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# The Ubiquitin Ligase HectH9 Regulates Transcriptional Activation by Myc and Is Essential for Tumor Cell Proliferation

**Sovana Adhikary,[1,6,7] Federica Marinoni,[2,6] Andreas Hock,[1,6] Esther Hulleman,[2,6] Nikita Popov,[1] Rudi Beier,[1] Sandra Bernard,[1] Micaela Quarto,[3] Maria Capra,[3] Stephan Goettig,[1] Ulrike Kogel,[5] Martin Scheffner,[5] Kristian Helin,[2,4,*] and Martin Eilers[1,*]**

[1]Institute for Molecular Biology and Tumor Research
University of Marburg
Emil-Mannkopff-Str.2
35033 Marburg
Germany

[2]European Institute of Oncology
Via Ripamonti 435
20141 Milan
Italy

[3]The FIRC Institute for Molecular Oncology
Via Adamello 16
20135 Milan
Italy

[4]Biotech Research & Innovation Centre
Fruebjergvej 3
2100 Copenhagen
Denmark

[5]Department of Biology
University of Konstanz
78457 Konstanz
Germany

## Summary

**The Myc oncoprotein forms a binary activating complex with its partner protein, Max, and a ternary repressive complex that, in addition to Max, contains the zinc finger protein Miz1. Here we show that the E3 ubiquitin ligase HectH9 ubiquitinates Myc in vivo and in vitro, forming a lysine 63-linked polyubiquitin chain. Miz1 inhibits this ubiquitination. HectH9-mediated ubiquitination of Myc is required for transactivation of multiple target genes, recruitment of the coactivator p300, and induction of cell proliferation by Myc. HectH9 is overexpressed in multiple human tumors and is essential for proliferation of a subset of tumor cells. Our results suggest that site-specific ubiquitination regulates the switch between an activating and a repressive state of the Myc protein, and they suggest a strategy to interfere with Myc function in vivo.**

## Introduction

The proto-oncogene *c-myc* encodes a transcription factor (Myc) that can both activate and repress transcription (Eisenman, 2001). Myc activates transcription as part of a binary complex with its partner protein, Max. The complex binds to specific DNA sequences, termed E boxes, and stimulates transcription of a large group of genes (Adhikary and Eilers, 2005). Myc represses transcription through the formation of a ternary complex that contains Max and the zinc-finger transcription factor Miz1. Free Miz1 binds to the core promoter of multiple Myc-repressed genes and activates these genes. Myc is recruited to these sites through interaction with Miz1 and represses transcription when tethered to Miz1.

Myc is a short-lived protein that is degraded through the ubiquitin-proteasome pathway (Salghetti et al., 1999). Two F box E3 ligases ubiquitinate Myc and contribute to Myc degradation: Fbw7 recognizes Myc that is phosphorylated at threonine 58 by glycogen synthase kinase 3 (Gsk3) (Welcker et al., 2004; Yada et al., 2004). Inhibition of Gsk3 by PI3-kinase or mutations of Myc at T58, which are found in lymphoma patients, stabilizes Myc (Yeh et al., 2004). The second E3 ligase, Skp2, binds to a conserved sequence element in the amino terminus of Myc (MycboxII), which is essential for transformation and transcriptional regulation. The signals that regulate interaction of Skp2 with Myc are currently unknown (Kim et al., 2003; von der Lehr et al., 2003).

Ubiquitination of transcription factors can control their activity independently of proteasomal degradation. For example, Met4, a bZIP factor that regulates a large number of genes predominantly involved in methionine biosynthesis, is ubiquitinated but not degraded in the presence of high intracellular levels of S-adenosylmethionine (Kaiser et al., 2000). Ubiquitination inactivates Met4 at least in part because it precludes recruitment of the coactivator, Cbf1 (Kaiser et al., 2000); in addition, binding of Met4 to a subclass of its target promoters is compromised by ubiquitination (Kuras et al., 2002). Ubiquitination does not necessarily inhibit transcription factors since ubiquitination of the HIVTat protein by Mdm2 augments its ability to activate transcription (Bres et al., 2003). Similarly, ubiquitination of Myc by Skp2 contributes to transcriptional activation, potentially by allowing Myc to recruit proteasomal subunits that have a proteolysis-independent role in transcriptional activation (Ferdous et al., 2001). Two signals are known to determine whether ubiquitination leads to degradation. Proteolytic substrates are modified by polyubiquitin chains, and a minimum chain length of about four ubiquitin residues appears to be required to target the attached protein to the proteasome (Flick et al., 2004). The lysine residue of ubiquitin, used for polyubiquitin chain formation, specifies the second signal. Whereas chains linked through lysine 48 usually lead to proteasomal degradation, those linked through lysine-63 of ubiquitin do not target proteins to the proteasome (Bres et al., 2003).

Here we describe a Hect-domain (*h*omologous to *E*6-AP *c*arboxy-*t*erminus; Huibregtse et al., 1995) ubiquitin ligase, which ubiquitinates Myc in a carboxy-terminal

*Correspondence: kristian.helin@bric.dk (K.H.); eilers@imt.uni-marburg.de (M.E.)
[6]These authors contributed equally to this work.
[7]Present address: Klinikum der Philipps Universität Marburg, Klinik für Hämatologie, Onkologie, und Immunologie, Marburg, Germany.

domain required for recruitment of the coactivator, p300. Our data support the hypothesis that the switch between transcriptional activation and repression by Myc is regulated by site-specific ubiquitination. HectH9 is overexpressed in multiple human tumors and is required for the proliferation of a subset of human tumor cells, closely paralleling the requirement for Myc among different cell lines. Our data suggest that interference with HectH9 provides a potential strategy to inhibit Myc function in human tumors.

## Results

### HectH9 Binds to Myc and Miz1 In Vivo

Miz1 is a zinc-finger transcription factor containing an amino-terminal POZ domain, which is a protein/protein interaction domain present in many zinc-finger proteins (Bardwell and Treisman, 1994). The POZ domain of Miz1 is required for transcriptional activation of the *p15ink4b* and *p21cip1* promoters and for suppression of cell proliferation (Herold et al., 2002).

To better understand the function of the Miz1 POZ domain, we performed a two-hybrid screen to identify novel interaction partners of Miz1. In this screen, we obtained multiple overlapping clones from a single gene, encoding a protein alternatively named HectH9, Lasu1, or KIAA0312 (Figure 1A). HectH9 belongs to the Hect-domain family of ubiquitin ligases ("*h*omologous to *E*6AP *c*arboxyl *t*erminus"), which are characterized by a conserved carboxy-terminal catalytic domain (Huibregtse et al., 1995). A protein corresponding to the carboxyl terminus of HectH9 has also been described as a DNA binding protein (Ureb1; Upstream-element binding protein 1; Gu et al., 1994); however, we have been unable to confirm a significant DNA binding activity of HectH9 (R.B., unpublished data). A human cDNA encoding a protein of 4374 amino acids was assembled from several EST clones. RT-PCR using primers against four different regions of the predicted cDNA confirmed that these sequences are indeed part of the same gene (Figure S1). To determine whether this cDNA encodes the full-length HectH9 protein, we transfected U2OS cells with a CMV-driven expression vector encoding an HA-tagged HectH9 protein (Figure 1B). Immunoblotting using antibodies directed against the HA-tag detected a protein of approximately 500 kDa in transfected cells. Both a polyclonal and different monoclonal antibodies raised against different regions of HectH9 detected an endogenous protein of 500 kDa in lysates of multiple human cell lines, which comigrated with the protein expressed from the cloned cDNA (Figure 1B). Based on these data, we conclude that we have isolated the full-length open reading frame of HectH9.

To test whether HectH9 binds to Miz1 in vitro, Miz1 and an amino-terminally truncated allele of HectH9 (ΔNHectH9, see below) were synthesized by coupled transcription-translation in a reticulocyte lysate in the presence of $^{35}$S-methionine (Figure 1C). After synthesis, lysates were mixed and immunoprecipitated either with antibodies directed against HectH9 or a control serum. Miz1 was present in HectH9 immunoprecipitates, whereas control precipitates contained much lower levels of Miz1. To test whether the POZ domain of Miz1 is required for binding, the experiment was repeated with ΔPOZ-Miz1. No ΔPOZ-Miz1 was recovered in HectH9 immunoprecipitates. Conversely, ΔNHectH9 was present in α-Miz1 immunoprecipitates when wtMiz1, but not when ΔPOZMiz1, was cotranslated with ΔNHectH9. Together, the data demonstrate that the POZ domain of Miz1 mediates binding to HectH9 in vitro. We also tested whether ΔNHectH9 binds to Myc in vitro and did not detect a stable interaction between ΔNHectH9 and Myc in these experiments.

To test whether Miz1 can bind to HectH9 in vivo, we transfected HeLa cells with expression vectors encoding Miz1 and HectH9. Since we were unable to consistently overexpress the full-length, 500 kDa HectH9 protein, all subsequent transient transfections were carried out using an expression plasmid encoding a protein corresponding to amino acids 2473–4374 of full-length HectH9 (ΔNHectH9). When coexpressed with ΔNHectH9, Miz1 was recovered in HectH9 immunoprecipitates. In contrast, control immunoprecipitates or HectH9 immunoprecipitates obtained from cells, which did not express ΔNHectH9, did not contain Miz1 (Figure 1D). No specific binding of Miz1ΔPOZ to ΔNHectH9 could be detected in vivo, consistent with the in vitro and two-hybrid data (not shown). When ΔNHectH9 and Myc were coexpressed, HectH9 immunoprecipitates contained significant amounts of Myc (Figure 1E). Myc was not recovered in control immunoprecipitates or in HectH9 immunoprecipitates from cells that did not express ΔNHectH9, demonstrating that Myc binds to ΔNHectH9 in vivo. Since no stable binding of Myc to ΔNHectH9 was detected in vitro, it is likely that additional proteins stabilize binding of Myc to ΔNHectH9 in vivo. We wondered whether endogenous HectH9 interacts with Miz1 and Myc and immunoprecipitated HeLa cell lysates with either α-HectH9 or control antibodies. Both Myc and Miz1 were coprecipitated with α-HectH9 antibodies but were absent from control immunoprecipitations (Figure 1F). Based on these results, we conclude that HectH9 interacts with both endogenous Miz1 and Myc in vivo. To test whether all three proteins form a ternary complex, we transfected cells with constant amounts of Myc and ΔNHectH9 and increasing amounts of Miz1 (Figure 1G). In these experiments, the amount of Myc recovered in HectH9 immunoprecipitates decreased with increasing amounts of Miz1, strongly suggesting that Myc and Miz1 compete for binding to HectH9.

### HectH9 Catalyzes K-63-Mediated Ubiquitination of Myc

To test whether HectH9 ubiquitinates either Miz1 or Myc, we transfected cells with expression plasmids encoding Myc, Miz1, ΔNHectH9, and a histidine-tagged ubiquitin. Transfected cells were lysed and ubiquitinated proteins were isolated by binding to Ni-agarose. Immunoblots of the eluates were probed with antibodies directed against Miz1 and Myc (Figure 2A and data not shown). In these experiments, we did not detect any activity of ΔNHectH9 toward Miz1 (data not shown). In contrast, ΔNHectH9 efficiently ubiquitinated Myc. Ubiquitinated forms of Myc accumulated up to a molecular weight of 250 kDa, demonstrating that ΔNHectH9 can

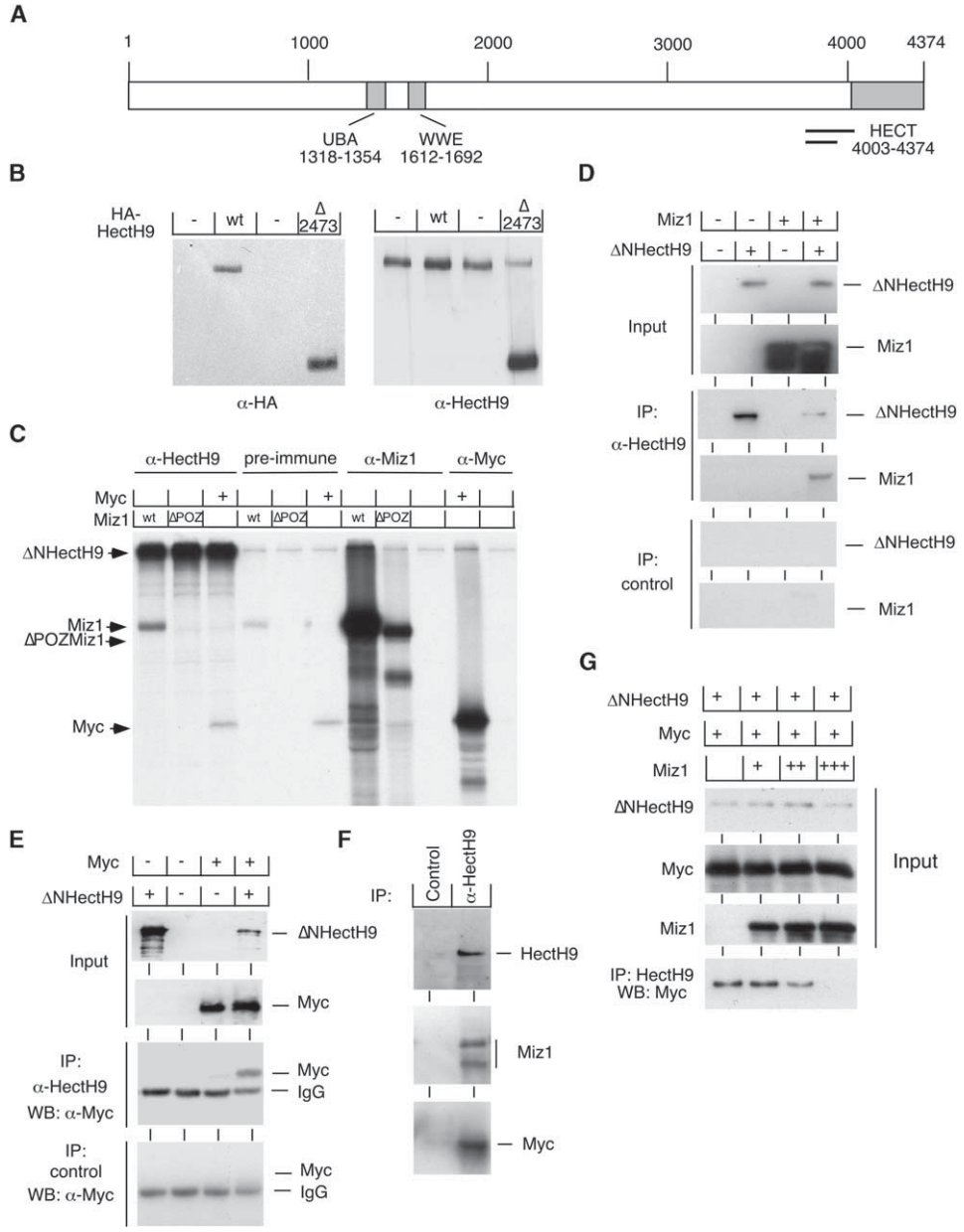



Figure 1. Myc and Miz1 Bind to HectH9

(A) Schematic diagram of HectH9. The diagram shows the carboxy-terminal catalytic domain (HECT) and the UBA and WWE domains. The sequences corresponding to the clones recovered in the two-hybrid screens are underlined. The full-length ORF of HectH9 is 4,374 amino acids. Numbers refer to the amino acids of HectH9.

(B) Comigration of endogenous and transiently expressed HectH9 proteins. U2OS cells were transfected with expression plasmids encoding HA-tagged cDNAs for full-length HectH9 or a truncated allele (2473–4374; ΔNHectH9). The blot on the left was probed with an HA antibody; the blot on the right was probed with a monoclonal antibody directed against HectH9.

(C) In vitro interaction of ΔNHectH9 and Miz1. Miz1, ΔPOZMiz1, and Myc were translated in a reticulocyte lysate in the presence of $^{35}$S-methionine; after synthesis, the lysates containing the proteins indicated at the top were mixed and precipitated with the indicated antibodies. Lysate containing ΔNHectH9 was present in all lanes. Shown is an autoradiogram of the precipitates.

(D) In vivo interaction of ΔNHectH9 and Miz1. HeLa cells were transiently transfected with expression plasmids encoding Miz1 and ΔNHectH9 as indicated; lysates were immunoprecipitated with either α-HectH9 or control antibodies. The blots were probed with antibodies against Miz1 and HectH9. The input corresponds to 10% of the lysate used for the immunoprecipitation.

(E) In vivo interaction of ΔNHectH9 and Myc. The experiment was carried out as in (D). The input corresponds to 7,5% of the lysate used for immunoprecipitation.

(F) Endogenous HectH9 interacts with Myc and Miz1. Lysates of HeLa cells were immunoprecipitated with antibodies directed against HectH9 or control antibodies. Immunoprecipitates were resolved on an SDS gel and probed with the indicated antibodies.

(G) Myc and Miz1 compete for binding to ΔNHectH9. HEK 293 cells were transfected with constant amounts of expression vectors encoding ΔNHectH9 and Myc and increasing amounts of a vector encoding Miz1. Cell lysates were immunoprecipitated with α-HectH9 antibodies and precipitates probed with α-Myc antibodies.

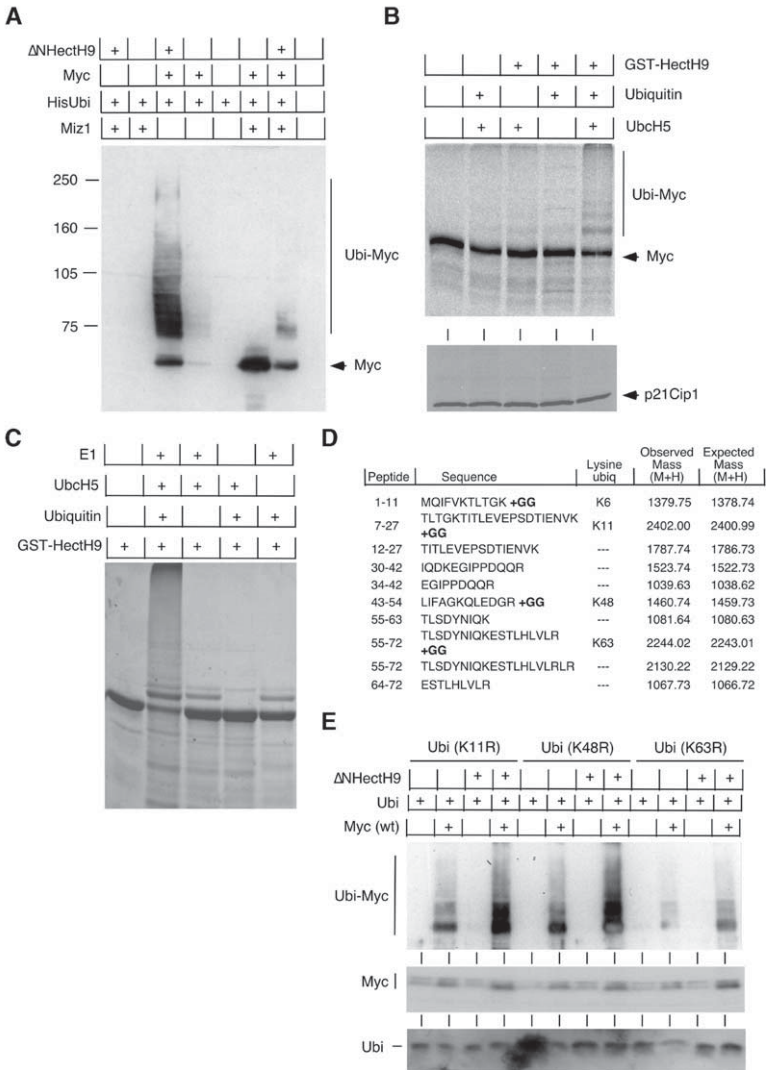



Figure 2. HectH9 Ubiquitinates Myc In Vivo and In Vitro

(A) In vivo ubiquitination of Myc by HectH9. HeLa cells were transfected with expression plasmids encoding the indicated proteins. Lysates were precipitated with Ni-agarose, and the precipitates were probed with antibodies against Myc.

(B) HectH9-mediated ubiquitination of Myc in vitro. In vitro-translated radio-labeled Myc (upper panel) or p21Cip1 (lower panel) was incubated with bacterially expressed GST-HectH9 (3228–4374) in the absence or presence of ubiquitin and the E2 protein UbcH5, as indicated.

(C) Auto-ubiquitination of GST-HectH9. The panel shows a Coomassie-stained gel of in vitro incubations using E1, UbcH5, HectH9, and ubiquitin as indicated.

(D) Mass-spectrometry of polyubiquitinated HectH9; ubiquitinated peptides derived from ubiquitin were identified by a mass increment of +114 Da, corresponding to the two C-terminal glycine residues of ubiquitin (+GG) that remain covalently attached to the respective lysine residue after trypsin digestion. The sequences of the identified peptides (the respective amino acid residues of ubiquitin are indicated) and the observed and the expected mass of the respective peptide are shown.

(E) Ubiquitination of Myc by HectH9 was carried out as in (A) in the presence of either wild-type ubiquitin or the indicated ubiquitin mutants.

catalyze the transfer of multiple ubiquitin moieties on Myc in vivo. Coexpression of Miz1 inhibited ubiquitination of Myc, most likely because of competition for binding to ΔNHectH9 (Figures 1G and 2A).

A mutant allele of ΔNHectH9, in which the catalytic cysteine residue is replaced with a serine, had a decreased ability to ubiquitinate Myc in vivo, suggesting that HectH9 itself is a catalytically active E3-ligase toward Myc (data not shown). To test whether HectH9 ubiquitinates Myc in vitro, we expressed a GST-fusion protein corresponding to amino acids 3228–4374 of full-length HectH9 in *E. coli*. Expression of the corresponding protein in HeLa cells led to efficient ubiquitination of Myc in vivo (data not shown). When incubated with in vitro-translated $^{35}$S-labeled Myc, purified GST-HectH9 efficiently ubiquitinated Myc in the presence of the E2 enzyme UbcH5 (Figure 2B, upper panel). In contrast, GST-HectH9 did not ubiquitinate p21Cip1, suggesting that the reaction toward Myc is specific (Figure 2B, lower panel). We note that in vitro ubiquitination of Myc by HectH9 does not require the formation of a complex that is stable enough for coimmunoprecipitation (see Figure 1B); similar observations have been reported for other E3-ligase/substrate interactions (Linares et al., 2003).

We noted that GST-HectH9 polyubiquitinated itself in vitro (Figure 2C). Mass spectrometry revealed that the ubiquitin moieties in these chains were linked through K48, K11, and K63 to each other, demonstrating that HectH9 is capable of assembling different types of polyubiquitin chains (Figure 2D). In vitro reactions showed that a point mutant of ubiquitin, in which K48 is replaced by arginine (UbiK48R), was efficiently ligated to Myc; conversely, a mutant that retained K48 but replaced all other lysines by arginine was not ligated to Myc (data not shown). In vivo, UbiK48R was ligated to Myc as efficiently as wild-type ubiquitin (Figure S2). In order to determine the type of polyubiquitin chain that HectH9 transfers to Myc in vivo, we performed ubiquitination assays using specific point mutants of his-tagged ubiquitin, in which either K11, K48, or K63 is replaced by arginine (UbiK11R, UbiK48R, and UbiK63R) (Figure 2F). Both UbiK11R and UbiK48R were ligated to Myc HectH9 as efficiently as wt ubiquitin; in

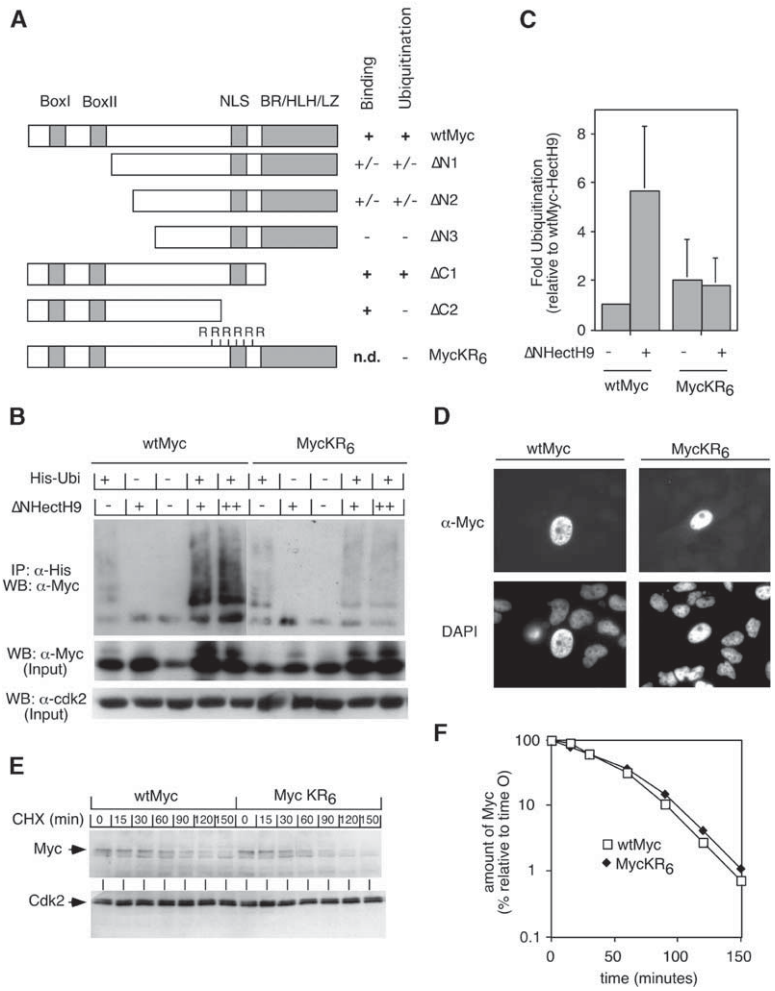



Figure 3. HectH9 Catalyzes Ubiquitination of Carboxyl-Terminal Lysines of Myc

(A) Summary of interaction and ubiquitination data for different Myc mutants. The panel shows a schematic drawing of the Myc mutants used in these experiments, indicated two conserved Mycboxes, the carboxy-terminal BR/HLH/LZ-domain and the nuclear localization signal (NLS). The symbols on the right indicated whether the corresponding mutant protein interacts with HectH9 and is ubiquitinated by ΔNHectH9.

(B) A mutant of Myc that has six lysines replaced by arginine ($MycKR_6$) shows reduced ΔNHectH9-mediated ubiquitination. The experiment was carried out as in Figure 2A. The top panel shows Ni-agarose precipitates of lysates of transfected cells, which were probed with an antibody directed against Myc. The lower panels show immunoblots of aliquots of the transfected cells with α-Myc and α-Cdk2 antibodies showing equal expression of Myc and $MycKR_6$ after transfection.

(C) Quantitation of ubiquitination of Myc and $MycKR_6$. The amount of ubiquitinated wtMyc in the absence of HectH9 was arbitrarily set to one. Data represent the mean ± SEM of three independent experiments.

(D) Indirect immunofluorescence pictures of transiently transfected HeLa cells documenting nuclear localization of both wtMyc and $MycKR_6$.

(E) Unaltered stability of Myc and $MycKR_6$. NIH3T3 cells were infected with retroviruses expressing either Myc or $MycKR_6$. Cells were treated with cycloheximide for the indicated times and lysates were probed with antibody 9E10, which is specific for human Myc.

(F) Quantitation of the results shown in (E).

contrast, HectH9 did not transfer UbiK63R to Myc. We concluded that HectH9 catalyzes the assembly of K63-linked polyubiquitin chains on Myc in vivo.

### Ubiquitination of Myc Is Required for the Transcriptional Activation of a Subset of Myc Target Genes

K63-linked polyubiquitin chains do not target proteins for proteasomal degradation; they instead regulate the function of the modified protein (Bres et al., 2003). As one approach to assess the functional consequences of HectH9-mediated ubiquitination of Myc, we mapped the lysine residues in Myc that are targeted by HectH9 (Figure 3). To do this, we measured in parallel binding to ΔNHectH9 and ubiquitination by ΔNHectH9 of a series of both N-terminal and C-terminal deletion mutants of Myc (Figures 3A and S3). In these experiments, full-length Myc bound efficiently to ΔNHectH9 and was efficiently ubiquitinated by ΔNHectH9, as described above (Figure 3A). Increasingly larger deletions from the amino terminus led to a progressive loss of both binding and ubiquitination, suggesting that the amino terminus of Myc is required for binding to ΔNHectH9 in vivo and that the loss of ubiquitination of amino-terminally deleted mutants of Myc by ΔNHectH9 is an indirect consequence of the reduction in binding of such proteins. In contrast, the analysis of two carboxy-terminal deletions (ΔC1Myc and ΔC2Myc) identified a region that was required for ubiquitination by ΔNHectH9 but not for binding, suggesting that it might contain the target lysines for ΔNHectH9. We therefore replaced the six lysines in this region by arginine in full-length Myc, generating $MycKR_6$, and repeated the ubiquitination assays using wtMyc and $MycKR_6$ in parallel. Ubiquitination of $MycKR_6$ by ΔNHectH9 was significantly reduced relative to wtMyc (Figures 3B and 3C). We conclude that HectH9 ubiquitinates Myc at least in part on one or more of the six lysine residues that are replaced by arginine in $MycKR_6$.

Immunofluorescence of transfected cells revealed that both wtMyc and $MycKR_6$ localized to the cell nucleus, suggesting that ubiquitination does not affect nuclear localization of Myc (Figure 3D). To test whether ubiquitination of Myc at these sites regulates Myc stability, we infected NIH3T3 cells with recombinant retroviruses that express wtMyc or $MycKR_6$. Pools of infected cells were treated with cycloheximide for different lengths of time (Figures 3E and 3F). Cell lysates were prepared and immunoblots were probed with the monoclonal antibody 9E10, which specifically recog-

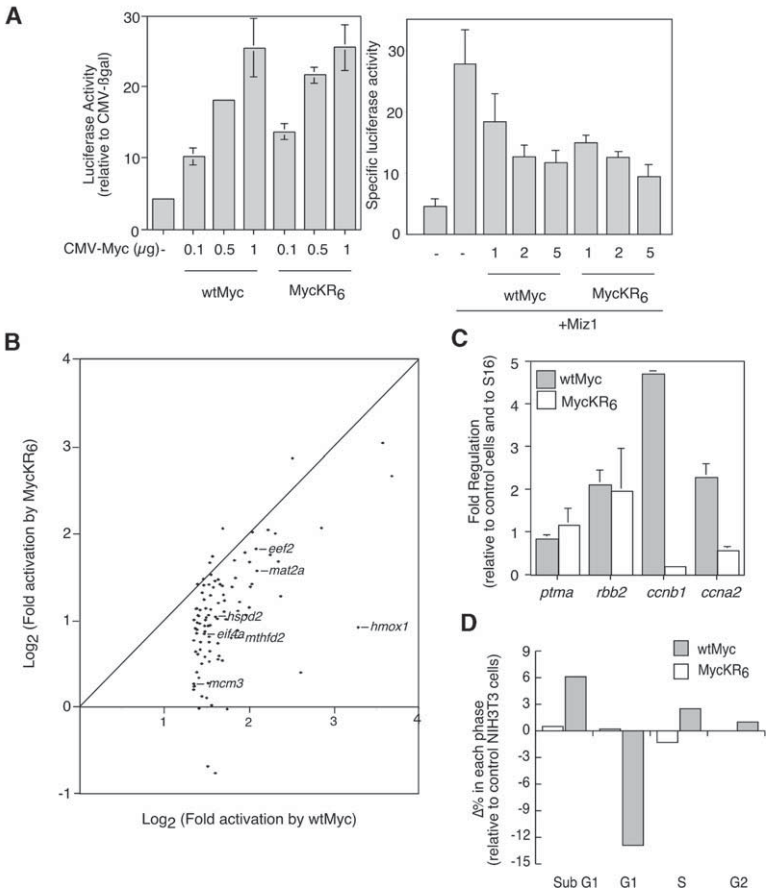



Figure 4. Biological Properties of Myc and $MycKR_6$

(A) Transient transfection assays demonstrating transcriptional activation of an E box-dependent reporter plasmid and repression of a Miz1-dependent reporter plasmid by Myc and $MycKR_6$. Data represent the mean ± SEM from three independent transfections for each condition.

(B) Relative expression levels of endogenous Myc target genes. NIH3T3 cells were infected with retroviruses expressing either Myc or $MycKR_6$ and serum-starved for 48 hr to deplete the endogenous Myc proteins. RNA was isolated and subjected to gene expression analysis. Shown are expression levels of the 100 most strongly Myc-induced genes, plotted for wtMyc on the *x* axis and for $MycKR_6$ on the *y* axis. The values are the $\log_2$ values of the expression in cells expressing wtMyc or $MycKR_6$ relative to control-infected cells.

(C) Real-time PCR analysis of selected target genes of Myc showing differential regulation of some, but not all, targets of activation by Myc and $MycKR_6$. Data represent the mean ± SEM from three determinations for each gene.

(D) FACS-analysis demonstrating induction of cell-cycle progression and apoptosis by Myc, but not $MycKR_6$, in serum-starved NIH3T3 cells.

nizes human Myc. In these experiments, we observed that the rate of degradation of wtMyc and $MycKR_6$ was identical (Figures 3E and 3F). To determine how ubiquitination affects Myc function, we initially performed transient reporter assays using an E box-dependent reporter plasmid derived from the prothymosin-α (*PTMA*) gene, a target for activation by Myc, and a reporter plasmid carrying the core *P15INK4B* promoter, a target for Miz1-dependent repression by Myc (Figure 4A). In these transient assays, we observed no difference between wtMyc and $MycKR_6$ in their ability to either activate or repress transcription. Thus, mutation of the six lysines to arginines in $MycKR_6$ does not lead to complete loss of function.

To determine whether $MycKR_6$ is affected in regulating Myc target genes, we performed a gene expression analysis of pools of mouse NIH3T3 fibroblasts infected with retroviruses expressing either wtMyc or $MycKR_6$. Immunoblots confirmed that the two cell pools expressed similar levels of Myc protein (Figure 3E). Cells were serum-starved for 48 hr to deplete endogenous Myc, and RNA was subsequently extracted and used to perform for gene-expression analysis (Figure 4B). Analysis of the entire dataset revealed no significant differences in the overall expression of all genes on the array (data not shown). In contrast, analysis of the genes that were induced by wtMyc relative to control cells showed that $MycKR_6$ activated the majority of these genes less efficiently than wtMyc (Figures 4B and S4). Analysis of the public database of Myc target genes revealed that this group of genes contained multiple known target genes of Myc (Figure S4). Functional annotation revealed that genes encoding proteins involved in cell proliferation, in cellular metabolism, and in protein synthesis were expressed at significantly lower levels in cells expressing $MycKR_6$ than in cells expressing wtMyc (Figures 4B and S4). Real-time PCR analysis confirmed that $MycKR_6$ activated some target genes to the same extent as wtMyc (see *rbb2*) but did not activate another subset of target genes that are activated by wtMyc (shown for *ccna2* und *ccnb1* in Figure 4C). Taken together, the data show that $MycKR_6$ is deficient in activation of a significant subset of Myc target genes in vivo. Consistent with these differences in transcriptional regulation, $MycKR_6$ was significantly less effective in promoting proliferation of serum-starved NIH3T3 cells (Figure 4D).

We speculated that the failure of $MycKR_6$ to activate endogenous target genes despite its ability to activate transient reporter plasmids reflected the failure to recruit a coactivator. Several potential coactivators of Myc have been described, most of which bind to the amino terminus of Myc, not immediately adjacent to the target lysines for HectH9 (Figure 5A). In contrast, the p300 and CBP histone acetyltransferases bind to the carboxyl terminus of Myc, and we therefore speculated

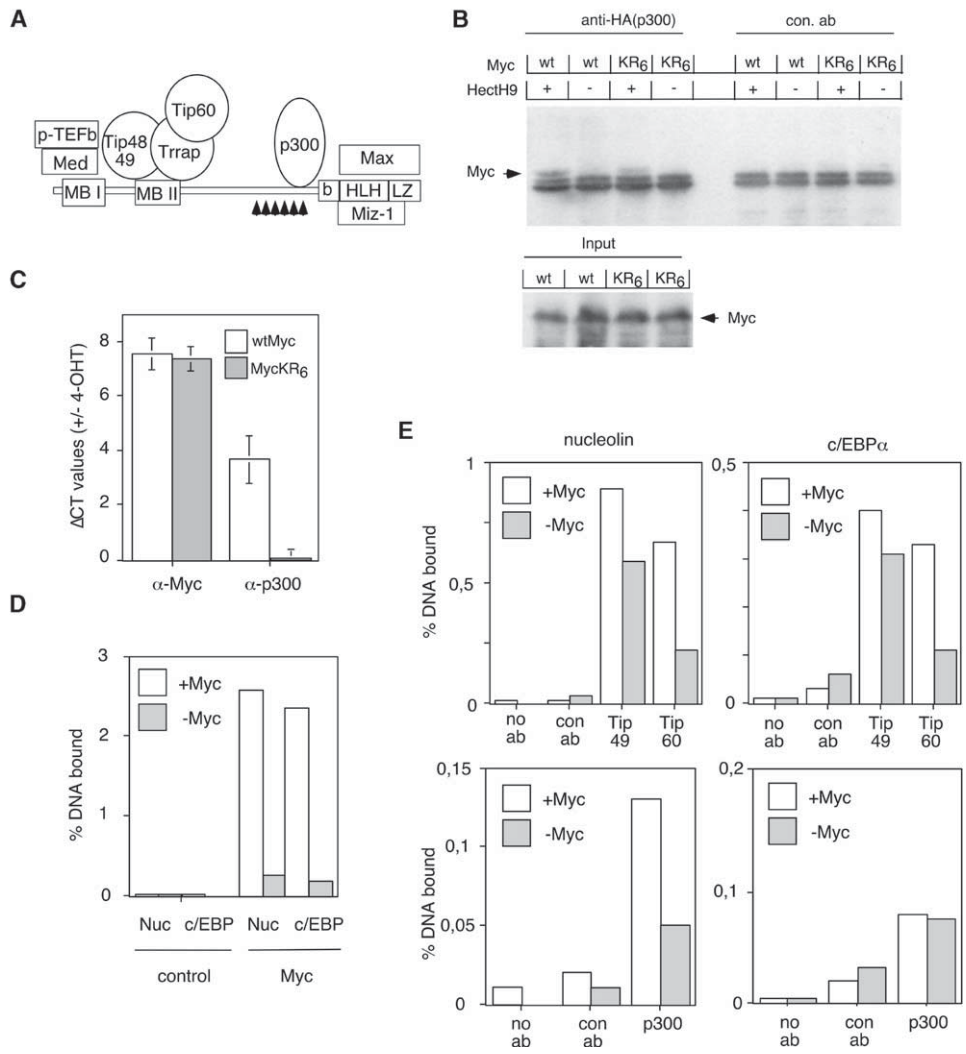



Figure 5. Differential Recruitment of p300 by Myc and MycKR$_6$

(A) Schematic diagram of Myc with known cofactors. Of the known coactivators, only binding of p300/CBP occurs in the carboxyl terminus of Myc.

(B) HectH9 stimulates binding of Myc to p300. HeLa cells were transfected with expression plasmids encoding HA-tagged p300, Myc or MycKR$_6$, and HectH9 where indicated. Lysates were precipitated either with α-HA or control antibodies and probed with antibodies directed against Myc. The bottom panel shows an input control.

(C) wtMyc but not MycKR$_6$ recruits p300 to an E box in vivo. NIH3T3-MycER and MycKR6ER cells were serum-starved for 48 hr and restimulated by addition of 200 nM 4-OHT for 6 hr. Chromatin immunoprecipitation experiments were performed with α-Myc and α-p300 antibodies and analyzed by real-time PCR using primers specific for the E box in the nucleolin promoter. The panel shows the change in CT values upon addition of 4-OHT for each antibody; the data represent the mean +/– SEM from three independent determinations.

(D and E) Myc recruits p300 selectively to E Boxes, not to Miz1 binding sites in vivo. Shown are chromatin-immunoprecipitation (ChIP) experiments with the indicated antibodies from a B cell line harboring a tetracyclin-regulatable Myc gene. ChIPs were carried out either in the presence or absence of Myc with the indicated antibodies. The percentage DNA bound was calculated from real-time PCR amplifying the precipitates with primers for the indicated genes. Data represent the average of two determinations.

that ubiquitination of Myc might be required for efficient recruitment of p300 (Vervoorts et al., 2003). To test this hypothesis, we cotransfected cells with HA-tagged p300 and either wtMyc or MycKR$_6$, either by itself or in the presence of HectH9. Lysates were immunoprecipitated with antibodies against the HA-tag of p300, and the precipitates probed with antibodies directed against Myc (Figure 5B). In these experiments, efficient complex formation between wtMyc and p300 was observed in the presence of HectH9 but not in its absence. The efficiency of complex formation was reduced for MycKR$_6$, suggesting that the lysine residues need to be ubiquitinated for complex formation to occur.

To demonstrate that MycKR$_6$ is deficient in recruitment of p300, we performed chromatin immunoprecipitation from cells expressing either MycER or MycKR$_6$ER using primers that measure occupancy at the E box localized in the nucleolin promoter, a target of E box-dependent activation by Myc (Figure 5C). Upon addition of 4-OHT, both MycER and MycKR$_6$ER effi-

ciently bound to the *nucleolin* promoter. In contrast, p300 was recruited by MycER but not by Myc$KR_6$ER. The data suggest that Myc$KR_6$ is deficient in transactivation of a subset of target genes because binding of Myc to p300 requires ubiquitination at one or more of the six lysines targeted by HectH9.

Miz1 inhibits ubiquitination of Myc by HectH9 (Figure 2A). If recruitment of p300 by Myc depends on ubiquitination by HectH9, then Myc should recruit p300 selectively to E box elements but not to Miz1 sites. We tested this model using a cell line that carries a tetracycline-inducible Myc (Schuhmacher et al., 2001). Chromatinimmunoprecipitations were performed before and after induction of Myc. As targets, we chose *nucleolin* and *c/EBP*$\alpha$, a Miz1-dependent target of repression by Myc, since initial experiments had shown that the amounts of Myc bound to both loci are comparable to each other and higher than for all other genes tested (Figure 5D). Addition of tetracycline led to a similar increase of Myc binding at both loci. Induction of Myc led to enhanced binding of the histone acetyl transferase Tip60, which is recruited to MycboxII domain as part of the TRRAP complex, at both loci (Figures 5D and 5E; Frank et al., 2003). Similarly, enhanced binding of Tip49, which also interacts with MycboxII, was seen at both the *nucleolin* and *c/EBP*$\alpha$ promoters, although the effects were not as strong (Wood et al., 2000). The data suggest that cofactors, which interact with MycboxII, are recruited to both E box targets and Miz1 binding sites in vivo. In contrast and consistent with our model, p300 is recruited to the *nucleolin* promoter but not to the *c/EBP*$\alpha$ promoter upon expression of Myc.

### HectH9 Is Required for Myc Function In Vivo

The phenotypes of the Myc$KR_6$ mutant might reflect the requirement for ubiquitination by HectH9 or, alternatively, reflect a HectH9-independent requirement for lysine residues at these sites. We therefore used shRNA vectors and siRNA to deplete HectH9 to provide independent evidence that HectH9 is required for Myc function in vivo.

We infected U2OS cells that express a MycER protein with either control retroviruses or retroviruses that direct the synthesis of shRNA molecules targeting HectH9. We designed three different shRNAs to HectH9; immunoblotting revealed that each of these viruses reduced the amount of endogenous HectH9 protein relative to control cells but did not affect expression of the MycER protein (Figure 6A). Two experiments were performed to assess how depletion of HectH9 affects Myc function: First, we activated MycER by addition of 4-OHT in serum-starved cells and isolated RNA either before or 24 hr after activation; we then performed a microarray experiment comparing gene induction by Myc in control to HectH9-depleted cells (Figures 6B and S5). Depletion of HectH9 by either of the shRNAs reduced the transactivation of many but not all target genes of Myc, consistent with the phenotype of the $KR_6$ mutant. While the genes that were most strongly induced by Myc in U2OS cells differed from those that were most strongly induced in NIH3T3 fibroblasts, the genes that were induced in U2OS cells again contained several known target genes of Myc, suggesting that depletion of HectH9 affected transcriptional activation of Myc; in addition, comparison of the array experiments revealed a significant number of genes that were affected both by $KR_6$ mutation and by depletion of HectH9, arguing that both affect Myc-dependent transactivation in a similar manner (Figures S6 and S7). In a second set of experiments, we compared the cell-cycle distribution of MycER cells to MycER cells depleted of HectH9 (Figure 6C). Activation of MycER promoted cell-cycle entry in serum-starved control cells. In contrast, depletion of HectH9 led to an accumulation of cells in the G1 phase of the cell cycle and abrogated Myc-induced cell-cycle entry (Figure 6C). Taken together, the data strongly suggest that HectH9 is required for target gene activation by Myc.

To extend these findings to the function of endogenous Myc in human tumor cells, we depleted HectH9 using siRNA oligonucleotides in HeLa cells, which express high levels of Myc (Figure 6D). Depletion of HectH9 did not affect the expression or stability of Myc (Figure 6D). To assess how depletion of HectH9 affects Myc function in these cells, we performed a microarray comparing cells depleted for HectH9 with control cells. Since we did not know which genes are regulated by endogenous Myc in these cells, we used a public database (http://www.myc-cancer-gene.org/) to identify known Myc target genes that were detectable on the microarray. Of these, 78 were downregulated by depletion of HectH9 (Figure S8); real-time PCR analysis confirmed the decrease in expression for individual Myc target genes (Figure 6E).

As in U2OS-MycER cells, depletion of HectH9 strongly inhibited proliferation and led to an accumulation of HeLa cells in the G1 phase of the cell cycle (Figure 6F). To determine whether this effect was due to inhibition of Myc function, we made use of the observation that deletion of Mnt, an antagonist of transactivation by Myc, can alleviate the requirement for transactivation by Myc in cell-cycle progression (Walker et al., 2005). We therefore used retroviral shRNA vectors to either deplete HectH9 or codeplete endogenous Mnt together with HectH9 in HeLa cells (Figure 6G). shRNA vectors against HectH9, but not control vectors, completed suppressed colony formation in HeLa cells. Depletion of Mnt did not enhance colony formation by itself, but it partially relieved the requirement for HectH9. Taken together, the data show that HectH9 is required for transactivation by MycER proteins and for transactivation by endogenous Myc proteins in human tumor cells.

### HectH9 Is Overexpressed in a Large Number of Primary Tumors

Deregulation of Myc expression contributes to the genesis of a large number of human tumors. We speculated, therefore, that the expression of HECTH9 might similarly be enhanced in human tumors. To test this, we performed tissue microarray experiments using multiple samples from primary colon patients of different stages (Figure 7A). In these experiments, expression of *HECTH9* closely correlated with tumor stage; while it was undetectable or expressed at very low levels in normal epithelium and in polyps, moderate or high ex-

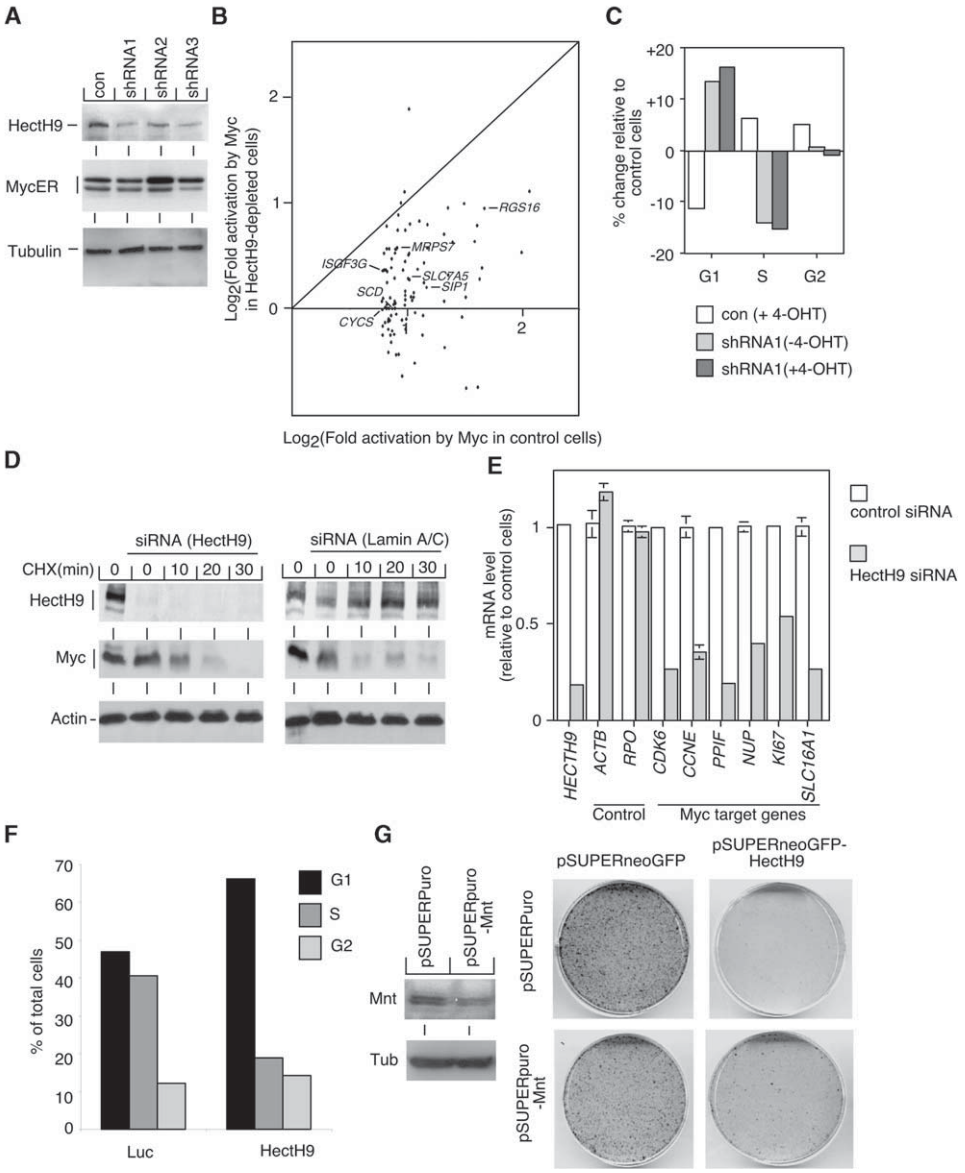



Figure 6. HectH9 Is Required for Myc Function

(A) Western blots documenting expression of HectH9, MycER, and, as control, β-tubulin in pools of U20S-MycER cells infected either with control vectors or different shRNA vectors targeting HectH9.

(B) Summary of microarray experiments documenting the fold induction of the 100 most strongly induced genes upon activation of Myc either in control cells (horizontal axis) or in cells expressing shRNA vectors targeting HectH9 (vertical axis). Data on the vertical axis represent the induction averaged from each of the individual shRNA pools; each array was performed in duplicate.

(C) FACSscan experiment showing the cell-cycle distribution of U2OS-MycER cells upon addition of 4-OHT and of MycER cells expressing shRNA1 before and after addition of 4-OHT relative to serum-starved U2OS-MycER cells.

(D) Western blots documenting expression and stability of HectH9 and Myc in HeLa cells transfected with siRNA directed against HectH9.

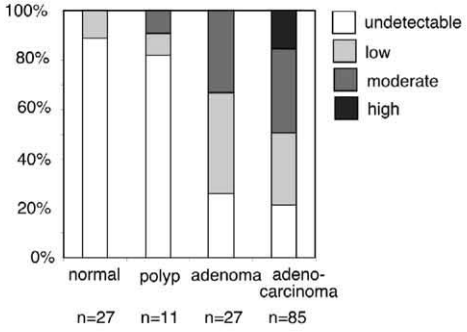
(E) Expression of HECTH9, of selected control genes, and of several Myc target genes in control HeLa cells and in HeLa cells depleted for HectH9 using siRNA. Data are calculated from a real-time PCR experiment and represent the mean ± SEM from three determinations for each gene.

(F) FACSscan experiment showing the cell-cycle distribution of exponentially growing HeLa cells and of cells depleted for HectH9.

(G) Colony assay documenting growth of HeLa cells stably infected with shRNA vectors either targeting Mnt or HectH9 or both. The panels on the left are Western blots documenting reduced Mnt levels upon infection with shRNA vectors targeting Mnt.

pression of *HECTH9* was detected in 9/27 adenomas and in 42/85 adenocarcinomas. Microscopic inspection of the colon samples confirmed that HECTH9 expression was confined to the tumor tissue (Figure 7B). Similarly, moderate or high expression of *HECTH9* was detectable in a high percentage of lung and breast carcinomas, while it was undetectable in normal breast (Figure 7C and data not shown). Most likely, this is due

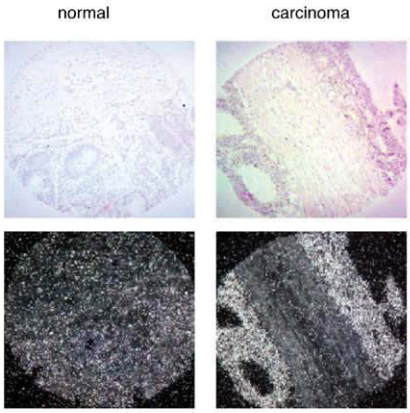
A




C

| Organ | Tumor Type | Incidence | Proportion |
|---|---|---|---|
| Breast | Carcinoma | 31/72 | 43% |
| Lung | Carcinoma | 18/39 | 46% |
| Colon | Carcinoma | 68/131 | 52% |
| Liver | Carcinoma | 2/11 | 18% |
| Pancreas | Carcinoma | 3/15 | 20% |
| Thyroid | Carcinoma | 1/11 | 9% |

B




D

| Cell Line | Relative mRNA expression | Growth arrest |
|---|---|---|
| | | |
| HeLa | 8x | + |
| MDAMB468 | 4x | + |
| T47D | 12x | + |
| MCF7 | 12x | - |
| BT549 | 1x | + |
| SKBR3 | 1x | + |
| IMR90 | 1x | - |
| WI38 | 1x | - |
| BT20 | 1x | - |
| CALU | 8x | - |
| DLD1 | 3x | - |
| SW480 | 3x | - |

E

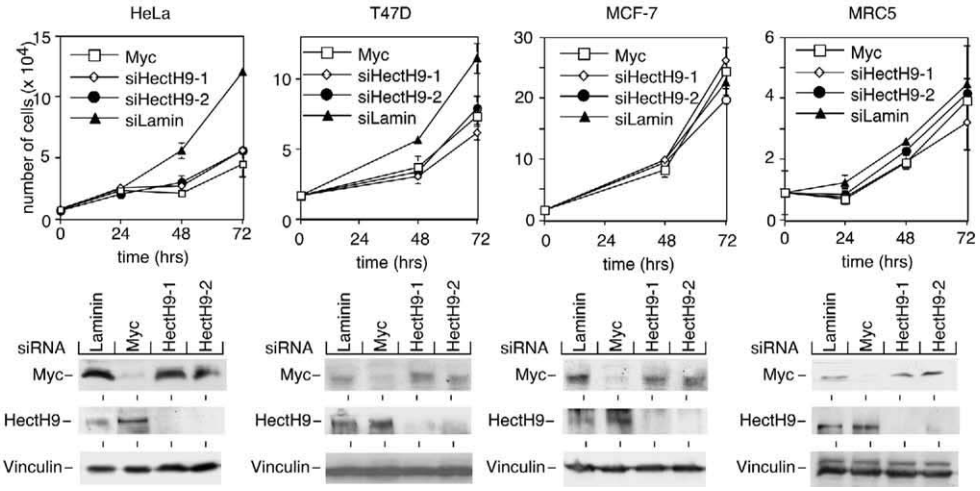



Figure 7. *HECTH9* Is Overexpressed in Human Primary Tumors and Is Essential for Growth of a Group of Cancer Cell Lines but Not of Normal Cell Lines

(A) Summary of in situ hybridization (ISH) on a tissue microarray (TMA) of colon tumors. Shown is the number of tumors with the indicated expression levels of *HECTH9* in relationship to tumor stage.

(B) Example of in situ hybridization data. Two samples from a tumor tissue and its normal counterpart of a colon TMA are shown (bottom panel). The bright field panel shows the morphology of the tissue sample as revealed by hematoxylin and eosin counterstaining. The dark field panel shows the ISH analysis of *HECTH9* expression levels in colon carcinoma and in a normal colon epithelium.

(C) *HECTH9* is highly expressed in a large number of primary human tumors. Summary table of *HECTH9* expression on the TMAs tested.

(D) Summary of expression data for *HECTH9* and effect of depletion by siRNA oligonucleotides on multiple human cell lines and primary cells.

(E) Effects of depletion of HectH9 and Myc on cell proliferation of three tumor cell lines and of MRC5 cells. The upper panels show the growth of transfected cells over a 72 hr time course after transfection; the lower panels document the degree of depletion of the indicated proteins. The data represent the mean of either two or three cell counts for each time point and condition.

to deregulation of HectH9 expression and not due to gene amplification, since in parallel FISH analysis we did not detect any evidence for amplification of the *HECTH9* locus (data not shown). Our in situ hybridization data are supported by inspection of available expression databases, which showed that *HECTH9* is higher in multiple solid tumors, including lung carcinoma, breast carcinoma, prostate carcinoma, glioblastoma, and colon carcinoma, relative to normal tissue (not shown).

To ascertain the relevance of HectH9 to tumor cell growth and proliferation, we measured expression of HECTH9 in human tumor cell lines; enhanced expression of HECTH9 relative to MRC5 diploid human fibroblasts was observed in multiple tumor cell lines (Figure 7D). We then used two different siRNA oligonucleotides (Figure 7E) and shRNA vectors (data not shown) to deplete endogenous HectH9. Similar to the observations made in HeLa cells, depletion of HectH9 inhibited the proliferation of several human tumor cell lines. In contrast, depletion of HectH9 had little effect on the proliferation of WI38 diploid human fibroblasts (Figure 7D). In order to test whether the different responses to depletion of HectH9 reflected a different requirement for Myc in cell proliferation, we selected three tumor cell lines and MRC5 human fibroblasts and either depleted Myc or HectH9 (Figure 7E). Depletion of either Myc or HectH9 inhibited proliferation of both HeLa and T47D cells. In contrast, depletion of neither Myc nor HectH9 arrested the proliferation of MCF-7 or MRC5 cells, although immunoblots revealed that the degree of depletion was similar for each protein in each cell line (Figure 7E). The findings show a close correlation between the requirement for Myc and that for HectH9 in cell proliferation. Taken together with the finding that loss of Mnt partially relieves the requirement for HectH9, the data strongly suggest that ubiquitination of Myc is the rate-limiting function of HectH9 in tumor cell proliferation.

## Discussion

The Myc protein forms at least two distinct protein complexes in vivo: an activating binary complex with its partner protein, Max, and a repressive complex, which contains in addition to Max the zinc-finger protein Miz1.

We have now found that the E3-ligase HectH9 ubiquitinates Myc and that Miz1 inhibits ubiquitination of Myc by HectH9. Several findings show that ubiquitination by HectH9 alters the transcriptional properties of Myc and suggest that it contributes to the switch between an activating and a repressive state of the protein: First, a mutant of Myc that lacks several of the lysines modified by HectH9 ($MycKR_6$) is compromised in its ability to activate multiple endogenous Myc target genes and to stimulate cell proliferation. Second, HectH9 stimulates binding of the coactivator p300 to Myc, while $MycKR_6$ is impaired in binding p300 and does not recruit p300 to an endogenous target gene. Third, inhibition of HectH9 impairs Myc-dependent gene activation and abrogates Myc-induced proliferation. Fourth, depletion of HectH9 in a human tumor cell line downregulates multiple Myc target genes and leads to an accumulation of cells in the G1 phase of the cell cycle.

Our own analysis and inspection of available databases show that HECTH9 is overexpressed in a subset of human tumors and that its expression correlates with tumor progression in primary colon tumor samples. Finding molecules that allow selective targeting of tumor cells is a key aim of tumor research. Myc is an attractive target molecule for such strategies since its deregulated expression contributes to the genesis of a high percentage of human tumors. In addition, inhibition of Myc function in vivo can induce regression even of advanced tumors (Jain et al., 2002). However, attempts to disrupt the Myc/Max interface using small molecules have yielded compounds with low affinity (Berg et al., 2002). In contrast, enzymes of the ubiquitin pathway have recently become amenable to pharmacological inhibition (Vassilev et al., 2004).

Our data indicate that depletion of HectH9 prevents the proliferation of a subset of human tumor cells but has little effect on the proliferation of primary cells. Neither the status of p53 nor deregulation of the E2F pathway determines sensitivity to depletion of HectH9 since both HeLa cells (which express papillomaviruses E6 and E7) and tumor cell lines, which express mutant p53, are inhibited by depletion of HectH9. In contrast, depletion of Mnt, an antagonist of transactivation by Myc, partially relieves the requirement for HectH9 in tumor cell proliferation, and the degree of inhibition by depletion of HectH9 correlates with the degree of inhibition by depletion of Myc in different cells. The findings strongly suggest that ubiquitination of Myc is the rate-limiting function of HectH9 in tumor cell proliferation and that inhibition of HectH9 function may provide a novel possibility to pharmacologically interfere with Myc's function in tumorigenesis.

Finally, we note that HectH9 is identical to p500ARF-BP1/Mule, a recently identified interaction partner for the ARF tumor suppressor protein (Chen et al., 2005). ARF inhibits the catalytic activity of p500ARF-BP1 toward one of its substrates, p53. To date, we have been unable to demonstrate that ARF inhibits ubiquitination of Myc by HectH9 (S.A., unpublished data). However, it is interesting to note that ARF inhibits transcriptional activation, but not repression, by Myc, similar to inhibition of HectH9 (Datta et al., 2004). It is conceivable that inhibition of HectH9 by ARF mediates p53-independent growth-arrest functions of ARF by limiting Myc function. If so, induction of ARF by Myc and subsequent inhibition of HectH9 may serve as a negative feedback loop that restrains excessive Myc function and enhanced expression of HectH9, or loss of ARF may disrupt this feedback loop in tumorigenesis.

## Experimental Procedures

### Cell Culture and Transfections

All cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM glutamine and 100 U/ml streptomycin. Transient transfections were carried out using standard $CaPO_4$-protocol. CMV-based expression plasmids encoding Myc and Miz1 have been described previously (Herold et al., 2002). Recombinant retroviruses were generated using pbabe-vectors or pRetrosuper vectors (Brummelkamp et al., 2002; Morgenstern and Land, 1990). Transient reporter assays were performed as described before using luciferase reporter plasmids that carry an E box element from the PTMA gene or the P21CIP1-core promoter

(Herold et al., 2002). To measure Myc stability in vivo, NIH3T3 cells were infected with retroviral constructs (pbabe-puro) expressing wtMyc or $MycKR_6$. After selection, cells were treated with 25 μg/ml cycloheximide for different times.

### Plasmid Constructs and Mutants

To identify the full-length sequence of HECTH9, we first assembled a putatively complete coding sequence using data of human ESTs and human genomic sequences available at the NCBI's Web site. The truncation mutants used for the mapping of target lysines of c-Myc have been described (Salghetti et al., 1999). Mutants of ubiquitin and Myc were constructed using PCR-based in vitro mutagenesis; to generate $MycKR_6$, lysines converting K298, K317, K323, K326, K341, and K355 were converted into arginines.

### Ubiquitination Assays

For in vitro ubiquitination, 2 μl of rabbit reticulocyte lysate-translated $^{35}$S-labeled Myc were incubated in the absence or in the presence of 200 ng of bacterially expressed GST-HectH9(3228–4374), 100 ng E1, 100 ng UbcH5, and 8 μg ubiquitin (SIGMA) in 40 μl volumes. In addition, reactions contained 25 mM Tris-HCl (pH 7.5), 60 mM NaCl, 5 mM DTT, 2 mM ATP, and 4 mM $MgCl_2$. After incubation at 25°C for 2 hr, total reaction mixtures were electrophoresed in 10% SDS-polyacrylamide gels and $^{35}$S-labeled Myc detected by fluorography. The ubiquitin-activating enzyme E1 and the ubiquitin-conjugating enzyme UbcH5 were expressed in the baculovirus system or in *Escherichia coli* BL21 by using the pET expression system as described (Huibregtse et al., 1995). In vivo ubiquitination assays were performed essentially as described by Welcker et al. (2004).

### Small-Interfering RNAs

siRNA and shRNA sequences targeting HectH9 and Mnt are available upon request. siRNA duplexes were prepared by annealing pairs of 21-ribonucleotides (Dharmacon Research), and they were transfected into different cell lines using OLIGOFECTAMINE (Invitrogen) according to the manufacturer's instructions. shRNAs were expressed from retroviruses generated using either pRetrosuper or pSUPERneoGFP (OligoEngine). As control, we transfected cells with siRNA duplex targeting firefly luciferase (LUC) or lamin A/C.

### Microarray Analysis

NIH3T3 cells were infected with either control retroviruses (pbabe-puro) or retroviruses wtMyc or $MycKR_6$. After selection, cells were starved in medium containing 0.1% calf serum for 48 hr. U2OS-MycER cells were infected either with p-Retrosuper or with p-Retrosuper-vectors expressing shRNA against HectH9 and serum-starved after selection in puromycin; MycER was activated by addition of 200 nM 4-OHT for 24 hr. For details about the array and procedures, see Supplemental Experimental Procedures. HeLa cells were transfected with either control RNAi or RNAi targeting HectH9, and RNA was isolated 36 hr after transfection; arrays were performed using the Affymetrix human genome U133 Plus 2.0 array.

### Tissue Microarrays

We prepared two multitumor tissue microarrays (TMAs; Kononen et al., 1998) and a colon progression TMA including normal epithelia, hyperplastic polyps, adenomas, and adenocarcinomas. In the colon progression TMA, we deposited, whenever possible, matched samples from the same patient. The *ISH* was performed as previously described (Rugarli et al., 1993). Levels of *HECTH9* expression in the in situ analysis were evaluated using a scale of 0–3, where 0 = negative and 3 = strong staining intensity.

### Antibodies

To detect HectH9, we raised and affinity-purified a polyclonal antiserum against a protein corresponding to amino acids 3773–4374 of HectH9. Moreover we generated several monoclonal antibodies to HectH9 using standard techniques. The antigen used corresponds to amino acids 2530 to 2596 of HectH9. In addition, the following antibodies were used: α-cdk2 (M2; Santa Cruz), α-Myc (9E10; N-262, Santa Cruz), α-Miz1 (10E2, 3F3, 11F4), rabbit or mouse α-HA (BAbCO), and mouse α-Vinculin (SIGMA).

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