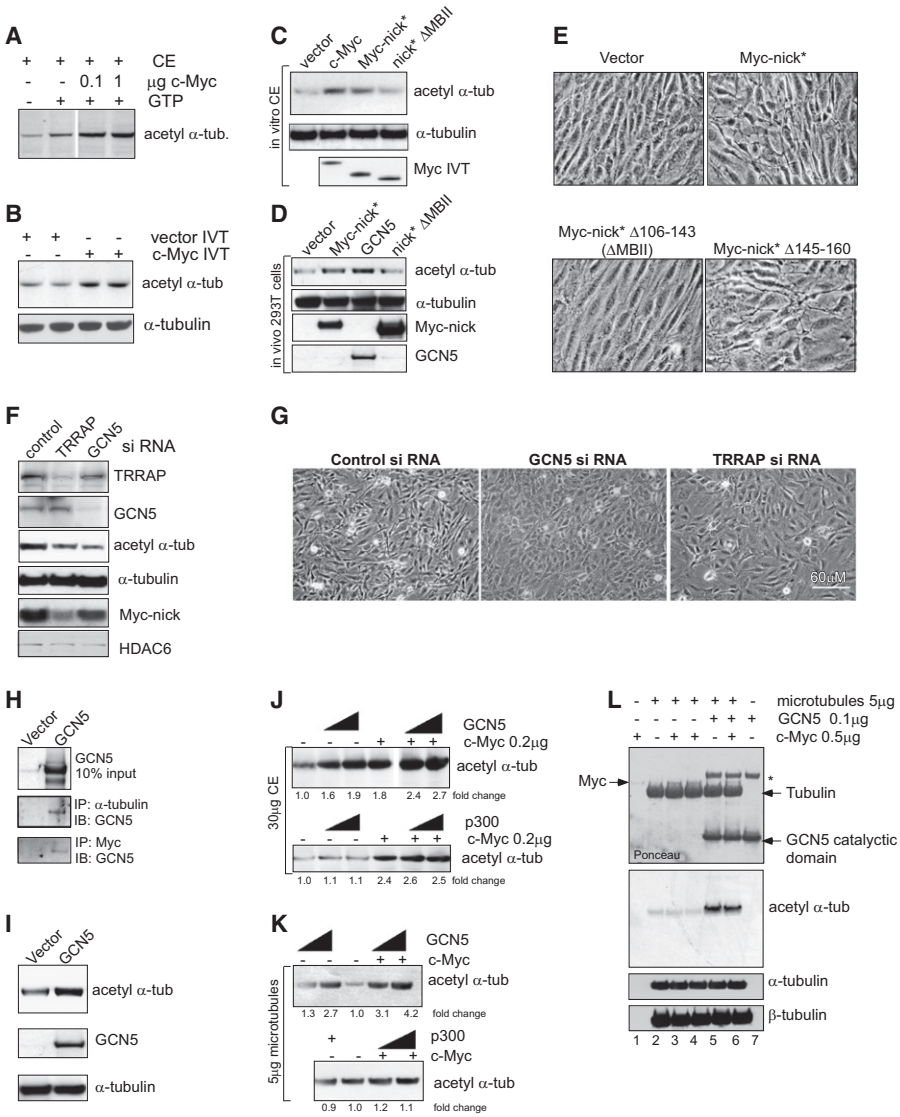

Figure 6. c-Myc and the HAT GCN5 Promote α-Tubulin Acetylation

(A) CE was incubated with recombinant c-Myc and c-Myc dilution buffer or (B) with c-Myc IVT or vector IVT for 1 hr at 37°C. The samples were immunoblotted as indicated.

(C) CE was incubated with IVT vector, c-Myc, Myc-nick, and Myc-nick ΔMBII (Δ106–143) for 30 min at 37°C, then processed for immunoblotting.

(D) 293T cells were transfected with empty vector, GCN5, Myc-nick, and Myc-nick ΔMBII and processed for immunoblotting after 48 hr.

(E) Rat1 *myc* null cells were infected as indicated and photographed 10 days after selection.

(F and G) Rat1 *myc* null cells expressing Myc-nick were transfected with 100 nM of control, TRRAP, or GCN5 siRNA and 76 hr later processed for immunoblotting (F) or photographed (G).

(H) CE of 293T cells transfected with empty or GCN5 vectors were immunoprecipitated with anti-α-tubulin or anti-Myc (143+274) and immunoblotted for GCN5.

(I) 293T cells were transfected with control or GCN5-expressing vectors and 48 hr later processed for immunoblotting.

(J–L) GCN5 induces acetylation of α-tubulin. (J) CE were incubated with 200 ng of Myc, 100 ng or 300 ng of GCN5 (upper panel), or 100 ng or 500 ng of p300 (lower panel). (K) Assembled microtubules were incubated with 100 ng or 500 ng of recombinant full-length GCN5 (upper panel) or p300 (lower panel) in the presence or absence of 200 ng of purified c-Myc. (L) Purified assembled microtubules were incubated with recombinant c-Myc and GCN5-catalytic domain. The asterisk indicates a nonspecific bacterial band copurified with GCN5-catalytic domain.

See also Figure S6.

cytoplasmic extracts (Figure 6J, upper panel) resulted in increased levels of acetylated α-tubulin. The addition of Myc further increased the levels of α-tubulin acetylation induced by GCN5 in cytoplasmic extracts (Figure 6J). Full-length recombinant GCN5 also induced the acetylation of α-tubulin present in assembled microtubules (Figure 6K) and synergized with c-Myc to promote further acetylation (Figure 6K, compare lanes 1 to 4 and 2 to 5). The GCN5 catalytic domain alone was

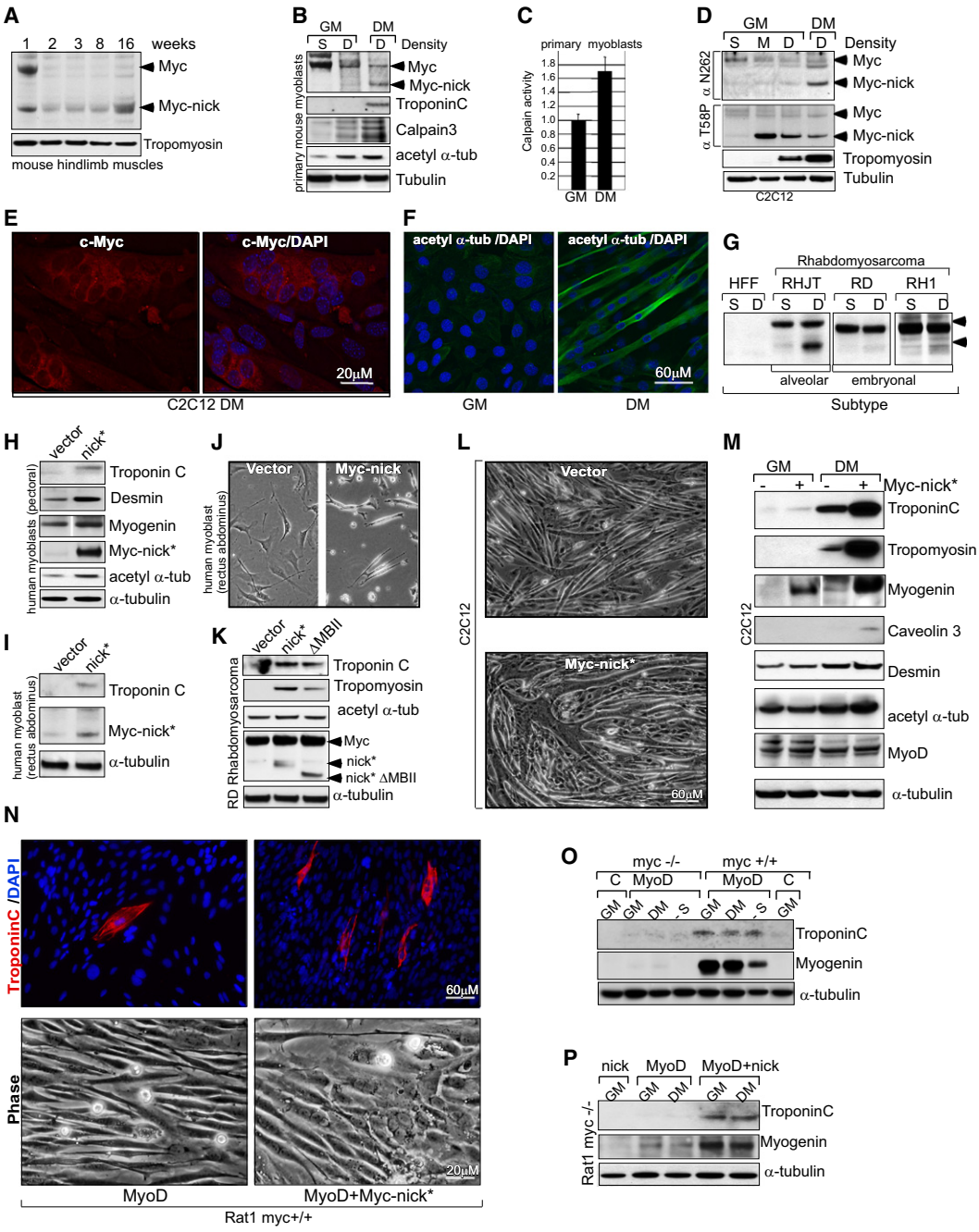

Figure 7. Myc-Nick Accelerates Muscle Cell Differentiation

(A) Hindlimb muscles dissected from 1-, 2-, 3-, 8-, and 16-week-old mice were processed for immunoblotting using N-terminal anti-Myc sera (anti 143+274).

(B) Mouse primary myoblasts isolated from hindlimb muscles of 8-week-old mice were cultured as sparse cultures for 24 hr or as dense cultures in the presence of either growth medium (GM) or differentiation medium (DM) for 3 days. Total cell extracts were immunoblotted for c-Myc (anti 143+274) and indicated proteins.

(C) Mouse primary myoblasts were cultured in GM or DM for 3 days, lysed in buffer A, and total calpain activity was measured using Suc-LLVY-AMC synthetic substrate (n = 2; calpain activity in DM was compared with calpain activity in GM; set to 1 ± standard error of the mean [SEM]).

(D) C2C12 mouse myoblasts cultured at sparse (S), medium (M), or high densities (D). Dense cultures were harvested or switched to DM for 7 days. Total cell extracts were immunoblotted for c-Myc using antibodies against total c-Myc (N262) or against phosphorylated T58 Myc, a signal for Myc degradation.

(E) C2C12 cells cultured in DM for 7 days and stained for endogenous c-Myc (anti-N262).

(F) C2C12 cells grown in the presence of GM or DM for 5 days and stained with anti-acetylated α-tubulin.

(G) Western blotting for Myc in rhabdomyosarcoma cell lines grown as dense or sparse cultures for 3 days.

(H and I) Human myoblasts expressing vector, or Myc-nick were cultured in DM and processed for immunoblotting after 4 days.

(J) Human myoblasts expressing vector or Myc-nick were cultured in DM and photographed after 2 days.

sufficient to induce α-tubulin acetylation, but no synergy with Myc was detected, most likely because association between these two proteins occurs outside of GCN5′s active site (Figure 6L). As a further control, we tested p300, a HAT that binds the C terminus of Myc (Faiola et al., 2005). p300 neither induced tubulin acetylation nor did it synergize with Myc (Figures 6J and 6K, bottom panels). These data indicate that GCN5 can specifically acetylate tubulin and that Myc augments tubulin acetylation by binding to tubulin and recruiting GCN5.

Myc-Nick Is Produced in Differentiating Cells and Tissues

When examining the expression of Myc-nick in adult mouse tissues, we found that brain, cerebellum, and skeletal muscle express significantly higher levels of Myc-nick than any other tissue (Figure 7A, Figure S7A). Interestingly, both neuronal and muscle differentiation require major cytoskeletal rearrangements that have been associated with increased microtubule stability and elevated levels of acetylated α-tubulin. For example, it has been demonstrated that the acetylation of α-tubulin by the Elp3 acetyltransferase is required for proper cortical neuronal migration and differentiation (Creppe et al., 2009). Increased levels of acetylated α-tubulin are found during myogenic differentiation (Gundersen et al., 1989). Importantly, inhibiting the activity of the deacetylases HDAC6 and Sirt2 (known to deacetylate α-tubulin) generates augmented levels of acetylated α-tubulin and promotes differentiation of myoblasts (Iezzi et al., 2004). Furthermore, the presence of the primary cilium, a microtubule-based antenna-like structure composed of acetylated α-tubulin, is essential for cardiomyocyte differentiation (Clement et al., 2009).

The extensive cytoskeletal changes that occur during muscle differentiation are regulated by calcium and calpains (Dedieu et al., 2004). The inhibition of calpain activity either by pharmacological inhibitors or by overexpression of Calpastatin (an endogenous inhibitor of calpains) blocks muscle cell differentiation (Dedieu et al., 2004). In addition, both types of ubiquitous calpains (m and μ) were shown to regulate muscle cell differentiation in vitro (Moyen et al., 2004). Moreover, mutations in the *calpain 3* gene (the muscle-specific calpain) cause limb girdle muscle dystrophy 2A (LGMD2A) (Richard et al., 1995). Finally, knocking out calpain r (the regulatory subunit shared by all calcium-dependent calpains) is lethal due to impaired cardiovascular development (Arthur et al., 2000).

To study the cleavage of Myc to Myc-nick during the process of muscle differentiation, we employed human primary myoblasts purified from pectoral girdle, mouse myoblasts purified from hindlimb muscles, and C2C12 cultured mouse myoblasts. When stimulated to differentiate, these cells displayed low levels of full-length c-Myc and high levels of Myc-nick when compared to undifferentiated cycling cells (Figures 7B and 7D; Figure S7B). Interestingly, Myc-nick levels were higher in a rhabdomyosarcoma cell line from the alveolar subtype (Figure 7G), a very aggressive tumor derived from partially differentiated muscle cells.

The increased levels of Myc-nick in differentiated primary mouse myoblasts correlate with an increase in calpain 3 levels (Figure 7B; Figure S7B) and in total calpain activity (Figure 7C). Similarly, when C2C12 cells were stimulated to differentiate, they also displayed an increase in total calpain activity (Figure S7C) and in the ability to cleave Myc in vitro (Figure S7D). Importantly, the levels of acetylated α-tubulin were also elevated during the process of differentiation (Figure 7B; Figures S7B and S7F). Acetylation of α-tubulin is accompanied by myoblast fusion to form multinucleated myotubes (Figure 7F; Figure S7F). When myoblasts are stimulated to differentiate they fuse into multinucleated myotubes. In addition to displaying increased levels of acetylated α-tubulin, these myotubes show decreased immunostaining for nuclear Myc and increased cytoplasmic Myc staining (Figure 7E). Conversely, undifferentiated satellite cells in the same culture display nuclear staining for Myc (Figure S7E). This is consistent with our results showing conversion of full-length Myc into predominantly cytoplasmic Myc-nick.

In C2C12 cells, concomitant with increased Myc-nick abundance, we observed a decrease in Myc-nick phosphorylation at threonine 58 (T58) after the switch to differentiation conditions (Figure 7D). Phospho-T58 mediates Fbw7-dependent degradation of Myc by the proteasome. Decreased phosphorylation at this site is consistent with the notion that stabilization of Myc-nick contributes to its elevated levels. Interestingly we found that Myc-nick phosphorylation at T58 is also reduced in adult muscle, brain, and cerebellum (Figure S7A and data not shown).

In summary, we found that during muscle differentiation there is an increase in Myc-nick, concomitant with an elevation in calpain activity and tubulin acetylation (Figures 7B–7D; Figure 7F; Figures S7B–7D; Figure S7F).

Myc-Nick Accelerates Muscle Differentiation

We examined the effects of ectopic expression of Myc-nick* in human primary myoblasts isolated from the pectoral girdle (Figure 7H) and rectus abdominus (Figures 7I–7J), from the human rhabdomyosarcoma cell line RD (Figure 7K), and in C2C12 cells (Figures 7L and 7M). In all cells we found that Myc-nick* expression accelerated muscle cell differentiation, augmented α-tubulin acetylation, and elevated expression of muscle-specific proteins (Figures 7H–7M).

(K) RD rhabdomyosarcoma cells expressing vector, Myc-nick, or Myc-nick ΔMBII (Δ106–143) were grown in DM for 4 days and processed for immunobloting.
(L) C2C12 cells expressing vector or Myc-nick were grown as dense cultures and stimulated to differentiate for 4 days and then photographed.
(M) C2C12 cells expressing vector or Myc-nick were grown to confluency and harvested or stimulated to differentiate for 3 days and then harvested. Total cell lysates were immunoblotted with antibodies against the indicated proteins.
(N) Rat1 ($myc^{+/+}$) cells expressing MyoD or MyoD + Myc-nick were cultured in DM for 3 days and photographed (lower panels) or stained for Troponin C and DAPI (upper panels).
(O) Rat1 ($myc^{+/+}$) or Rat1 *myc* null ($myc^{-/-}$) cells expressing vector (lane C) or MyoD were cultured in GM, DM, or serum-free medium (lane −S) for 4 days and processed for immunobloting.
(P) Rat *myc* null ($myc^{-/-}$) cells expressing Myc-nick, MyoD, or MyoD + Myc-nick were grown in GM or DM for 3 days and processed for immunoblotting.
See also Figure S7.

The expression of Myc-nick* in C2C12 myoblasts promoted an increase in cell fusion not only in confluent cultures (Figure 7L; Figure S7H) but also in sparse cultures (Figure S7G). The role of Myc-nick in promoting muscle cell differentiation is partially dependent on MBII, as the differentiation of RD and C2C12 cells by Myc-nick can be delayed but not inhibited by the deletion of MBII (Figure 7K; Figure S7I).

To examine the requirement for Myc-nick production during differentiation further, we first tested calpain inhibitor XII and found that it reduces differentiation and tubulin acetylation (Figure S7J). Next we employed c-Myc mutant (Δ291–300), which is deficient in cleavage to Myc-nick. Similar to full-length WT c-Myc, this mutant can induce proliferation, apoptosis, fibrillarin expression, and phosphorylated H2AX in Rat1 *myc* null cells (Figures S7L–S7M). However the Δ291–300 c-Myc mutant significantly reduces C2C12 cell differentiation when compared to c-Myc, even though the proteins are expressed at equal levels. Whereas full-length c-Myc only blocks the fusion of C2C12 myoblasts, the c-Myc mutant (Δ291–300) with reduced ability to generate Myc-nick dramatically reduces the expression of muscle markers in addition to blocking fusion (Figures S7N–S7O).

Myc-Nick Renders Rat1 *myc* Null Cells Competent to Differentiate

MyoD has been long known to induce transdifferentiation in diverse cell types. We found that MyoD induces expression of muscle-specific markers in Rat1 fibroblasts but not in Rat1 *myc* null fibroblasts (Figure 7O), indicating that *myc* is necessary for the transdifferentiation process. Expressing Myc-nick together with MyoD in $myc^{+/+}$ Rat1 fibroblasts further elevated the levels of muscle-specific markers and promoted cell fusion compared to MyoD alone (Figure 7N). Importantly, we find that in Rat1 *myc* null cells, the coexpression of Myc-nick (Figure S7K) permitted transdifferentiation to muscle in response to MyoD (Figure 7P). This experiment indicates that Myc-nick is sufficient for MyoD-induced transdifferentiation, supporting the idea that conversion of full-length Myc to Myc-nick is important in MyoD-induced differentiation.

DISCUSSION

Recent studies have demonstrated that Myc directly regulates transcriptional activation through the three RNA polymerases, as well as transcriptional repression, and DNA replication (Eilers and Eisenman, 2008). Here we identified and characterized Myc-nick, a novel form of Myc performing transcription-independent functions in the cytoplasm. One of these functions is to regulate α-tubulin acetylation in cooperation with the HAT GCN5. Moreover, Myc-nick levels are elevated in differentiated muscle tissues and Myc-nick overexpression accelerates muscle cell differentiation.

Despite its widespread expression, Myc-nick has not been previously characterized. There are several likely reasons for this. First, the most commonly used antibodies against Myc (such as 9E10) recognize its C terminus and would not detect Myc-nick. Second, the proportion of Myc-nick to Myc increases when cells are grown as confluent cultures, conditions that are not commonly employed when studying Myc proteins. Third, most studies use total protein or nuclear extracts and have not analyzed the cytoplasmic pool of Myc. Fourth, Myc-nick may have been mistaken for the similarly sized MycS protein (Hann et al., 1988), a nuclear localized product of internal translation initiation.

Calpain Cleavage as a Posttranslational Functional Switch

We found that Myc-nick is generated in the cytoplasm by calpain-mediated cleavage of full-length Myc. Cytoplasmic cleavage by calpains has been reported for over 100 proteins including many cytoskeletal proteins, membrane receptors, and transcription factors (Tompa et al., 2004). This number is probably an underestimate because the lack of a clearly defined consensus cleavage site for calpains makes the identification of novel calpain substrates difficult. For most substrates the role of calpain cleavage is not known. However, there are several noteworthy examples of calpain cleavage operating as a functional switch (Abe and Takeichi, 2007; Yousefi et al., 2006). Here we have shown that the cleavage of Myc by calpain converts this predominantly nuclear transcription factor into Myc-nick, a cytosolic factor that regulates α-tubulin acetylation. Based on our findings and the examples in the literature, we surmise that the partial proteolytic cleavage of proteins by calpains functions as an irreversible posttranslational modification.

A Role for Myc-Nick in α-Tubulin Acetylation

We have shown that Myc-nick mediates the acetylation of α-tubulin by forming a complex with microtubules and the HAT GCN5. GCN5 has been long known to associate with nuclear Myc through the highly conserved Myc box II, a region retained in Myc-nick. Although tubulin acetylation was first described 20 years ago, little is known about the enzymes that catalyze this reaction. Recently Elp3, a histone acetyltransferase, was demonstrated to be critical in acetylation of α-tubulin in cortical projection neurons, an event linked to neural differentiation and migration (Creppe et al., 2009). We surmise that GCN5 represents another acetyltransferase targeting tubulin. Deacetylation of α-tubulin is mediated by both HDAC6 and Sirt2 (Hubbert et al., 2002; Matsuyama et al., 2002; North et al., 2003). However the increase in acetylated α-tubulin by Myc-nick does not occur through modulation of HDAC activity (Figures S6E and S6F).

A connection between α-tubulin acetylation and calpain activation was previously suggested by two independent studies. First, calcium depletion, which inactivates calpains, was shown to decrease the levels of acetylated α-tubulin in epithelial cells (Ivanov et al., 2006). Second, the ectopic expression of calpain 6 causes microtubule stabilization, elevates the levels of acetylated α-tubulin, and impairs cytokinesis in HeLa cells (Tonami et al., 2007). Our findings suggest that the induction of α-tubulin acetylation by calpains could be mediated at least in part by Myc-nick and GCN5.

The Role of Myc-Nick in Terminal Differentiation

The role of Myc family members in differentiation is complex. Endogenous Myc is strongly downregulated during terminal differentiation of many cell types. Moreover, ectopic expression of Myc has been shown to block terminal differentiation. The

ability of Myc to negatively regulate differentiation is consistent with its role in maintaining pluripotency in ES cells and in the generation of induced pluripotent stem (iPS) cells (Cartwright et al., 2005; Takahashi et al., 2007). However, Myc has also been implicated in promoting both proliferation and differentiation in specific cellular contexts such as in progenitor cells of the skin (Gandarillas and Watt, 1997; Gebhardt et al., 2006), B lymphocytes (Habib et al., 2007), and hematopoietic stem cells (Wilson et al., 2004). Our data suggest that whereas full-length Myc blocks differentiation at the transcriptional level, Myc-nick may be involved in promoting differentiation through transcription-independent mechanisms. In agreement with this hypothesis there have been numerous reports of Myc antigenicity localized predominantly in the cytoplasm of differentiated cells (see Introduction). In addition, we show that ectopic expression of Myc-nick accelerates muscle cell differentiation and renders Rat1 *myc* null cells competent to differentiate into muscle following introduction of MyoD.

A number of studies have shown a strong correlation between muscle cell differentiation, calcium influx, and calpain activation (Dedieu et al., 2004; Kumar et al., 1992). In addition, limb girdle muscular dystrophy 2A (LGMD2A) is caused by mutations in calpain 3 that affect its activity (Richard et al., 1995). During the process of normal differentiation, myoblasts elongate and fuse into syncytial myotubes. An early event during this process is the remodeling of the microtubule cytoskeleton, involving disassembly of the centrosome and the alignment of stable microtubules into a parallel array along the long axis of the cell. We observed an increase in the levels of acetylated α-tubulin (an indicator of microtubule stabilization and cytoskeletal reorganization) and an elevation in Myc-nick abundance during muscle cell differentiation. Induction of α-tubulin acetylation and microtubule stabilization are likely to be important events in terminal differentiation especially when cells must establish and maintain a new shape. Acetylation of α-tubulin was also demonstrated to regulate cortical neuron differentiation and migration (Creppe et al., 2009).

We propose a model for the regulation of Myc by calpains and for the function of Myc-nick. During proliferation, full-length Myc is rapidly synthesized and transported to the nucleus where it transcriptionally activates growth- and proliferation-related genes and represses genes involved in differentiation. External cues that stimulate differentiation, such as cell-cell contact, and calcium influx can lead to the activation of calpains. Activated calpains interact with and may retain Myc in the cytoplasm where it is cleaved to produce Myc-nick. As the cleavage removes the nuclear localization sequence (NLS) and the DNA- and Max-binding domains, Myc-nick is predominantly cytoplasmic and transcriptionally inactive. Indeed we have shown that Myc-nick, when expressed in cells devoid of full-length Myc, does not stimulate proliferation, growth, or apoptosis—the transcription-dependent functions of full-length Myc. In such Myc-nick-expressing cells we instead observe morphological changes and increased tubulin acetylation, which are unique to Myc-nick. During muscle differentiation Myc-nick appears to be stabilized by dephosphorylation of threonine 58 within the Myc box I phosphodegron. We propose that one of the functions carried out by Myc-nick in the cytoplasm involves binding tubulin and recruiting GCN5 to mediate α-tubulin acetylation. Myc-nick is also likely to have additional functions and partners in the cytoplasm as the deletion of MBII in Myc-nick, which should block the interaction with GCN5, only partially reduced the ability of Myc-nick to promote muscle differentiation. We predict that the functions carried out by Myc-nick in the cytoplasm cooperate to promote cytoskeletal changes that can further drive terminal differentiation. In our model, the cleavage of Myc by calpains has two roles. First, it helps diminish nuclear Myc abundance preventing newly synthesized Myc from entering the nucleus, therefore eliminating the transcriptional blockade to differentiation caused by Myc. Second, the production of Myc-nick influences cytoskeletal organization and facilitates terminal differentiation. We predict that Myc-nick will play a general role linking *myc* to both nuclear functions and cytoplasmic organization during differentiation of a wide variety of cell types.

EXPERIMENTAL PROCEDURES

Retroviral Infection and Transfection

For retroviral production, 293T cells were cotransfected with pBabe-puro vector expressing Myc clones and amphotropic helper. Infected cells were selected with 2 μg/ml puromycin for 4 days. Rat1 *myc* null cells were harvested 10–14 days after infection and C2C12 cells were stimulated to differentiate after selection. For overexpression experiments, 293T (transfected with Fugene-Roche) cells were harvested 3 days after transfection with a change in culture medium 24 hr before harvesting. Sparse cultures (S) are about 20% confluent, medium cultures (M) are about 40%–60% confluent, and dense cultures (D) are grown as confluent cultures for 2–4 days.

Total Cell Lysates and Nuclear and Cytoplasmic Fractionation

For total extracts, cells were lysed in either boiling sample buffer or in RIPA buffer. For cellular fractionation, cells were lysed in buffer A on ice for 20 min, centrifuged for 3 min, and the supernatant employed as the cytoplasmic fraction. The pellet was resuspended in buffer B, rotated for 20 min, sonicated, and centrifuged for 10 min. The supernatant was employed as the nuclear fraction. Ten to twenty micrograms of total extracts or 10 μg of nuclear and 30 μg of cytoplasmic extracts were probed overnight with indicated antibodies. See Extended Experimental Procedures for composition of buffers.

Immunofluorescence

Cells were grown on glass coverslips and fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 5 min, and blocked with Image IT FX signal enhancer (Invitrogen) for 30 min. Primary and secondary antibodies were diluted in PBS at 1:200 and incubated for 1 hr.

In Vitro Cleavage of Myc

All cDNAs were cloned into pCS2^{+} and were transcribed from the SP6 promoter using the Promega wheat germ system, in the presence of cold (Promega) or ^{35}[S]-labeled methionine (Perkin Elmer) according to the manufacturer's instructions. For the in vitro cleavage experiments, Myc (1 μl IVT Myc or 1 μg recombinant Myc) was incubated with 30 μg of nuclear or cytoplasmic extracts in 20 μl of buffer G at 37°C and the samples were processed for autoradiography or western blot as specified. Nuclear and cytoplasmic fractions were dissolved in the same buffer and adjusted to identical salt concentration for these experiments. For Myc cleavage using recombinant calpains, 1 μl IVT c-Myc or 0.25 μg of purified c-Myc was incubated with the indicated amounts of calpains in the presence of 20 μl buffer G. Total calpain activity was measured using a Calbiochem kit.

In Vitro Tubulin Acetylation Assays

CE (30 μg) or purified assembled microtubules (1 μg) were incubated with 1 μl of IVT c-Myc or IVT vector or with recombinant Myc for 1 hr in the presence of

acetylation buffer. Assembled microtubules were incubated at 30°C and CE at 37°C.

See Extended Experimental Procedures for buffer composition, constructs, siRNA sequence, pharmacological inhibitors, antibodies, cell lines, and culture media.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.cell.2010.06.037.

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