Myc-Mediated Transcriptional Repression by Recruitment of Histone Deacetylase

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Abstract

Myc is a transcription factor that features prominently in cancer. The oncogenicity of Myc stems from its ability to regulate expression of genes required for cell growth and proliferation. Although the mechanisms through which Myc activates transcription have been extensively studied, less is known about how Myc represses transcription. Recently, we reported that a conserved element within Myc–MbIII— is important for transcriptional repression. Here, we investigate the mechanism through which MbIII contributes to repression. We show that Myc represses transcription of target genes Id2 and Gadd153 by a process that involves histone deacetylation. We show that MbIII is important for repression of these genes and present evidence that this element contributes to repression by recruiting the histone deacetylase HDAC3 to the Id2 and Gadd153 promoters. These results describe a mechanistic role for MbIII in transcription, and reveal that recruitment of HDAC3 is a process by which Myc represses gene activity. [Cancer Res 2008;68(10):3624–9]

Introduction

Myc is a helix-loop-helix transcription factor that regulates cell growth and proliferation (1). Capable of acting as both a transcriptional activator and repressor, Myc controls the expression of a battery of genes required for protein synthesis, DNA replication, apoptosis, and cell metabolism. Consistent with its potent growth-promoting properties, Myc can drive oncogenic transformation, and deregulated Myc expression and activity is a hallmark of many human cancers (1).

Because the amino terminus of Myc contains a potent transcriptional activation domain, initial studies on Myc focused on its role as a transcriptional activator. These studies revealed that the ability of Myc to recruit histone acetyltransferases (HAT) to chromatin is crucial for transcriptional activation and led to the notion that Myc acts as a "permissive factor" (2). Despite the initial focus on activation, subsequent studies revealed that Myc-driven transcriptional repression is critical for its oncogenic activity (1). Less, however, is known about how Myc represses transcription. One mechanism of repression, which has been documented for the p21 gene, involves Myc-dependent antagonism of Miz-1 (3), together with recruitment of the DNA methyltransferase 3a, leading to Cpg hypermethylation within the locus (4). Whether this is the only mechanism through which Myc can repress transcription, or whether other pathways exist, is unknown.

We recently reported that a conserved, but little studied, element within Myc–Myc box III (MbIII)—is important for the ability of Myc to repress transcription (5). Here, we investigate how MbIII contributes to this process, and present evidence that MbIII functions, in part, by recruiting the histone deacetylase (HDAC) HDAC3 to chromatin. These findings reveal a previously unanticipated mode of transcriptional repression by Myc and underscore the role of Myc in regulating transcription—positively and negatively—via histone acetylation.

Materials and Methods

Antibodies and primers. Immunochemicals used were anti-Myc (N262; Santa Cruz), antiacetyl H4 (06-866; Upstate), anti-RNA polymerase II (pol II; H224; Santa Cruz), anti-HDAC3 (H-99; Santa Cruz), anti-HAM (6), anti-FLAG-M2 (A2220; Sigma), anti-VP16 (Clontech), anti–HA-horseradish peroxidase (HRP; 3F10; Roche), anti–T7-HRP (Novagen), and anti–FLAG-M2-HRP (A8592; Sigma). Primer sequences available on request.

Cell culture and treatments. Rat1 cells expressing FLAG-tagged estrogen receptor (ER)-Myc fusion proteins were described previously (5). To generate tetracycline-repressible (Tet-Off)—based cell lines, wild-type (WT)-Myc or ΔMbIII-Myc sequences were cloned into pRevTRE (Clontech) and retrovirally transduced, together with pRevTet-Off-IN (Clontech), into Rat1 cells. For RNA and chromatin immunoprecipitation (ChIP) analyses, Rat1 cells were grown until confluence and then serum deprived by growth in DMEM containing 0.1% fetal bovine serum for 48 h. For ER-Myc—expressing cells, Myc was activated by addition of 4-hydroxy-tamoxifen (4HT; 1 μmol/L) for either 4 (ChIP) or 8 h [quantitative reverse-transcription PCR (RT-QPCR)]. Where indicated, 200 ng/mL trichostatin A (TSA) was added to the medium with 4HT. Tet-Off-Myc cells were treated with 2 μg/mL tetracycline for 2 or 4 h; alternatively, cells were treated with the HDAC inhibitor MS-275 (0.3 to 4 μmol/L) for 8 h. RNA was then prepared and RT-QPCR was performed as described (5).

ChIP. ChIP assays were performed using the protocol described at Upstate.com.1 Coprecipitating DNA was quantified using quantitative PCR (QPCR) by comparison to input sample signals as described (2). Enrichment values for each PCR amplicon were calculated versus a control primer set for a region of the PCNA gene that does not contain a Myc-binding site (2). Probe coordinates are given relative to the 5’-end of the first exon of each gene. Co-occupancy of HDAC3 with Myc on chromatin was determined using a protocol similar to that described (7). Primary and secondary immunoprecipitation (IP) steps were each performed using anti-HDAC3 and anti-Myc antibodies, respectively.

Short-hairpin RNA-mediated gene knockdown. ER-WT-Myc Rat1 cells were retrovirally transduced with pLMP vectors (ref. 8; R. Dickins and S. Lowe, CHSL) targeted to each of the rat HDAC genes. Three different
short-hairpin RNAs (shRNAs), analyzed in pools, were used for each HDAC. After selection of transduced cells, RNA was collected and RT-QPCR was performed.

Co-IP. Co-IP experiments were performed as described (9). HEK293 cells were transfected with the indicated combinations of pCGT7-Max (10), pFLAG-HDAC3 [E. Seto, Moffitt Cancer Center; ref. 11], pUC119, and either pCGN-HAM-Myc-WT, pCGN-HAM-Myc-ΔMbII, or pCGN-HAM-Myc-ΔMbIII (5). Immune complexes were captured using HAM (Myc IP) or FLAG (HDAC3 IP) antibodies, and Western blot was used to detect either HAM-tagged or FLAG-tagged proteins.

Cell viability assays. Cell viability assays were performed as described (5), except that apoptosis was induced by doxorubicin. ER-Myc–induced cell death was calculated as the percentage increase in cell death observed for ER-Myc–expressing cells over cells expressing vector alone.

Results

MbIII broadly contributes to Myc-dependent repression. Our previous work showed that MbIII is important for the ability of Myc to repress several target genes, including Gadd45, Gas1, and p21 (5). To determine whether MbIII contributes to the regulation of other Myc-repressed genes, we activated ER-regulated WT and ΔMbIII forms of Myc (5) in Rat1 cells and used RT-QPCR to measure transcript levels from 50 putative Myc-repressed genes selected from the Myc Cancer Gene Database. Of these 50 genes, only 8 were repressed by WT-Myc >2-fold in Rat1 cells (Table 1). In each case, however, repression was clearly diminished by deletion of MbIII. In some cases, deletion of MbIII blocked repression (e.g., Casp1), whereas in some cases (e.g., Id2, p15, and p21), the ΔMbIII deletion resulted in a partial loss of repression. Interestingly, activation of gene expression (Nuc and Cad) was unaffected by loss of MbIII. From these data, we conclude that MbIII is dispensable for gene activation in Rat1 cells but broadly contributes to Myc-mediated gene repression.

Loss of MbIII reduces the ability of Myc to promote histone deacetylation. We next sought to understand the mechanism through which MbIII contributes to Myc-dependent repression. For this analysis, we used two parallel inducible Myc systems: one in which Myc is expressed as an ER-fusion (as above) and another in which Myc is expressed under the control of the Tet-Off promoter. The ER-Myc fusion system has been described and expresses comparable levels of WT-Myc and ΔMbIII-Myc proteins (5). The Tet-Off system was developed here. In Tet-Off cells, WT-Myc and ΔMbIII-Myc are expressed at equivalent levels and associate similarly with chromatin, as determined by ChIP (Supplementary Fig. S1). We established these two systems to control for any differences in Myc activity that may result from the specific mode of Myc expression. Our goal was to use the ER-Myc and Tet-Off-Myc settings to identify—by ChIP—transcriptionally relevant factors that are recruited to chromatin by WT Myc but less-so by the ΔMbIII mutant. We focused our analysis on the Id2 and Gadd153 genes because they are bona fide targets of Myc (12, 13) and respond similarly to WT-Myc and ΔMbIII-Myc in both systems (Supplementary Table S1), although repression is generally more profound in the ER-Myc setting.

Gadd153 is repressed by Myc at a stage preceding recruitment of RNA pol II (13), and our analysis of pol II recruitment by ChIP in both settings confirmed this report (Fig. 1A). Induction of WT-Myc or ΔMbIII-Myc produced little if any change in the levels of Gadd153 promoter–associated pol II. Interestingly, a different pattern was observed at Id2 (Fig. 1B), where induction of WT-Myc resulted in a significant decrease in pol II recruitment to the Id2 promoter. Moreover, unlike Gadd153, pol II levels at Id2 were sensitive to deletion of MbIII, with the ΔMbIII mutant showing an impaired ability to reduce pol II–promoter occupancy. These results are consistent with the notion that Id2 is repressed by Myc at a stage before pol II recruitment, and that MbIII is important for modulating levels of pol II at the Id2 promoter.

Given the disparate manner in which Myc seems to repress Gadd153 and Id2, it is curious that MbIII contributes to the regulation of both genes. This observation suggested to us that MbIII participates in repression via a mechanism capable of influencing transcription at multiple different stages. We therefore reasoned that alterations at the level of chromatin—which could control accessibility of DNA to both initiation and elongation factors—might be such a mechanism. Because histone H4 acetylation is a means through which Myc activates transcription (2), we hypothesized that loss of this modification might accompany Myc-dependent repression. To test this hypothesis, we used ChIP to probe for changes in H4 acetylation status at both Gadd153 and Id2. This analysis (Fig. 1C–D) revealed that WT Myc does indeed signal loss of H4 acetylation at an upstream site (~1466) in the Gadd153 promoter (adjacent to predicted Myc-binding sites; ref. 14), and at the canonical E-box (~1348) within Id2.

Importantly, this reduction in H4 acetylation was partially reversed by deletion of MbIII, demonstrating that this element contributes to Myc-induced changes in H4 acetylation. As controls, we also found that: (a) alterations in pol II levels at Id2 (Fig. 1E), and of H4 acetylation at Gadd153 (Fig. 1F) and Id2 (Fig. 1G), were reversed by addition of tetracycline to suppress Myc expression; and (b) that both WT-Myc and ΔMbIII-Myc behaved similarly with respect to pol II binding and H4 acetylation at Nuc and Cad (Supplementary Fig. S2). Taken together, these results show that Myc-dependent repression of Gadd153 and Id2 correlates with reduced histone H4 acetylation, and that MbIII is important for this process.

Myc-dependent repression of Gadd153 and Id2 requires HDAC activity. The correlation we observed between Myc-mediated repression and loss of H4 acetylation suggested to us

| Table 1. Genes repressed by Myc in the Rat1 ER-Myc system |
|----------------|----------------|----------------|
| Gene           | ER-WT-Myc      | ER-ΔMbIII-Myc  |
| Id2            | 2.0 ± 0.31     | 65 ± 7.3       |
| Gadd153        | 38 ± 9.9       | 144 ± 37       |
| Gadd45a        | 50 ± 8.0       | 130 ± 22       |
| p15            | 0.71 ± 0.21    | 68 ± 9.5       |
| p21            | 27 ± 8.1       | 77 ± 8.5       |
| Casp1          | 1.6 ± 0.21     | 118 ± 20       |
| CryaB          | 15 ± 3.4       | 42 ± 11        |
| Klf4           | 26 ± 6.2       | 53 ± 9.7       |
| Nuc            | 186 ± 17       | 260 ± 22       |
| Cad            | 286 ± 5.7      | 261 ± 15       |

NOTE: RNA was isolated from Rat1 cells expressing activated ER-WT-Myc or ER-ΔMbIII-Myc, and RT-QPCR was used to measure the relative expression of eight genes that were repressed 2-fold or more by the WT-Myc protein. cDNA levels are expressed relative to those in empty vector-expressing cells (100%). Results are based on the average of three experiments ± SE.
that HDAC activity may contribute to both processes. We therefore asked whether chemical inhibition of HDACs leads to derepression of Id2 and Gadd153 and restoration of H4 acetylation (Fig. 2). These experiments revealed that treatment of cells with the HDAC inhibitors TSA (Fig. 2A) or MS-275 (Supplementary Fig. S3) reversed Myc-driven repression at both genes. Relief of inhibition was accompanied by an increase in the level of H4 acetylation and pol II recruitment to Id2 in the presence of Myc (Fig. 2B), suggesting that HDAC activity plays a direct, and mechanistic, role in the ability of Myc ability to repress gene activity.

Although HDACs display redundant activities (15), we reasoned that it might be possible to identify an HDAC that is preferentially required for repression of Id2 and Gadd153. We therefore used shRNA-mediated gene silencing (8) to knockdown the expression of each of the TSA-sensitive HDACs in Rat1 cells expressing ER-WT-Myc. There are 11 TSA-sensitive HDACs. We found that HDACs 6 and 11 are not detectably expressed in Rat1 cells (data not shown), and we were unable to successfully target HDAC9; we thus focused on the remaining eight HDACs. We transduced ER-Myc cells with shRNAs against each HDAC and used RT-QPCR to monitor for changes in Myc-dependent Id2 and Gadd153 repression (Fig. 3). This analysis revealed that despite modest levels of knockdown for each HDAC (~50% Supplementary Table S2), knockdown of both HDAC3 and HDAC4 partially reversed the repression of Id2 and Gadd153. Knockdown of HDAC1 also partially relieved repression, albeit to a level lower than that of the other two HDACs. Although repression was not completely reversed by knockdown of HDAC3 and HDAC4, the increase in Id2 and Gadd153 expression was robust.

![Figure 1](image_url).

**Figure 1.** Myc-dependent loss of histone H4 acetylation at the Gadd153 and Id2 regulatory regions. A and B, Rat1 cells expressing activated ER–Myc or Tet-Off–Myc were subject to ChIP analysis using antibodies against pol II (A) or acetylated histone H4 (B). C and D, Rat1 cells expressing Tet-Off–Myc (or vector control) were left untreated or treated with 2 μg/mL tetracycline for the indicated times before ChIP analysis using antibodies against pol II (C) or acetylated histone H4 (D). In all cases, co-precipitating DNAs were quantified by Q-PCR using the indicated primer sets (probe), and enrichment was calculated relative to a probe in the PCNA gene. Columns, mean of three experiments; bars, SE.
Figure 2. Myc-dependent repression of id2 and Gadd153 is reversed by HDAC inhibition. A, Rat1 cells expressing activated ER-WT-Myc or ΔMbIII-Myc (or vector control) were treated with 200 ng/mL TSA (where indicated) for 8 h, RNA was isolated, and RT-QPCR was used to measure levels of id2 and Gadd153 cDNA. B, ChIP analysis was performed, using an antibody against acetylated H4 (left) or pol II (right), on cells treated with TSA as in A but for 4 h. Enrichment of signal at the id2 E-box (~1348) is shown for acetylation; pol II signals were quantified at the proximal promoter (probe –245). Columns, mean of two experiments; bars, SE.

Gadd153 expression was clear, and at a similar level to that reported in studies examining the contribution of HDAC3 to repression by nuclear hormone receptors (11). These data suggest that HDACs 3 and 4, and possibly HDAC1, contribute to Myc-dependent repression of Gadd153 and id2.

Interestingly, HDAC3 and HDAC4 are functionally connected, and it has been reported that HDAC4 requires HDAC3 for its action (16). Because of this central role of HDAC3, we focused our subsequent analyses on this HDAC. We first asked whether we could detect a physical interaction between endogenous Myc and HDAC3. Myc was recovered from extracts of HEK293 cells by IP, and coprecipitating HDAC3 was detected by Western blotting. As seen in Fig. 4A, HDAC3 could be specifically recovered in the Myc immune complexes (lane 4), but not complexes recovered with an irrelevant control antibody (lane 3), revealing that endogenous Myc and HDAC3 physically associate. We estimate that as much as 0.5% of HDAC3 can be recovered in complex with Myc; we do not know whether this interaction is direct or mediated by additional protein factors.

To determine if the interaction between Myc and HDAC3 is influenced by MbIII, we coexpressed HA-tagged Myc (WT or ΔMbIII), its partner protein Max (T7-tagged), and flag-tagged HDAC3 in HEK293 cells, and immunoprecipitated flag-HDAC3 under nondenaturing conditions (Fig. 4B). As a control, we also directly immunoprecipitated HA-Myc. Under these conditions, WT-Myc associated with HDAC3 (lane 3), and this association was reduced by deletion of MbIII (lane 6), demonstrating that MbIII contributes to this interaction. Importantly, recovery of HDAC3 also resulted in the specific corecovery of T7-tagged Max, consistent with the notion that HDAC3 recognizes Myc within the context of the Myc-Max heterodimer. We repeated this analysis in the “reverse” orientation (recovering Myc and probing for HDAC3; Fig. 4C) and obtained similar results—Myc associated specifically with HDAC3 (lane 2), and this association was reduced by loss of MbIII. Deletion of MbI, in contrast, which is not required for repression, had no detectable effect on HDAC3 association (lane 3).

Our data are consistent with a model in which Myc interacts with HDAC3, and that this interaction recruits HDAC3 to the id2 and Gadd153 loci to effect repression. If this is correct, then we would expect that Myc and HDAC3 co-occupy sites on chromatin in a manner that depended on MbIII. Our studies had shown that both Myc and HDAC3 could independently be detected at the id2 promoter by ChIP in the presence of Myc (data not shown), but to probe for true co-occupancy, we performed “sequential ChIP,” in which we recovered chromatin fragments associated with HDAC3, and then performed a subsequent round of IP using antibodies against Myc. This experiment (Fig. 4C–D) revealed that HDAC3 and Myc do indeed co-occupy the id2 and Gadd153 regulatory regions. Critically, co-occupancy of Myc and HDAC3 was reduced upon deletion of MbIII, demonstrating that MbIII is important for modulating the association of HDAC3 with these target genes.

The ability of Myc to induce cell death is modulated by HDAC3. Our previous analyses revealed that MbIII is important for the ability of Myc to function as an oncogene, both in vitro and in vivo (5). Interestingly, loss of MbIII increases the ability of Myc to promote cell death, arguing that MbIII contributes to tumorigenesis by tempering the proapoptotic activity of Myc. If MbIII functions in part via HDAC3, and if this interaction is biologically relevant, we would expect that knocking down HDAC3 expression will recapitulate the effects of removing MbIII from within Myc. To test this notion, we compared the ability of WT-Myc and ΔMbIII-Myc to drive apoptosis in cells that either expressed HDAC3 or had HDAC3 knocked down by shRNA (Fig. 5). In this experiment, cells were treated with two concentrations of doxorubicin (17), and cell death was scored by trypan-blue exclusion. Consistent with our previous study (5), we found that the ΔMbIII form of Myc was significantly better at promoting cell death than the WT protein. Satisfyingly, knockdown of HDAC3 promoted the ability of WT-Myc to induce cell death.
to induce apoptosis, to a level indistinguishable from the of the ΔMbIII deletion mutant. This result suggests that the differences in proapoptotic activity between WT-Myc and ΔMbIII-Myc may be due to differences in their ability to functionally interact with HDAC3. If so, we would expect that the ΔMbIII mutant would be insensitive to HDAC3 knockdown in this assay. Indeed, we find that this is the case, and that HDAC3 knockdown has little if any effect on apoptosis that is signaled by the ΔMbIII mutant. Together, these results lead us to conclude that functional interactions between Myc and HDAC3 modulate the ability of Myc to promote cell death.

**Discussion**

Although Myc was initially described as a transcriptional activator, subsequent studies have shown that the ability of Myc to repress transcription is also critical for its function in normal and cancer states (1). To date, most studies on the mechanism through which Myc inhibits transcription have focused on its ability to inhibit activation by Miz-1 (3). Our recent finding that MbIII is important for Myc-mediated repression (5) provided us with the opportunity to study other ways in which Myc can function in this capacity. Using the Gadd153 and Id2 genes as platforms for our analyses, we have found that: (a) repression of these genes by Myc is associated with a loss of histone H4 acetylation; (b) HDAC activity, particularly from HDAC3, contributes to repression; (c) Myc recruits HDAC3 to these genes in an MbIII-dependent manner; and (d) the functional interaction of Myc with HDAC3 plays an important role in the ability of Myc to promote apoptosis. These findings suggest a model in which Myc recruits HDAC3, and possibly other HDACs, to repress the expression of genes important for regulating the balance between cell proliferation and cell death.

From our genetic screen, HDAC3 seems to make the most prominent contribution to Gadd153 and Id2 repression (Fig. 3). HDAC3 is interesting because it is a core component of the nuclear receptor corepressor complexes N-Cor and SMRT (18), and because silencing of HDAC3 is sufficient to induce apoptosis in a variety of cell systems (Fig. 5; ref. 19). We speculate, therefore, that HDAC3 plays an important role in Myc activity by tempering the ability of Myc to induce apoptosis. Given that knockdown of HDAC3 recapitulates the effects of deleting MbIII in our assays and that MbIII plays a critical role in cellular transformation by Myc (5), we speculate that HDAC3 will also be important for the overall ability of Myc to function as an oncoprotein.

It is important to note that HDAC3 is unlikely to be the sole HDAC that is relevant to Myc-dependent repression. In our hands, treatment of Rat1 cells with TSA reverses Myc-mediated Gadd153 and Id2 repression (Fig. 2), whereas knockdown of HDAC3 results in a partial restoration of Gadd153 and Id2 expression (Fig. 3). Additionally, our genetic screen revealed that HDAC4 and HDAC1 may also contribute to gene repression, albeit to a lower level than HDAC3 (Fig. 3). The HDAC4 result can be explained by the fact that it typically functions in complex with HDAC3 (16). The HDAC1 result could reflect functional redundancy among the HDACs. In genome-wide analyses in Drosophila cells, for example (20), RNAi-mediated knockdown of HDAC1 or HDAC3 produces overlapping changes in gene expression profiles, suggesting that these two enzymes have partially redundant functions. Furthermore, recent studies have revealed a role for HDAC1 in Myc-dependent repression at the HIV-1 (21) and transglutaminase (22) genes. We suspect that multiple HDACs will be involved Myc-dependent gene expression.
repression, and that MbIII will contribute to recruitment of several different HDACs to chromatin.

The involvement of histone deacetylation in Myc-dependent repression establishes the regulation of histone acetylation status as a common theme in the way in which Myc activates and represses gene activity. The involvement of a single process—histone acetylation—in both aspects of the transcriptional function of Myc raises the possibility that Myc be able to both repress and activate some sets of target genes. Indeed, Id2 is an example of such a gene, being activated in some cell systems (12) and repressed in others (Fig. 1; ref. 23). The ability of Myc to interact with both HATs and HDACs (via MbIII) raises the possibility that it could simultaneously recruit both sets of enzymes to target genes, with the ultimate transcriptional outcome being determined by the balance of acetylase or deacetylase activities at the promoter.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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