**Oncogenic Protein MTBP Interacts with MYC to Promote Tumorigenesis**

Brian C. Grieb, Mark W. Gramling, Maria Pia Arrate, Xi Chen, Stephen L. Beauparlant, Dale S. Haines, Hua Xiao, and Christine M. Eischen

**Abstract**

Despite its involvement in most human cancers, MYC continues to pose a challenge as a readily tractable therapeutic target. Here we identify the MYC transcriptional cofactors TIP48 and TIP49 and MYC as novel binding partners of Mdm2-binding protein (MTBP), a functionally undefined protein that we show is oncogenic and overexpressed in many human cancers. MTBP associated with MYC at promoters and increased MYC-mediated transcription, proliferation, neoplastic transformation, and tumor development. In breast cancer specimens, we determined overexpression of both MYC and MTBP was associated with a reduction in 10-year patient survival compared with MYC overexpression alone. MTBP was also frequently co-amplified with MYC in many human cancers. Mechanistic investigations implicated associations with TIP48/TIP49 as well as MYC in MTBP function in cellular transformation and the growth of human breast cancer cells. Taken together, our findings show MTBP functions with MYC to promote malignancy, identifying this protein as a novel general therapeutic target in human cancer.

**Introduction**

c-MYC (MYC) is an oncogenic transcription factor that has conserved function across species and cell types. MYC is overexpressed/dysregulated in ~70% of human malignancies, often correlating with poor patient outcomes (1–4). Although recently Myc was shown to act as a global transcriptional amplifier (5, 6), it is known to function by binding promoters where it transcriptionally activates or represses many genes that control critical cellular processes (1, 7, 8).

Specifically, Myc promotes proliferation by transcriptionally repressing cell-cycle inhibitors, such as p15, p21, and p27, whereas transcriptionally activating genes, such as nucleolin (NCL), ornithine decarboxylase (ODC), carbamoyl-phosphate synthetase 2/aspartate transcarbamylase/dihydroorotase (CAD), and cyclin D2 (CCND2) that are needed for ribosomal assembly, polyamine generation, pyrimidine synthesis, and cell-cycle progression, respectively (1, 8). As such, Myc overexpression induces proliferation and transformation; cells initially inhibit these processes through activation of apoptosis or senescence (1, 4). Myc-mediated transcription is regulated, in part, by transcriptional cofactors. For example, the nuclear ATPases Tip48 (Pontin/RUVBL2) and Tip49 (Reptin/RUVBL1), that form hexomers or dodecamers, bind Myc and are necessary for Myc-mediated in vitro transformation (9–12). Despite the identification of these and other cofactors, a clear understanding of how Myc activity is regulated and the cofactors involved remains unresolved. Moreover, because Myc has proven difficult to directly target therapeutically, identifying proteins that regulate Myc function could provide novel therapeutic approaches for the treatment of cancers that rely on MYC.

The 104 kDa Mdm2-binding protein (MTBP) was originally identified in a yeast-two hybrid screen binding to Mdm2, a negative regulator of p53 (13). However, subsequent data demonstrated Mtbp does not regulate Mdm2 in vivo (14, 15). Instead, data suggested Mtbp may function in proliferation, as Mtbp expression increased in response to pro-proliferative factors and siRNA knockdown of Mtbp reduced proliferation regardless of p53 status (15, 16). In addition, we reported Mtbp heterozygosity limited the ability of Myc to promote proliferation and activate transcription of pro-proliferative target genes. Mtbp heterozygosity also delayed Myc-induced lymphomagenesis in mice (15). Here, we determined Mtbp is oncogenic and identified novel interactions between Mtbp and Tip48, Tip49, and Myc. Through these associations, Mtbp increased Myc-mediated transcription, proliferation, and transformation, while inhibiting Myc-induced apoptosis. Collectively, our data show MTBP is an oncogenic protein and a novel regulator of MYC.

**Authors’ Affiliations:** Departments of 1Pathology, Microbiology and Immunology and 2Biostatistics, Vanderbilt University Medical Center, Nashville, Tennessee; 3Department of Biochemistry, Temple University; 4Fels Institute for Cancer Research and Molecular Biology, Temple University; 5Institute for Physiology, Michigan State University, East Lansing, Michigan; and 6Department of Pathology, Microbiology and Immunology and 7Biolistics, Vanderbilt University Medical Center, Nashville, Tennessee

**Corresponding Author:** Christine M. Eischen, Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, C3321 MCN, 1161 21st Avenue South, Nashville, TN 37232. Phone: 615-322-3234; Fax: 615-343-1633; E-mail: christine.eischen@vanderbilt.edu

doi: 10.1158/0008-5472.CAN-13-2149
©2014 American Association for Cancer Research.
Materials and Methods

Cell culture, vectors, transfection, and infection
NIH3T3, HEK293T, H1299, HCC1806, MDA-MB-231, and Raji cells were cultured as described by the American Type Culture Collection (ATCC). HCC1806, MDA-MB-231, immortalized human mammary epithelial (HMLE) cells, rat fibroblasts, and human retinal epithelial cells were provided by Drs. J. Piettempol (Vanderbilt University, Nashville, TN), J. Sedivy (Brown University, Providence, RI), or D. Cortez (Vanderbilt University; refs. 17–19). Mouse embryonic fibroblasts (MEF) were isolated and cultured as previously described (20). Cell lines were recently obtained from ATCC or authenticated by STR profiling or similar method. Vectors are listed in supplemental information. H1299, NIH3T3, and 293T cells were transfected with FuGene 6 (Promega), Lipofectamine 2000 (Invitrogen), and calcium-phosphate, respectively. Cells were infected with retroviruses, as previously described (20).

Proliferation, cell cycle, apoptosis, and transformation assays
To measure proliferation, 1,000 to 5,000 cells were plated (triplicate) and MTT or MTS (Cell Titer 96 AQueous One Solution Proliferation Assay; Promega) assays were performed per manufacture's protocol. Viable cells were counted at intervals with Trypan Blue Dye. Cell cycle (Dean-Jett-Fox analysis) and apoptosis (sub-G1 DNA content) were evaluated by flow cytometry, following DNA staining with propidium iodide. To allow for Myc-induced apoptosis, cells were cultured under low serum (1%) conditions. Cleaved caspase-3 levels were evaluated by Western blot analysis. Foci formation after culturing cells for 7 days at low density was evaluated as described (21). Soft agar assays were performed as previously described (22).

Mice
Female athymic nude mice (Harlan) were injected subcutaneously (flanks) with NIH3T3 fibroblasts expressing Myc, Mtbp, both Myc and Mtbp, or vector controls. Tumor volume was calculated from electronic caliper measurements. Upon sacrifice, tumors were extracted, photographed, and weighed. All experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee and followed all federal and state rules and regulations.

Immunoprecipitation and Western blotting
Cells or tumors were lysed as previously reported (13,20,22–24). Equal amounts of protein were resolved by SDS-PAGE and Western blotted or were first immunoprecipitated using anti-Flag (M2; Sigma), anti-HA (F7; Santa Cruz Biotechnology), anti-Mtbp (K20; Santa Cruz), or isotype control antibodies as previously described (25). Antibodies against Flag (M2; Sigma), HA (F7; Santa Cruz or Roche, 115831600), Mtbp (B5; Santa Cruz), TIP48 [36569; Ab-Cam for Fig. 6A or from Dr. M. Cole (Dartmouth, Hanover, NH) for Fig. 2B], TIP49 (from Dr. M. Cole), Myc (C33; Santa Cruz Biotechnology or 06-340; Upstate Biotechnology), cleaved caspase-3 (D175; Cell Signaling Technology), and β-actin (AC15; Sigma) were used for Western blot analysis. For Fig. 2G, the whole cell lysates represent 2.5% of protein used for the immunoprecipitations.

Identification of MTBP binding proteins by mass spectrometry
Whole cell extracts from H1299 cells infected with retroviruses encoding Flag-tagged MTBP or GFP control were prepared and immunoprecipitated with anti-Flag immunofinity matrix (M2; Sigma) as described previously (23). Immunoprecipitates were eluted with Flag peptides, resolved by SDS-PAGE, and stained with silver as previously described (26). Silver-stained protein bands were excised and subjected to in-gel trypsin digestion. Peptides were analyzed by LC/MS-MS (see Supplementary Data).

In vitro binding assay
In vitro binding assays were performed as previously described (13), using MTBP and control MGA2 translated in rabbit reticulocyte lysate in the presence of 35S-methionine (TNT T7 Reticulocyte System; Promega) and recombinant GST and GST-tagged TIP48, TIP49, and MYC generated in bacteria and purified on glutathione beads. Complexes were allowed to form in 50 mmol/L Tris-HCl at pH 8.0, 200 mmol/L KCl, 2.5 mmol/L MgCl2, 1% Triton X-100 and 5% glycerol, and 0.1 mmol/L DTT with protease inhibitors. Samples were resolved by SDS-PAGE and detected by fluorography.

Immunofluorescence
p53-null MEFs grown on glass coverslips were processed, imaged, and analyzed as previously reported (22). Anti-Mtbp (K20; Santa Cruz), anti-Tip48 (Ab-Cam 36509), and/or isotype controls, followed by Alexa Fluor 594 and Alexa Fluor 488 (A11058 and A21206; Invitrogen) were used.

Chromatin immunoprecipitation
HEK293T cells were transfected with vectors encoding Flag-Mtbp, Flag-Mtbp mutants, Flag-Myc, HA-Myc, or empty vector control. Raji cells were used to chromatin immunoprecipitate (ChIP) endogenous MTBP and MYC. ChIP protocol from Upstate Biotechnology was followed except Raji cells were crosslinked for 1 to 2 hours. DNA was sheared into ~500 bp pieces with sonication (VirSonic 600). After removing aliquots of each for input controls, the remainder was immunoprecipitated with anti-Flag (M2; Sigma), anti-Mtbp (K20; Santa Cruz), anti-Myc (N262; Santa Cruz), or isotype control antibodies. For anti-Flag ChIP, no SDS was used. Sequential ChIP for Myc (anti-HA, F7; Santa Cruz) and then Mtbp (anti-Flag), was performed as previously described (27), except using formaldehyde as a cross-linking agent and sonication to shear DNA. Quantitative PCR of precipitated DNA described below.

Quantitative real-time PCR
NIH3T3 cells infected with an murine stem cell virus retrovirus encoding MycER (28) and transfected with nontargeting control siRNA or Mtbp siRNA (SMARTpool ON-TARGETplus; Thermo-Scientific) or vectors encoding Mtbp or Mtbp mutants were treated with 1 µmol/L 4-hydroxytamoxifen (4-OHT; Sigma) or ethanol vehicle control for 0, 6, or 8 hours. Total RNA was isolated, cDNA was generated, and quantitative real-time PCR (qRT-PCR) for Myc target genes was performed as previously described (15). For ChIP, qRT-PCR was performed.
MTBP Regulates MYC Oncogenic Activity

Figure 1. Mtbp is oncogenic. A, cell lysates of NIH3T3 cells expressing Flag-tagged Mtbp, Myc, or empty vector control were Western blotted. MTS assays (left) were performed, and viable cells were counted (right) at 24-hour intervals (*, $P < 0.01$, Myc or Mtbp vs. vector). B, cells described in A were placed in culture at low density and foci were quantified 7 days later (**, $P < 0.001$). C, Western blots and soft agar assays of NIH3T3 cells expressing RFP and YFP, Myc and RFP, or Mtbp and YFP; colonies quantified after 21 days (*, $P < 0.0001$; **, $P = 0.0036$). D, cells described in C were injected subcutaneously into nude mice at day 0, and tumor volume was measured at intervals (*, $P < 0.03$, RFP + YFP vs. Myc; **, $P < 0.05$, RFP + YFP vs. Mtbp). Number of mice indicated by n. Error bars are standard deviation (A–C) or standard error of the mean (D). P values determined by Student t tests.

Results

Mtbp has oncogenic activity

An Mtbp haploinsufficiency suppressed Myc-induced proliferation and lymphomagenesis (15), suggesting Mtbp functions as a pro-proliferative factor. In support of this theory, analysis of public mRNA expression and copy number data showed MTBP is overexpressed and/or amplified in many human cancers (Table 1 and Supplementary Table S1). To evaluate whether Mtbp overexpression contributes to cancer, the biologic effects of Mtbp overexpression were investigated. Similar to Myc, Mtbp overexpression significantly enhanced proliferation in NIH3T3 fibroblasts (Fig. 1A). This pro-proliferative effect of Mtbp was evident in fibroblasts and epithelial cells and in cells from different species (human, mouse, and rat; Supplementary Fig. S1), indicating a conserved function of Mtbp. In addition, elevated Mtbp levels increased foci formation of cells cultured at low density (Fig. 1B). Mtbp expression also significantly augmented soft agar colony formation, although the increase was moderate compared with that induced by the powerful oncogene Myc (Fig. 1C).

To further examine the oncogenicity of Mtbp, NIH3T3 fibroblasts overexpressing Mtbp were injected into the flanks of athymic mice, and tumor growth was assessed. Mtbp overexpressing fibroblasts formed palpable tumors by 36 days that continued to grow, whereas none of the negative controls had developed tumors by day 44 (Fig. 1D). As expected, Mtbp-induced tumor development was not as robust as that driven by Myc. However, the increased proliferative capacity and promotion of cellular transformation, in vitro and in vivo, indicate Mtbp is oncogenic.

Myc and the Myc transcriptional cofactors, Tip48 and Tip49, associate with Mtbp

Because MTBP has no identified functional domains that explain its oncogenic activity, we utilized an unbiased biochemical approach to identify proteins that bind MTBP. Flag-tagged MTBP was expressed in H1299 cells, and immunoprecipitated under stringent conditions. The resolved proteins were visualized by silver stain and identified by mass spectrometry as with primers specific for MYC-binding sites in promoter regions or up/downstream regions; values are relative to respective vector or IgG control and input DNA. Primer sequences are listed in supplement.

Patient data

Cancer patient survival and gene expression data were accessed from The Cancer Genome Atlas (TCGA; https://tcga-data.nci.nih.gov/tcga/) January to April 2013 (breast) and November 2013 (colon and lung adenocarcinoma). For Kaplan–Meier survival curves, normalized RNA-Seq data (version 2, level 3) were used as gene expression values, and the median was used to classify samples into high and low expression groups. Gene copy number alteration data were obtained from the cBioPortal for Cancer Genomics (http://www.cbioportal.org/public-portal/) May 2013. MTBP mRNA expression data in normal and cancer samples and statistics were obtained from Oncomine (www.oncomine.org) June 2013.
MTBP (104 kDa), the MYC transcriptional cofactors TIP49 (49 kDa) and TIP48 (48 kDa), and HSP70 (70 kDa; Fig. 2A and Supplementary Fig. S2). HSP70 was not investigated further as it is known to bind overexpressed proteins (29). Immunoprecipitation of the same lysates confirmed endogenous TIP48 and TIP49 co-immunoprecipitated with MTBP (Fig. 2B). Immunoprecipitations with tagged proteins further demonstrated the MTBP and TIP48/TIP49 interaction (Fig. 2C). Binding assays revealed in vitro translated MTBP, but not the control MGA2 yeast transcription factor, bound to both GST-tagged TIP48 and TIP49 (Fig. 2D). Mtbp was also localized to the nucleus and shared an overlapping nuclear distribution with Tip48 (Fig. 2E). Therefore, TIP48/TIP49 are novel MTBP binding proteins and likely directly bind MTBP.

Given Tip48/Tip49 are Myc transcriptional cofactors and directly bind Myc (10), we tested whether Mtbp interacted with Myc. Flag-tagged Mtbp and HA-tagged Myc co-immunoprecipitated one another (Fig. 2F). We detected endogenous association between MTBP and MYC in 2 cell lines driven by MYC and harboring amplified MYC (HCC1806 human breast carcinoma and Raji Burkitt lymphoma cells; Fig. 2G). However, in vitro binding assays did not show binding between MTBP and MYC (data not shown), indicating their interaction is likely not direct. Mtbp did co-immunoprecipitate Myc lacking an N-terminal region (MycΔ20–48; Fig 2H), but not a mutant lacking the Myc Box II (MBII) domain (MycΔ118–152). Therefore, the MBII domain, which is required for binding to Tip48/Tip49 and critical for Myc transcriptional and oncogenic activity (10), is
required for Mtbp association. In addition, the results indicate Mtbp associates indirectly with Myc by binding directly to Tip48/Tip49.

Mtbp associates with Myc at promoters

Through interactions with 2 Myc transcriptional cofactors and chromatin, we postulated Mtbp would associate with chromatin. To test this, we first separated chromatin-bound from soluble proteins (24). MTBP was primarily detected in the chromatin-bound fraction where MYC and histones reside, whereas little MTBP was in the soluble fraction with the ERK1/2 kinases (Fig. 3A). We then tested whether Mtbp associated with promoter regions bound and transcriptionally regulated by Myc (1, 8). Mtbp or Myc was expressed in 293T cells. ChIP with antibodies specific for Myc or Mtbp, but not immunoglobulin controls, showed the promoter regions of ODC, CAD, NCL, and CCND2 (genes Myc transcriptionally activates) were enriched, but not upstream or downstream elements (Fig. 3B and data not shown). Mtbp also immunoprecipitated the promoter regions of p21, p15, and p27; genes transcriptionally repressed by Myc (Fig. 3C). Similarly, endogenous MTBP was present at MYC-regulated promoters in Raji cells (Fig. 3D). Notably, sequential ChIP of Myc first followed by Mtbp showed enrichment at both Myc transcriptionally activated and repressed promoter regions (Fig. 3E), demonstrating the 2 proteins occupy the same sites concurrently. Thus, Mtbp and Myc interact together at Myc-targeted promoters.

Mtbp enhances the oncogenic activity of Myc

Because Mtbp and Myc associate together at promoters and both are overexpressed in cancers, we evaluated the effects of Mtbp overexpression on Myc-induced transcription. Mtbp was overexpressed in NIH3T3 cells expressing a 4-OHT regulatable form of Myc, MycER (28). Within 8 hours following MycER activation, cells overexpressing Mtbp showed enhanced induction of pro-proliferative Myc-regulated genes compared to cells with empty vector control (Fig. 4A), indicating increased Mtbp levels augment Myc transcriptional activity. These data are consistent with our previous study showing that reduced levels of Mtbp, because of a haploinsufficiency, resulted in decreased Myc-induced transcription of pro-proliferative genes (15) and data presented below.

To test whether Mtbp cooperates with Myc to promote proliferation, Myc, Mtbp, or both were overexpressed in NIH3T3 cells and proliferation measured. Although Myc and Mtbp individually increased proliferation rates over cells with
vector control, a large, significant increase in proliferation was observed in cells expressing both Mtbp and Myc (Fig. 4B). This cooperative effect was also observed in immortalized human mammary epithelial cells (Supplementary Fig. S3A). Cell-cycle analysis revealed a decrease in the percentage of cells in G0–G1 and an increase of cells in S-phase when both Mtbp overexpression promotes Myc oncogenic activity. A, MycER-expressing NIH3T3 cells were transfected with an empty vector or a vector encoding Flag-Mtbp. Western blots of whole cell lysates were performed. In addition, MycER was activated in the fibroblasts with 4-OHT for 0 or 8 hours. qRT-PCR was performed in triplicate for the indicated Myc target genes. All samples were normalized to β-actin (\( P = 0.0032; * *, \( P = 0.016; ** *, \( P = 0.0004)). B, NIH3T3 cells were transfected with empty vector or vectors encoding the indicated proteins. Western blots of WCLs were performed. MTS assays were performed at 24-hour intervals (\( P < 0.001 \text{ Myc vs. Myc + Mtbp}). C–E, NIH3T3 cells infected with two bicistronic retroviruses encoding the indicated proteins were analyzed. C and D, flow cytometry analysis after propidium iodide staining of DNA. The percentage of cells in each phase of the cell cycle was determined by Dean–Jett–Fox analysis (gray line; \( P = 0.012, \text{RFP + YFP vs. Myc}; ** *, \( P = 0.0296, \text{RFP + YFP vs. Mtbp}; * *, \( P = 0.0191, \text{RFP + YFP vs. Myc + Mtbp); E}, \text{representative histograms shown (C). The proportion of cells with sub-G1 DNA content was measured following 1% serum culture conditions for 24 hours (D): \( P = 0.0025, \text{RFP + YFP vs. Myc}; ** *, \( P = 0.0018, \text{Myc vs. Myc + Mtbp}). Western blots were performed (D). E, cells were subjected to soft agar colony assay, and colony number was quantified after 10 days. Error bars are standard deviation. P values determined by Student t tests.
Myc and Mtbp were co-overexpressed; this was particularly evident when growth factors were limiting, but was also observed when cells were in 10% serum (Fig. 4C and Supplementary Fig. S3B). Because this difference in S-phase may not fully account for the considerable increase in cell number with Mtbp and Myc co-overexpression, we also evaluated apoptosis. Within 24 hours of culturing the cells in low serum conditions, which allows Myc to induce apoptosis, there was a significantly reduced percentage of cells with sub-G1 DNA and lower levels of cleaved caspase-3 when Mtbp and Myc were co-overexpressed, compared with cells overexpressing Myc alone (Fig. 4D). Mtbp overexpression alone had no effect on apoptosis. To determine if Mtbp also modulates Myc transforming activity, soft agar assays were performed. Co-overexpression of Mtbp and Myc significantly increased colony formation over that of Myc alone in NIH3T3 and human mammary epithelial cells (Fig. 4E and Supplementary Fig. S3A). Therefore, Mtbp promotes Myc-driven proliferation and in vitro transformation by enhancing the proliferative capacity of Myc and inhibiting Myc-induced apoptosis.

Mtbp increases Myc-induced in vivo transformation and cooperates with MYC to decrease breast cancer patient survival

To evaluate whether Mtbp and Myc cooperate in transformation in vivo, NIH3T3 cells expressing Mtbp, Myc, or both were injected subcutaneously into athymic mice and tumor growth was monitored. Compared with cells overexpressing Myc or Mtbp alone, cells co-overexpressing Mtbp and Myc formed palpable tumors sooner, and the tumors grew larger faster (Fig. 5A) and weighed more upon extraction at day 34 (Fig. 5B). These data indicate Mtbp enhances the ability of Myc to promote cellular transformation in vivo.

To determine if the cooperation observed between Mtbp and Myc is reflected in human malignancy, we evaluated a breast cancer patient population. MYC is a critical contributor to breast tumorigenesis and progression, and increased MYC transcriptional activity, which we observed with Mtbp overexpression (Fig. 4A), was recently linked to poor patient outcomes (2, 3). Analysis of RNA-sequencing data from 844 breast cancers in TCGA showed patients with breast cancers that had high expression of both MYC and MTBP mRNA exhibited significantly reduced 10-year survival compared with those that were MYC high and MTBP low (P = 0.0314; Fig. 5C), indicating MTBP levels influence the impact of MYC on patient prognosis. This trend was also observed in patients with colon and lung (adenocarcinoma) cancer (Supplementary Fig. S4). In addition, evaluation of TCGA copy number alterations in 20 different human cancers revealed that among those that had amplified MYC, MTBP was frequently co-amplified (Supplementary Table S1; refs. 30 and 31). This occurred even though MTBP and MYC are 7.2 megabases apart at 8q24.12 and 8q24.21, respectively (Supplementary Fig. S5). Notably, in 200 of 913 (21.9%) breast carcinomas with amplified MYC, 85% co-amplified MTBP (Supplementary Table S1). Thus, patient data suggest increased expression of both MTBP and MYC is selected for during tumorigenesis and can negatively impact patient survival.
C-terminus of Mtbp associates with and inhibits Myc

Because breast cancer patient data indicated MTBP and MYC co-overexpression reduces 10-year survival, identifying the domains of MTBP required for interaction with TIP48/TIP49 and MYC should provide insight for novel therapeutic interventions. Therefore, because sequence analysis revealed no potential functional motifs to guide mutation generation, we divided Mtbp (aa 1-894) into thirds. The central (aa 299-597)
and C-terminal (aa 597-894) Mtbp mutants were detectable by Western blot analysis, whereas the N-terminal mutant (aa 1-298) was not (Fig. 6 and data not shown), suggesting it was unstable. Immunoprecipitations showed endogenous Tip48 co-immunoprecipitated with full-length Mtbp and the C-terminal Mtbp mutant, but not the central domain mutant (Fig. 6A). The commercially available Tip49 antibody was not of sufficient quality to conclusively determine Tip49 association. However, given the interaction between Mtbp and both Tip48 and Tip49 (Fig. 2) and reports that Tip48 and Tip49 form heterocomplexes (11), Mtbp likely binds Tip48/Tip49 complexes through its C-terminus. In addition, consistent with our data indicating Mtbp associates with Myc by binding Tip48/Tip49, the C-terminal Mtbp mutant, but not the central domain Mtbp mutant, co-immunoprecipitated Myc (Fig. 6B). Furthermore, ChIP analyses showed the C-terminal Mtbp mutant, but not the central domain Mtbp mutant, enriched Myc-regulated promoter sequences (Fig. 6C). Therefore, the C-terminus of Mtbp is sufficient to mediate the interaction with Tip48/Tip49, Myc, and Myc-bound promoters.

To determine whether the C-terminal Mtbp mutant can impact Myc activity, we evaluated Myc-induced transcription, proliferation, and transformation. In contrast to full-length Mtbp, the C-terminal Mtbp mutant blunted MycER-induced proliferation, and transformation. Mtbp, the C-terminal Mtbp mutant blunted MycER-induced proliferation, and transformation. In contrast to full-length Mtbp, the central Mtbp mutant had no significant effect. Similarly, MTBP knockdown in human or murine cells reduced proliferation (Supplementary Fig. S7), consistent with previous reports (15, 16). To expand our analysis, we further evaluated the C-terminal Mtbp mutant using human breast carcinoma cell lines. HCC1806 cells contain amplified MYC and MDA-MB-231 cells are MYC-dependent (32, 33). In both, the C-terminal Mtbp mutant significantly reduced proliferation compared with the central domain mutant or empty lentivirous control (Fig. 6G and Supplementary Fig. S8). Thus, the C-terminal Mtbp mutant seems to function as a dominant negative inhibitor of Myc, resulting in reduced Myc-mediated transcription, proliferation, and transformation.

### Discussion

Several studies suggested Mtbp has a role in cancer development and possibly progression, but its function remained unresolved (13–15). Initially, Mtbp was thought to regulate the protein from which it received its name, Mdm2, a negative regulator of p53 (13). However, studies utilizing genetically engineered mice did not support this function of Mtbp in vivo (14, 15). Here, we make the unexpected discovery that Mtbp has oncogenic functions and reveal a new mechanism by which Mtbp promotes proliferation and transformation. We show Mtbp is part of a Tip48/Tip49 complex that binds Myc and regulates Myc-mediated transformation (Supplementary Fig. S9). Our data also show Mtbp is a novel transcriptional regulator of Myc that enhances Myc-dependent activation of genes necessary for proliferation and transformation. Mtbp also redirects Myc activity away from apoptosis. Therefore, this study reveals Mtbp is a novel regulator of Myc and significantly advances knowledge into Myc-induced transformation. These results position MTBP as a possible novel drug target for the 70% of human cancers that depend on MYC for continued growth and survival.

Although Myc has been studied for 30 years, its specific functions and the proteins that regulate it continue to be elucidated (1, 4). Myc has been shown to associate with several transcriptional cofactors that regulate its transformation activity, including Tip48 and Tip49, TRRAP, Tip60, GCN5/PCAF, CBP/p300, INI1, Skp2, and others, and yet, their regulation of Myc-mediated transcription is not fully understood (1, 4, 10, 34). For Tip48 and Tip49 specifically, they form a hexamer/dodecamer, bind Myc, and facilitate Myc-mediated transformation (9–12). Although Tip48 and Tip49, through interactions with Myc, are critical for cell growth and proliferation during Drosophila and Xenopus development (9, 12), their precise function in relationship to Myc remains unresolved. With mass spectrometry, we identified TIP48 and TIP49 as novel interacting proteins of MTBP. In vitro binding experiments indicated MTBP likely binds TIP48 and TIP49 directly and...

### Table 1. MTBP mRNA is overexpressed in many human cancers

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Fold change</th>
<th>P value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast ductal carcinoma (N = 78, C = 639)</td>
<td>1.87–2.66</td>
<td>1.3E–02 to 3E–23</td>
<td>(40–42)</td>
</tr>
<tr>
<td>Cervical carcinoma (N = 8, C = 20)</td>
<td>2.12</td>
<td>1.8E–06</td>
<td>(43)</td>
</tr>
<tr>
<td>Colorectal carcinoma (N = 97, C = 273)</td>
<td>1.60–3.71</td>
<td>4.6E–04 to 1.2E–19</td>
<td>(44, 45)</td>
</tr>
<tr>
<td>Gastric adenocarcinoma (N = 50, C = 91)</td>
<td>1.43–2.25</td>
<td>3.1E–04 to 1.9E–10</td>
<td>(46)</td>
</tr>
<tr>
<td>Glioblastoma (N = 23, C = 81)</td>
<td>2.25</td>
<td>4.8E–04</td>
<td>(47)</td>
</tr>
<tr>
<td>Lung adenocarcinoma (N = 65, C = 45)</td>
<td>1.54</td>
<td>2E–07</td>
<td>(48)</td>
</tr>
<tr>
<td>Lung large cell carcinoma (N = 65, C = 19)</td>
<td>2.28</td>
<td>1.9E–06</td>
<td>(48)</td>
</tr>
<tr>
<td>Lung squamous cell carcinoma (N = 65, C = 27)</td>
<td>1.62</td>
<td>1.9E–08</td>
<td>(48)</td>
</tr>
<tr>
<td>Prostate carcinoma (N = 8, C = 13)</td>
<td>1.65</td>
<td>9.1E–04</td>
<td>(49)</td>
</tr>
<tr>
<td>Squamous cell carcinoma (N = 4, C = 11)</td>
<td>1.97</td>
<td>9.5E–04</td>
<td>(50)</td>
</tr>
</tbody>
</table>

NOTE: MTBP mRNA expression data in cancer (C) compared with normal (N) tissue from Oncomine; P values determined by Student t test.
and MYC indirectly. The association of Mtbp with Myc required the MBII domain of Myc, which is the domain that Tip48 and Tip49 directly bind (10). Furthermore, we detected Mtbp in complex with Myc at promoters transcriptionally activated or repressed by Myc, where others have also observed Tip48 and Tip49 (9, 11, 12, 34). The C-terminus of Mtbp was necessary for interaction with both Tip48 and Myc and for association with Myc-binding sites in promoters. Therefore, Mtbp, through its C-terminus, is in a transcriptional protein complex with Tip48-Tip49-Myc (Supplementary Fig. S9), a complex critical for Myc-induced transformation (10).

Mtbp alone was less oncogenic than Myc, an established powerful oncopogene (1, 4). However, when Mtbp and Myc were co-overexpressed, there was a dramatic increase in proliferation and in both in vitro and in vivo transformation. These results, together with the reduction in Myc-mediated apoptosis observed with increased levels of Mtbp, indicate Mtbp inhibits this negative consequence of Myc overexpression and enhances Myc-regulated proliferation and transformation. Moreover, we previously reported an Mtbp haploinsufficiency decreased Myc-mediated B-cell proliferation and delayed Myc-driven lymphoma development (15), indicating reduced levels of Mtbp inhibited the proliferative and transforming functions of Myc. Here, we present new mechanistic data that explains both the overexpression and the reduced expression effects of Mtbp on Myc activity and tumorigenesis. Specifically, our results show Mtbp is in a transcriptional complex with Myc by binding Tip48/Tip49, and Mtbp interacts with Myc at promoters and significantly enhances Myc-mediated transcription of pro-proliferative genes, leading to increased proliferation and transformation (Supplementary Fig. S9). This was revealed, in part, with the C-terminal fragment of Mtbp that mediates the interaction with the Tip48–Tip49–Myc complex that appeared to function as a dominant negative inhibitor of Myc. Our previous observation that an Mtbp haploinsufficiency led to reduced Myc-mediated transcription (15) and additional Mtbp knockdown data shown here also support the conclusion that Mtbp directly facilitates Myc-induced transcription. In addition, Myc controls the expression of many of its regulators. We previously reported that Mtbp seems to be a direct transcriptional target of Myc (15), and again here we detect increased Mtbp expression following Myc overexpression (Fig. 1A); these data suggest Mtbp is in a feedforward regulatory loop with Myc (Supplementary Fig. S9). Therefore, the data strongly indicate Mtbp modulates Myc transcriptional function through their association, and together they promote proliferation and tumorigenesis.

The pro-proliferative and oncogenic behavior of Mtbp and its cooperation with Myc are further supported by data showing MTBP expression is increased in many human cancers (see Table 1 and Supplementary Table S1; ref. 15). Moreover, we determined MTBP, which is 7.2 megabases apart from MYC on chromosome 8q24, is frequently selected for co-amplification with MYC, which should provide a previously unappreciated proliferative and transforming advantage to cells. There are negative consequences for patients with MTBP and MYC co-overexpression as evidenced by the significantly reduced 10-year survival for breast cancer patients with tumors with high levels of both MTBP and MYC compared with those with low levels of MTBP and high levels of MYC. A similar trend was also observed for patients with colon and lung cancer. In addition, the C-terminal mutant of Mtbp, which inhibited Myc activity, suppressed expansion of MYC-dependent breast carcinoma cell lines. Collectively, these results indicate MTBP is utilized by cancer cells to make MYC a more effective oncogene. Although lower levels of MTBP have been associated with increased metastasis of tumor cells (14, 35), cancer cells were shown to downregulate proliferation and MYC in favor of movement (36). One report does show decreased MTBP expression in a subset of head and neck cancer correlated with reduced survival (37). However, the pro-proliferative function of Mtbp is supported by data that Mtbp induces proliferation in cells from multiple species. Moreover, Mtbp levels are low in G0 and increase as cells progress through S-phase and into M-phase (15). MTBP was also recently linked to DNA replication origins and mitotic progression (16, 38). Therefore, although MTBP may have different roles in distinct cell types and tumor contexts, it seems to be pro-proliferative and a positive contributor to tumorigenesis in the majority of cell types evaluated.

Our studies also likely have broader implications. Although the focus of this manuscript has been on the regulation of Myc by Mtbp as part of a complex with Tip48/Tip49, Tip48/Tip49 are reported to bind several proteins, including the E2F1 and β-catenin transcription factors as well as the INO80 and Tip60 complexes (11). Therefore, Mtbp may regulate other proteins that bind Tip48/Tip49, and these too could contribute to tumorigenesis. However, Tip48/Tip49 remain incompletely characterized and require significant additional research to further define their cellular functions. Moreover, in addition to its role in transcription, Myc has been shown to function in DNA replication by associating with the prereplication complex and facilitating DNA replication initiation (1, 4, 39). Of note, MTBP was recently reported to interact with a DNA replication protein and be involved with DNA replication origins (38). Therefore, Mtbp could have functions in DNA replication with or without Myc, in addition to its role in transcription with Myc. Future studies are needed to address these possibilities and explore other roles for MTBP in human cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: B.C. Grieb, D.S. Haines, C.M. Eischen
Development of methodology: B.C. Grieb, M.P. Arrate, D.S. Haines, H. Xiao, C.M. Eischen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.C. Grieb, M.W. Gramling, M.P. Arrate, S.L. Beaul parlant, C.M. Eischen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.C. Grieb, M.W. Gramling, X. Chen, D.S. Haines, H. Xiao, C.M. Eischen
Writing, review, and/or revision of the manuscript: B.C. Grieb, M.W. Gramling, D.S. Haines, H. Xiao, C.M. Eischen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Xiao, C.M. Eischen
Study supervision: D.S. Haines, C.M. Eischen
MTBP Regulates MYC Oncogenic Activity

Acknowledgments

The authors thank B. Metge for technical assistance; Dr. M. Cole for the Myc vectors and Tip48 and Tip49 antibody; members of the Eischen lab and Dr. S. Hiebert for helpful discussion and review of the manuscript.

Grant Support

This work was supported by F30 AG 039164 (B.C. Grieb), the Vanderbilt MSTP T32GM007347 (B.C. Grieb), T32CA119925 (M.W. Gramling), R01CA148950 (C.M. Eischen), the Vanderbilt Breast Cancer SPORCE CA099131, CTSA ULTR008445 from the National Center for Advancing Translational Sciences, and the NCI Cancer Center Support Grant P30CA064853 utilizing the Flow Cytometry and the Translational Pathology Shared Resources and the Mass Spectrometry Research Core.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 29, 2013; revised February 4, 2014; accepted April 14, 2014; published OnlineFirst May 1, 2014.

References


www.aacajournals.org Cancer Res; 74(13) July 1, 2014 3601

Downloaded from cancerres.aacajournals.org on June 10, 2017. © 2014 American Association for Cancer Research.
Oncogenic Protein MTBP Interacts with MYC to Promote Tumorigenesis

Brian C. Grieb, Mark W. Gramling, Maria Pia Arrate, et al.

Cancer Res 2014;74:3591-3602. Published OnlineFirst May 1, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-13-2149

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/05/01/0008-5472.CAN-13-2149.DC1

Cited articles  This article cites 50 articles, 21 of which you can access for free at: http://cancerres.aacrjournals.org/content/74/13/3591.full#ref-list-1

Citing articles  This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/74/13/3591.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.