Sin3b Interacts with Myc and Decreases Myc Levels*

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Pablo Garcia-Sanz†,*, Andrea Quintanilla‡, M. Carmen Lafita‡, Gema Moreno-Bueno§, Lucia García-Gutierrez*, Vedrana Tabor*, Ignacio Varela‡, Yuzuru Shiio**, Lars-Gunnar Larsson†, Francisco Portillo†‡, and Javier Leon†‡

From the †Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC), Consejo Superior de Investigaciones Científicas, Universidad de Cantabria, Sociedad para el Desarrollo de Cantabria and the Departamento de Biología Molecular, Universidad de Cantabria, Santander 39011, Spain, the ‡Instituto de Investigaciones Biomédicas Alberto Sols, Consejo Superior de Investigaciones Científicas, Instituto de Investigación Hospital Universidad La Paz (IdiPaz), Facultad de Medicina, Universidad Autónoma de Madrid, 28046 Madrid, Spain, the **Fundación M. D. Anderson Internacional, Madrid, Spain, the †Department of Microbiology, Tumor and Cell Biology, Karolinska Institute, Stockholm SE-17177, Sweden, and the **Greehey Children’s Cancer Research Institute, The University of Texas Health Science Center, San Antonio, Texas 78229-3900

Background: Myc is an oncogenic transcription factor that is frequently deregulated in cancer, and Sin3b is a transcriptional regulator that recruits histone deacetylases.

Results: A new interaction between the protein Sin3b and Myc that leads to Myc down-regulation is described.

Conclusion: Sin3b-Myc interaction regulates Myc levels and activity.

Significance: Sin3b adds a second level of control of Myc by directly regulating Myc levels.

Myc expression is deregulated in many human cancers. A yeast two-hybrid screen has revealed that the transcriptional repressor Sin3b interacts with Myc protein. Endogenous Myc and Sin3b co-localize and interact in the nuclei of human and rat cells, as assessed by co-immunoprecipitation, immunofluorescence, and proximity ligation assay. The interaction is Max-independent. A conserved Myc region (amino acids 186–203) is required for the interaction with Sin3 proteins. Histone deacetylase 1 is recruited to Myc–Sin3b complexes, and its deacetylase activity is required for the effects of Sin3b on Myc. Myc and Sin3a/b co-occupied many sites on the chromatin of human leukemia cells, although the presence of Sin3 was not associated with gene down-regulation. In leukemia cells and fibroblasts, Sin3b silencing led to Myc up-regulation, whereas Sin3b overexpression induced Myc deacetylation and degradation. An analysis of Sin3b expression in breast tumors revealed an association between low Sin3b expression and disease progression. The data suggest that Sin3b decreases Myc protein levels upon Myc deacetylation. As Sin3b is also required for transcriptional repression by Mxd-Max complexes, our results suggest that, at least in some cell types, Sin3b limits Myc activity through two complementary activities: Mxd-dependent gene repression and reduction of Myc levels.

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‡ Supported by grants from the Cancerfonden and the Karolinska Institutet. Present address: Dept. of Medical Biochemistry and Biophysics, Karolinska Inst., Stockholm SE-17177, Sweden.

§ To whom correspondence may be addressed. Tel.: 34-914-972732; Fax: 34-914-975353; E-mail: fportillo@iib.uam.es.

¶ To whom correspondence may be addressed. Tel.: 34-942-201952; Fax: 34-942-266399; E-mail: leoni@unican.es.

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The mechanism through which Sin3b represses transcription involves the recruitment of histone deacetylases (HDACs) types 1 and 2 (28–30). Sin3-containing complexes, besides HDAC1 and HDAC2, contain other proteins to form a transcription regulatory complex (reviewed in Refs. 19, 29, and 31). Compared with Sin3a interactions, much less is known about Sin3b interactions. There are differences in the protein interactions described for each Sin3 family member (32–35) and the phenotypes of Sin3a- and Sin3b-deficient mice, with the Sin3a knock-out mice showing an earlier lethality than Sin3b knock-out mice (35). Here we show an interaction between Myc and Sin3b that results in impaired Myc transcriptional activity. The Myc region involved in the interaction is a small conserved region (Myc box III). Myc-Sin3b interaction leads to Myc deacetylation and destabilization, and co-expression of Sin3b leads to reduced Myc levels.

EXPERIMENTAL PROCEDURES

Breast Tumor Samples and Immunohistochemical Analysis—A total of 106 infiltrating ductal breast carcinoma tumors from the archive of the Pathology Department of the M. D. Anderson Cancer Center (Madrid, Spain) were studied. All of the tumors were grade 3. Patients underwent surgery between 2006 and 2007. The mean patient age at surgery was 57.8 years (range, 33 to 82 years). According to the TNM (tumor-node-metastasis) classification for staging, 32 of the tumors were stage I, 35 were stage II, and 34 were stage III–IV. Among the tumors, 17.5% were stage II, and 34 were stage III–IV. The standard ethical procedures of the Spanish regulation (Ley de Investigación Biomédica) were followed. All participants in this study signed informed consent forms, and the study was approved by the Institutional Review Board of the M. D. Anderson Cancer Center (Madrid, Spain). Sin3b immunohistochemical staining was performed by the labeled streptavidin-biotin (EnVision™+ kits, Dako) method with a heat-induced antigen retrieval step. Briefly, sections were immersed in boiling 10 mM sodium citrate, pH 6.0, for 3 min in a pressure cooker. A polyclonal antibody against human Sin3b was used. The antibodies used are listed in Table 1. The primary antibodies were omitted in the negative controls. Sin3b staining was scored as positive when nuclear localization was observed in at least 10% of the tumor cells and as negative when expressed in less than 10% of the tumor cells. For the meta-analysis of breast cancer and Sin3b correlation, the breast microarray and clinical data were used to determine the statistical significance of the relationships between SIN3B expression and survival or metastasis. Values of \( p < 0.05 \) were considered statistically significant. These analyses were carried out using the SPSS 17.0 software.

Yeast Two-hybrid Screening—The Matchmaker system 3 (Clontech) was used for the yeast two-hybrid screen. The bait protein consisted of the full-length human Myc protein subcloned in the pGBK7T vector (containing a GAL4 DNA-binding domain). A pACT2 vector-based mouse NIH3T3 fibroblast cDNA library (Mouse Embryonic Fibroblast Matchmaker, Clontech) was used to screen for prey proteins. Yeasts (AH109 strain) were transformed with the EasyComp transformation kit for Saccharomyces cerevisiae (Invitrogen). Positive colonies were isolated based on their capacity to express the markers Ade2 and His3, which allowed them to grow on SC–AH1LW, a selection medium lacking tryptophan, leucine (selection markers for pGBK7T- and pACT2-based plasmids, respectively), adenine, and histidine. pT1D and pVA3 plasmids (Clontech) were used as an interaction positive control in the yeast two-hybrid system. The pLAM5′ plasmid (Clontech) was used as a control to detect fortuitous protein-protein interactions. pACT2-Myc vector consisted of full-length human Myc subcloned in the pACT2 vector, which encodes for the GAL4 transactivation domain. The clones were also grown in SC–LW medium, which lacks tryptophan and leucine (the selection markers for pGBK7T- and pACT2-based plasmids, respectively), to rule out the possibility that the absence of growth in the selection medium SC–AH1LW was due to an inefficient transformation.

Cell Culture and Transfections—HEK293T and K562 cells were from ATCC. K562/S is a K562 subclone able to grow attached to plastic (36). The P493-6 cell line consists of immortalized human B cells carrying a tetracycline-repressible Myc transgene (37). K562 cells and derivatives were grown in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (Lonza), 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin. Rat1a cells were a gift from Chi Dang (University of Pennsylvania) (38). Rat1aMyc was generated by transfection of Rat1a cells with pCEFL-Myc (36) and selection with 0.4 mg/ml G418 (Sigma). UR61 cells derive from rat pheochromocytoma (39). Rat1a and UR61 cells were cultured in DMEM (Invitrogen) supplemented with serum and antibiotics as described above. The vectors used were as follows: pME185-Myc-FLAG, pME185-MycD1–98-FLAG, pME185-MycD106–143-FLAG, pME185-MycD157–262-FLAG, pME185-MycD186–203-FLAG, pME185-MycD244–271-FLAG, and pME185-MycD348–439-FLAG (39). pcDNA3-Myc-HA was constructed in this work, using the Myc cDNA from pCEFL-Myc (36), pcDNA3-Sin3b-FLAG and pcDNA3-Sin3b-HA were constructed from pcDNA3-Sin3b, encoding full-length murine Sin3b cDNA (a gift from Gregory David, New York University Medical Center, NY). Other vectors used for transfections were: pcDNA3-HDAC1-FLAG, pcDNA3-HDAC2-FLAG, and pS2-Cin3a-myc (22) and the corresponding empty vectors pME185, pcDNA3, pCEFL, and pSc2. For transient transfection of HEK293T cells for protein immunoprecipitation and immunoblot analysis, typically 750,000 cells were transfected using FuGENE 6 transfection reagent (Roche Applied Science) following the manufacturer’s instructions and were lysed 24 h after transfection. In order to silence Sin3b expression in Rat1aMyc cells and in K562 cells, two siRNA duplexes (Mission siRNA, Sigma-Aldrich, 100 pmol each; RNA sequences available upon request) were transfected to each cell line by using
TABLE 1
Primary antibodies used
Use in immunoblot (IB), immunofluorescence (IF), immunoprecipitation (IP), proximity ligase assay (PLA), immunohistochemistry (IHQ), and chromatin immunoprecipitation (ChIP) experiments is indicated. The dilution of the antibodies used for immunoblot was 1:1000.

<table>
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<th>Antigen</th>
<th>Type</th>
<th>Source</th>
<th>Reference no.</th>
<th>Use (dilution)</th>
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<td>Cell Signaling Technology</td>
<td>9681</td>
<td>IB</td>
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<td>1-19, sc-1616</td>
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<td>IB</td>
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<td>HA</td>
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<td></td>
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<tr>
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Table 2

Sequences of the PCR primers used
The forward primer is listed first, and the reverse primer is listed second. All primers were used at 0.3 μM.

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<td>CDK4 (human)</td>
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<td>ChIP</td>
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<tr>
<td>HES1 (human)</td>
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</tr>
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<td>LDHA (human)</td>
<td>TCTGACTCAGCGCTCTATG and AGACAGGCAACGCCAGCA</td>
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<tr>
<td>MYC (human)</td>
<td>AGAGCTCCAGCCTCCCTCTCTT and GGGTTCCCAACCTGGGATCTT</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>RPS14A (human)</td>
<td>CAAGGAGGAAAGAAAGAAAGG and GAGAAGCTCCATCTGGTAGAC</td>
<td>RT-qPCR</td>
</tr>
<tr>
<td>Sin3b (rat)</td>
<td>GCTGATGTCACCTGGGACGAC and CTATCTTCATGGCTATTTAGG</td>
<td>ChIP</td>
</tr>
<tr>
<td>SRD5A1 (human)</td>
<td>GAAACTCCAGCGCCTTCTCT and GTTTTCCAACTCCGGGATCTT</td>
<td>ChIP</td>
</tr>
</tbody>
</table>

Lipofectamine 2000 (Invitrogen) for Rat1aMyc and by electroporation in an Amaza electroporator for K562 cells. Sin3b RNA levels were analyzed 24 h after transfection. K562 cells were transiently transfected with 5 μg of pcDNA3-Sin3bHA plasmid or empty pCDNA3 vector. When indicated, the cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma), trichostatin A (TSA, Sigma), and vorinostat sodium fluoride (5 mM) and 

Pull-down Assay—For the pull-down assay, DH5α cells (Invitrogen) were transformed with pGEX-Myc-GST (glutathione S-transferase) plasmid (41) and grown to exponential phase at 37 °C until an A600 of 0.6–0.8 was reached. Myc-GST expression was induced with 0.5 mM IPTG for 3 h at 37 °C. The cell pellet was resuspended in BFX buffer (20 mM Tris, pH 7.4, 1 M NaCl, 0.2 mM EDTA, 1% Triton X-100, and protease-phosphatase inhibitors) and sonicated. After centrifugation (13,000 rpm for 30 min), the supernatant was collected and incubated for 1 h at 4 °C with glutathione-Sepharose 4B beads (GE Healthcare) equilibrated previously with PBS and BFX buffer. Beads were washed with GST–FISH buffer (10% glycerol, 50 mM Tris, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, and 2 mM MgCl2) and
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incubated for 2 h at 4 °C with a protein extract obtained from HEK293T cells transfected with pcDNA3-Sin3b-HA (and the corresponding pcDNA3 vector) 24 h earlier. After four washes with GST-FISH buffer, the proteins were collected and analyzed by immunoblotting. For the GST-Sin3b and recombinant Myc pull-down assay, the first 1170 nucleotides (390 amino acids) of murine Sin3b cDNA were subcloned into pGEX-4T vector (GE Healthcare). This region encompasses the first three PAH domains at the N terminus of the protein (NSin3b). To this end, pcDNA3-Sin3b was digested with BamHI and the resultant fragment subcloned in the pGEX-4T vector (containing the proteins) were collected and analyzed by immunoblotting.

Luciferase Reporter Assays—The luciferase reporters used were as follows: pGL3-E-box, carrying four E-box elements in the pGL3 vector; and pGL3-E-BoxMut, carrying four mutated E-boxes (42). Cells were transfected using FuGENE 6 transfection reagent (Roche Applied Science) with 30 ng of the reporter plasmid and 30 ng of pcDNA3-Sin3b-HA (and the corresponding empty vector) and grown to exponential phase at 37 °C until an A600 nm of 0.6–0.8 was reached. GST-Sin3b expression was induced with 0.5 mM IPTG for 3 h at 37 °C. The cell pellet was resuspended in BFX buffer and sonicated. After centrifugation (13,000 rpm for 30 min), the supernatant was collected and incubated for 1 h at 4 °C with Pierce glutathione magnetic beads (Thermo Scientific), previously equilibrated with binding/wash buffer (125 mM Tris, pH 8, and 150 mM NaCl). Beads were washed with wash buffer and incubated for 2 h at 4 °C with 50 ng of Myc recombinant protein. After three washes with binding/wash buffer, the beads were resuspended in Western blot loading buffer (containing SDS and β-mercaptoethanol) and boiled at 95 °C for 5 min, and the supernatant fractions (containing the proteins) were collected and analyzed by immunoblotting.

Chromatin Immunoprecipitation (ChIP)—Cells were fixed with 1% formaldehyde for 10 min at room temperature, and fixation was stopped by incubation with 0.12 M glycine for 10 min at room temperature. The cells were washed with PBS and lysed in 1% SDS lysis buffer. Cross-linked chromatin was fragmented by sonication to an average size of 500 bp and immunoprecipitated (500,000 cells/antibody). Antibodies and cell lysates were incubated overnight at 4 °C and then for 4 h at 4 °C with protein G-coupled magnetic beads (Dynabeads, Invitrogen) blocked with salmon sperm DNA (1 h at 4 °C). The protein-DNA cross-link was reversed in elution buffer (1% SDS and 0.1 M NaHCO3) and RNase overnight at 65 °C. The eluted material was treated with proteinase K for 3 h at 45 °C, and the DNA
was purified using the QIAquick PCR purification kit (Qiagen). Quantitative PCR (SYBR Green PCR kit, Bio-Rad) was performed in a Bio-Rad iCycler iQ5 apparatus. The immunoprecipitations were carried out with anti-Myc and anti-Sin3b antibodies or nonimmune rabbit IgG as a negative control (Table 1). The cells were transfected with vectors for Myc-HA and Sin3b-FLAG, and after transfection, the lysates were immunoprecipitated with anti-FLAG to detect Sin3b-bound proteins. The immunoprecipitates were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies as indicated. Immunoprecipitations with immunoglobulin G were conducted as a negative control (not shown). C, the cells were transfected with vectors encoding Myc-FLAG and Sin3b-HA, and after transfection, the lysates were immunoprecipitated with anti-FLAG to detect Myc-bound proteins. The immunoprecipitates were analyzed by Western blotting as in B. D, cellular Sin3b binds to recombinant Myc. Bacterially expressed Myc-GST was mixed with lysates of HEK293T cells transfected with pCDNA3-Sin3b-HA or empty vector (EV). Myc-GST was incubated with glutathione-Sepharose, and the pulled down material was analyzed by immunoblotting with the indicated antibodies. Sin3b expression in the input lysates is shown at the right. E, recombinant Sin3b and Myc proteins interact in vitro. A fusion protein consisting of GST and the N-terminal domain of murine Sin3b (first 390 amino acids (NSin3b)) and Myc purified protein were incubated with glutathione bound to magnetic beads. The levels of Myc, GST-NSin3b, and GST were determined in the inputs and in the pulled down material by immunoblotting.

Genome-wide Chip-seq Analysis—DNA binding sites for Sin3a and Max proteins in the K562 cell line identified by the ENCODE/HAI1B project were downloaded from the University of California, Santa Cruz (UCSC), database. Similarly, DNA binding sites for the c-Myc protein in K562 and GM12878 cell lines identified by the ENCODE/Open Chrom project (release of September 2012) were downloaded from the UCSC database. In order to restrict the analysis to only bona fide binding sites, in the case of Sin3a and Max, only those binding sites present in both technical replicates were used. In the case of Myc, binding sites from two human hematopoietic cell lines (K562 and GM12878) were compared and the analysis was restricted to the common binding sites. To detect those binding sites present in gene promoters, gene information from the RefSeq database was downloaded from the UCSC database (GRCh37/hg19 RefSeq release 55), and information 1 kb upstream and downstream of the transcription starting site for each gene (as anno-
tated in the UCSC browser) was extracted. All downloaded files were homogenized to bed format using perl and awk scripts written in-house (available upon request). Finally, all comparisons between the number of binding sites of different proteins were performed using the intersectBed tool from the BedTools suite (44). A minimum of 10% of sequence overlap between different binding sites was required. The normalization of binding sites/Mb was estimated considering 51.3 Mb of total promoter sequence (according to RefSeq) and 3 Gb of total genomic sequence. RNA-seq BAM files from ENCODE/Caltech corresponding to two non-strand-specific RNA 75-bp paired-end experiments in the K562 cell line were downloaded from the UCSC database. RPKM (reads per kb per million mapped reads) were calculated using Cufflinks v2.0.2, and the mean of the two replicates were used for forward analysis. Only genes with Myc, Sin3a, or both in their promoter (defined as above) and with expression levels of $\frac{\text{RPKM}}{\text{H11022}}$ 5 RPKM were selected.

Accumulated frequencies were normalized against the number of expressed genes in each case and plotted against the RPKM.

RESULTS

Sin3b Identified as a Myc Interactor in a Yeast Two-hybrid Assay—To identify new Myc interactors, the ORF of human Myc was used as bait in a two-hybrid assay in the S. cerevisiae AH109 strain. The vector pGBK7-Myc was co-transformed with a NIH3T3 library based on the pACT2 vector. pGBK7-Myc with empty vector did not give positive colonies (Fig. 1A, left). Also, the co-transformation of pACT2-Myc and pLAM5 (which encodes for lamin C) did not allow growth in restriction medium (Fig. 1A, right), indicating that the positive clones obtained in the screening were not artifacts due to fortuitous interactions. Sixty positive clones (i.e. growing in selective medium SC – AHLW) were detected in our screens. We focused on one of them, clone M168, which encoded a region of Sin3b, a transcriptional co-repressor known to interact with Mxd proteins. The region of clone M168 comprised amino acids 5–194 of the mouse Sin3b protein (Fig. 1A). This region of Sin3b encompasses the PAH1 domain (amino acids 30–100) and part of the PAH2 domain (amino acids 145–230) (Fig. 1A, bottom panel). Next, we confirmed this interaction in animal cells. Expression vectors for tagged Myc and full-length Sin3b were transfected into HEK293T cells, and the lysates were immunoprecipitated for Sin3b and Myc using antibodies against the corresponding tags. The results showed that Myc was present in the immunoprecipitates for Sin3b (Fig. 1B), and Sin3b was present in the immunoprecipitates for Myc (Fig. 1C). The results of the two-hybrid assay indicated a direct interaction between Myc and Sin3b. To confirm this finding, extracts of HEK293T cells transfected with Sin3b were incubated with...
full-length Myc-GST recombinant protein purified from bacteria. After pull-down, Sin3b was readily detected as bound to Myc (Fig. 1D). Finally, we assayed the binding between recombinant Myc and Sin3b proteins. The results showed that a fusion protein consisting of GST and the three first PAH domains of Sin3b (termed GST-NSin3b) could pull down Myc protein (Fig. 1E). The GST protein, used as a control, did not bind Myc in this experiment. These results support the hypothesis of a direct interaction between Myc and Sin3b.

Interaction between Endogenous Myc and Sin3b Proteins—
We next asked whether endogenous Myc and Sin3b interacted in the cells. We selected for this purpose human myeloid leukemia cells K562, as they are known to express high levels of Myc and also high levels of endogenous Sin3b. We conducted immunoprecipitation with anti-Myc, and Sin3b was detected in the Myc immunoprecipitates (Fig. 2A). The results showed that at least a fraction of endogenous Myc and Sin3b proteins interacted in K562 cells. Most of Myc and Sin3b localized in the cell nuclei, and the expectation would be that the Myc-Sin3b complexes would also be nuclear. By immunofluorescence we showed that Myc and Sin3b co-localized in the cell nuclei (Fig. 2B). The total expression of Myc and Sin3b was assessed by immunoblot (Fig. 2C). As a control, we used cells treated for 24 h with 20 nM TPA, a treatment that reduces Myc expression (45). To confirm the interaction between endogenous Myc and Sin3b and the cell compartment in which the interaction took place, we performed isPLA in K562/S cells. This technique allows the visualization of interactions of endogenous proteins and their localization in the cell, avoiding possible artifacts caused by protein overexpression or protein misfolding as in the FRET technique (43, 46). We used rabbit anti-Sin3b and mouse anti-Myc primary antibodies and secondary antibodies conjugated to probe oligonucleotides. The bound secondary antibodies guided the
formation of circular DNA strands when bound in close proximity. The circular DNA strands in turn served as templates for localized rolling-circle amplification, allowing the visualization of Myc-Sin3b complexes. We conducted isPLA in growing cells and in TPA-treated cells. As positive controls we also tested the interaction between Myc and Max and between Mxd1 and Sin3b. The expression of Mxd1 in these cells was assessed by immunoblotting (Fig. 2C). The results showed a clear positive isPLA signal in the nuclei of the cells. This signal was dramatically reduced in those cells in which Myc levels were abated by TPA treatment (Fig. 2D). Quantification of the corresponding signals is shown in Fig. 2E. Consistent with the lower Myc expression in TPA-treated cells, the level of interaction was also reduced in these cells. In contrast, the signal of Sin3b-Mxd1 was consistent with the lack of effect of TPA on Sin3b and Max levels (Fig. 2, B and C). Overall, the results demonstrated that endogenous Myc and Sin3b interact in the cell nuclei.

Myc Interacts with Sin3b through Myc Box III—The data from the yeast two-hybrid assay suggested that Sin3b interacted with Myc through the first two PAH domains (Fig. 1). To dissect the Myc regions involved in the interaction with Sin3b, we transfected HEK293T cells with Sin3b and a collection of six Myc mutants (Fig. 3A, upper panel) and conducted co-immunoprecipitations to identify the mutants with bound Sin3b. The results (Fig. 3A, lower panel) showed that the mutants MycD157–262 and MycD186–203 did not bind to Sin3b. Therefore, we concluded that the region comprising amino acids 186–203 was required for the interaction. The mutant lacking the helix-loop-helix/leucine zipper domain, which is responsible for the interaction with Max, was able to interact with Sin3b. To confirm that the interaction was independent of Max, we conducted the immunoprecipitation in PC12-derived UR61 cells, which lack a functional Max gene, expressing instead a truncated and rearranged Max (39, 47). Expression vectors for tagged Myc and Sin3b were transfected into UR61 cells, and after the immunoprecipitation of the protein lysate with anti-FLAG (for Myc) and anti-HA (for Sin3b), the presence of both proteins in the immunoprecipitated fraction was investigated by immunoblotting. The results showed that Myc and Sin3b co-immunoprecipitate in UR61 cells (Fig. 3B). Endogenous Myc and Sin3b of UR61 cells also co-immunoprecipitated (Fig. 3B, right panel). Overall, these results confirmed that Max was not required for Myc to interact with Sin3b.

Sin3b Antagonizes Myc Transactivation Activity—Sin3b functions as a transcriptional repressor through a mechanism involving recruitment of HDACs. Therefore, we tested whether Sin3b modified the transcriptional activity of Myc. We performed luciferase assays using a reporter carrying a minimal promoter with four Myc-binding E-boxes upstream of the luciferase gene. As a control reporter, we used the same Myc-responsive reporter construct but with mutated E-boxes, which are unable to bind to Myc-Max. The results showed that co-expression of Sin3b clearly reduced transactivation of the promoter by Myc in HEK293T cells (Fig. 4A).

We then studied the effect of Sin3b on the transactivation elicited by wild-type Myc and the Myc mutant MycD186–203, which is unable to bind to Sin3b. The results showed that although the MycD186–203 mutant activated the luciferase reporter to a similar extent as wild-type Myc, Sin3b was unable to impair transactivation by the Myc mutant (Fig. 4B), suggesting that direct interaction was required for the repression of Myc transcriptional activity by Sin3b. The results described above indicated that the Myc mutant lacking the Max interaction domain could still bind to Sin3b. To investigate whether Max was required for Sin3b-induced impairment of Myc transactivation activity, we carried out luciferase assays in the UR61 cell line. It was reported previously that Myc activates this reporter construct in the absence of Max in UR61 cells (39). We showed that Sin3b
efficiently impaired Myc-dependent transactivation in UR61 cells, i.e. in the absence of Max (Fig. 4C).

**Sin3a Also Binds Myc—** We then investigated whether Myc also bound to Sin3a, the other member of the Sin3 family. We transfected Myc and Sin3a expression vectors into HEK293T cells, and co-immunoprecipitation assays were carried out as described above. The results showed that Myc also formed complexes with Sin3a (Fig. 5A). We compared the effects of Sin3a and Sin3b on Myc-mediated transactivation. The results of the luciferase assays indicated that Sin3a and Sin3b were similarly efficient in reducing the transcriptional activity of Myc (Fig. 5B). Further, similar to Sin3b, Sin3a showed no effect on the transcriptional activity of the MycD186–203 mutant (Fig. 5C), suggesting that the 186–203 region of Myc was responsible not only for Sin3b but also Sin3a binding.

**Histone Deacetylase Also Is Recruited by Sin3b-Myc Complexes—** As described in the Introduction, Sin3 proteins are known transcriptional repressors, and the mechanism of action involves the recruitment of HDACs to induce a less active chromatin conformation. To find out whether the activity of HDACs was involved in the decrease of the transcriptional activity of Myc, we first used the HDAC inhibitors TSA and vorinostat. In the presence of these inhibitors, Sin3b was unable to repress Myc activity (Fig. 6A). To confirm the involvement of the HDACs, we asked whether the forced expression of HDACs enhanced the repressive effect of Sin3b on Myc transcriptional activity. The cells were transfected with vectors for Myc-FLAG and Sin3b-HA, and after transfection, the lysates were immunoprecipitated with anti-FLAG (to detect Myc) and anti-Sin3a antibodies. Only the band corresponding to Sin3a-myc is shown. Immunoprecipitation experiments with IgG were conducted as negative control. 8, HEK293T cells were transfected with the indicated vectors or the empty vector (EV). Twenty-four hours after transfection, the effect of Sin3a on Myc transcriptional activity was analyzed by a luciferase assay using a promoter-luciferase construct containing four Myc-responsive elements. Data were normalized to the β-galactosidase activity. Data are mean values ± S.E. of three independent experiments and were measured in duplicates. C, the Myc-(186–203) region is required for Sin3a-mediated repression. HEK293T cells were transfected with the indicated vectors or empty vector and the E-box luciferase reporter. 24 h after transfection the Myc transcriptional activity was analyzed as described in the legend for Fig. 4. Data are mean values ± S.E. of six independent experiments. **, p < 0.01.
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Myc and Sin3 Co-localize on Chromatin—As an important fraction of Myc in the cell nuclei is bound to chromatin, we asked whether Sin3 can also be found at Myc-bound chromatin sites. Therefore we employed ChIP assays to analyze Myc-Sin3b co-localization on some Myc target sites. We used the P493-6 human lymphoblastoid cell line, which displays conditional Myc expression with the Tet-off system (37) (Fig. 7A). The ChIP assays, conducted on several E-box-containing promoter regions described previously as binding Myc (48), showed that this co-occupancy also occurred in promoters, although the sites with Myc without Sin3a were more frequent (Fig. 7B). Taken together, the results strongly suggest that a fraction of Myc bound to chromatin also interacts with Sin3a. Interestingly, the coincidence of Myc and Sin3a in the proximal promoters does not correlate with a lower expression with respect to genes with Myc alone (Fig. 8C).

Sin3b Deacetylates Myc and Promotes Myc Degradation—The fact that we did not find correlation between the presence of Sin3a/b and transcriptional repression by Myc in most genes led us to the hypothesis that the major effect of Sin3-HDAC binding was not at the level of transcriptional repression. Myc has been shown to be acetylated, which regulates Myc stability (49–52). On the other hand, it has been reported that Sin3a induces deacetylation of Myc protein resulting in decreased Myc stability (53). We asked whether this was also the case with Sin3b. HEK293T cells were transfected with expression vec-

ments were performed in HEK293T cells, and the results showed that HDAC1 was present in the complexes with Sin3b and Myc (Fig. 6C, left panel). Moreover, endogenous HDAC1 co-immunoprecipitated with both endogenous Myc and Sin3b in K562/S cells (Fig. 6C, right panel).

Moreover, the genome-wide pattern of Myc and Sin3a (but not Sin3b) binding in several human cell lines has been determined by ChIP-seq by the ENCODE project consortium (data available at the University of California Santa Cruz browser). The comparison of Myc and Sin3a binding patterns on K562 cell line revealed that Sin3a is present in ~20% of the Myc binding sites on the genome (Fig. 8A). Mxd proteins may also recruit Sin3a to the same sites, and thus the actual fraction of Sin3a bound to Myc-binding regions is unknown. To assess whether the Myc-Sin3a co-localization on chromatin was higher in promoters (defined as 1 kb upstream and downstream from the transcription start site of the genes), we quantified the binding sites normalized to Mb of genomic DNA. The results show that this co-occupancy also occurred in promoters, although the sites with Myc without Sin3a were more frequent (Fig. 8B).

FIGURE 7. Co-localization of Myc and Sin3b in chromatin. A, repression of Myc expression by tetracycline in P493-6 cells. Cells were treated for 24 h with 0.1 μg/ml tetracycline (Tet), and the levels of Myc were determined by immunoblot. B, ChIP of Myc and Sin3b on the E-box-containing regions of NCL, CDK4, SRD5A1, and HES1 genes (39). P493-6 cells were treated with tetracycline for 72 h and then washed, and grown in medium without tetracycline for another 12 h to induce Myc expression. The data are mean values of five experiments normalized against the values obtained with IgG for each amplicon. C, mRNA expression of the indicated genes in cells with induced Myc (black bars) versus uninduced cells. Cells were treated as described in B, D, co-occupancy of Myc and Sin3b on DNA assayed by re-ChIP. ChIP for Myc (left panel) and sequential ChIP for Sin3b of Myc-bound chromatin (right panel) were performed on the E-box-containing regions of the promoters of CAD and LDHB genes of K562 cells. Data are mean values of two independent experiments.
Sin3b levels upon transfection of siRNAs in human K562 cells or Rat1a fibroblasts resulted in the up-regulation of Myc protein (Fig. 9D). We asked whether the deacetylation of Myc mediated by Sin3b-HDAC modified the stability of Myc protein. HEK293T cells were co-transfected with Sin3b and Kat2a expression vectors, and the protein stability was assayed by cycloheximide treatment. The immunoblot results showed a faster Myc degradation in Sin3b-expressing cells (Fig. 9E). Altogether, the data suggest that HDAC1 is recruited to Myc-Sin3b complexes, which leads to the deacetylation and subsequent destabilization of Myc.

**Low Sin3b Correlates with Breast Cancer Progression**—The expression of Sin3b is reduced in many cancer types and most clearly in breast cancer (ONCOMINE database and Ref. 54). Moreover, the meta-analysis of Sin3b mRNA expression in data sets available in public breast carcinoma database showed that low Sin3b expression was associated with a decrease in overall free survival and in distant metastasis-free survival (Table 3 and Fig. 10A). These observations, along with the above results, prompted us to explore the correlation between Sin3b and Myc protein levels in breast cancer. We analyzed Sin3b and Myc expression by immunohistochemistry in 102 infiltrating ductal breast carcinomas. We observed a lack of expression of Sin3b in 57% of the samples. Interestingly, we detected a strong association between low Sin3b expression in the primary tumors and distant metastasis (Fig. 10B). These observations indicated that Sin3b expression could be considered as a prognosis marker in breast cancer. In addition, we found that tumors with high Sin3b expression showed a tendency to present low Myc protein levels, although the association was weak (55.8% Myc-low versus 44.2% Myc-high, p = 0.06).

**DISCUSSION**

We have described here a direct interaction between Myc and the transcriptional corepressor Sin3b. We initially discovered this interaction by yeast two-hybrid screening. Furthermore, Myc and Sin3b co-immunoprecipitated in human HEK293T and rat UR61 cells upon transfection of expression vectors. Endogenous Myc and Sin3b also interacted in K562 cells, as shown by co-immunoprecipitation and iPLA. Moreover, we also detected the interaction of Sin3b with purified recombinant Myc, and we confirmed this interaction using purified Myc protein and a purified fusion Sin3b protein consisting of GST and the N-terminal region of Sin3b. Interestingly, in a previous report on two-hybrid screen designed to search Sin3b interacting peptides, two interacting peptides were found with a sequence similar to Myc Box III (55) (not shown). Although Sin3a did not appear in our initial two-hybrid screen, Sin3a also co-immunoprecipitated with Myc in cells and exerted the same effects on Myc-dependent transcription as did Sin3b. We also showed that Sin3b and Myc bind to the same promoter regions. Also, the genome-wide ChIP-seq data available in the UCSC genome browser reveal that Sin3a and Myc co-localize in a relevant fraction of chromatin sites. Taken together, the data strongly argue in favor of a direct interaction between Myc and Sin3b.

The Sin3b region found in the two-hybrid positive clone corresponds to amino acids 5–194, encompassing most of the
PAH1 and PAH2 domains (amino acids 30–230). Myc mutational analysis showed that the Sin3b-interacting region includes amino acids 186–203. This region essentially corresponds to the conserved Myc Box III (56). Interestingly, Max is not required for Sin3b binding to Myc, and Sin3b impaired Myc transcriptional activity on E-box-carrying promoters. However, Sin3b and Sin3a showed no effect on the transactivation mediated by the Myc mutant lacking the region required for Sin3 interaction (amino acids 186–203).

It has been reported that Myc represses some genes through the recruitment of HDACs and that Myc Box III is required for this activity (57). We found that the Myc region involved in this repression was the same region required for Sin3b and Sin3a interaction.

### TABLE 3

Correlation of Sin3b expression with breast cancer survival and metastatic capacity

The breast microarray and clinical data were obtained from the International Cancer Research database. Several independent data sets of breast carcinoma series were analyzed. A statistical survival analysis of breast samples was performed using ROCK and SPSS software. The results corresponding to three studies are shown.

<table>
<thead>
<tr>
<th>Breast cancer data set source</th>
<th>Low expression of Sin3b correlated to decreased overall survival</th>
<th>Low expression of Sin3b correlated to decreased distant metastasis-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van de Vijer et al. (59)</td>
<td>0.026</td>
<td>0.032</td>
</tr>
<tr>
<td>Perreard et al. (60)</td>
<td>0.041</td>
<td>0.021</td>
</tr>
<tr>
<td>Chin et al. (58)</td>
<td>0.036</td>
<td>0.001</td>
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* Log-rank test.
Therefore, we propose that Sin3 mediates the interaction of Myc with HDAC1, although interaction with other protein deacetylases cannot be ruled out. This proposal is based on these findings: (a) the co-expression of HDAC and Sin3b enhanced the repressive effect on E-box-containing reporters; (b) HDAC inhibitors (e.g. TSA and vorinostat) impaired the effect of Sin3b on Myc-mediated transcription; and (c) overexpression of HDAC alone did not modify Myc transcriptional activity. Therefore, Sin3b acts as a negative modulator of Myc transcriptional activity.

Several concomitant mechanisms could explain the repression of Myc activities by Sin3b. One would be the recruitment of Mxd protein to the promoter to exert transcriptional repression. This model is depicted in Fig. 11A. Although this mecha-
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nism must operate in vivo, by itself it cannot explain our results, as the D186–203 Myc mutant did not interact with Sin3b and yet transactivated an E-box reporter even when Sin3b was co-expressed. Thus, the increase in Mxd-Sin3b-HDAC complexes on chromatin was not the main mechanism responsible for the diminished transcriptional activity of Myc in the presence of Sin3b.

A second explanation would be that the effect of Sin3b depends on histone deacetylation in the regulatory region of Myc-bound genes, leading to transcription repression (schematized in Fig. 11B). However, our ChIP results argue against this hypothesis as the major mechanism. In human leukemia cells, we found that both Myc and Sin3b bound to some promoters regardless of whether the gene was induced or repressed (Fig. 7), and Sin3b binding at these sites was not related to histone acetylation marks (48). Also, the combined analysis of ChIP-seq and RNA-seq data generated by the ENCODE consortium revealed that in K562 cells, the coincidence of Myc and Sin3a on the proximal promoters did not correlate with a lower expression with respect to genes with Myc alone (Fig. 8). Thus, our data do not support a direct role of Sin3b in transcriptional repression of Myc target genes. Given that the binding of Myc and Sin3b to chromatin can be transient, we cannot rule out an effect of Sin3b binding to Myc that leads to gene repression via histone deacetylation. Actually, Myc-mediated repression of some genes (e.g. GADD45 and ID2) in Rat1a cells was impaired by co-transfection of Sin3b siRNA (not shown). However, taken together, the ChIP data are not consistent with this mechanism being the major one responsible for the decrease in Myc activity when Sin3b is co-expressed.

A third explanation is that Sin3b leads to reduced Myc levels. It is reported that Sin3a induces Myc deacetylation (53), and we have confirmed the same effect for Sin3b. Although the underlying mechanisms are not clear yet, it has been reported that acetylation of Myc by acetyltransferases (e.g. GCN5, Tip60, and p300/CBP) usually results in an increase in protein stability (49–53), although Myc deacetylation by Sir1 results in increased stability (52). We showed that Sin3b decreased Myc levels, whereas Sin3b depletion augmented Myc levels. The simplest explanation is that Sin3b interaction contributes to Myc deacetylation leading to reduced Myc levels, as schematized in Fig. 11C. In agreement with the findings in cultured cells, we also found an association between high Sin3b and low Myc in human breast cancer samples (although the data were not statistically significant). In summary, the data suggest that, at least in some cell types, Sin3b controls Myc levels through a mechanism that involves protein deacetylation and destabilization. Further work is required to dissect the underlying mechanisms. On the other hand, transcriptional repression by Mxd of Myc target genes can also be mediated by Sin3b. Thus, Sin3b may reduce Myc activity through two parallel pathways: the canonical Sin3b-Mxd interaction, resulting in Myc target gene repression, and the reduction of Myc levels, as shown in this work. The effect of Sin3b on Myc activity opens possibilities of therapeutic intervention for Myc-driven tumors.

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