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Oncogenic protein MTBP interacts with MYC to promote tumorigenesis

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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 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 to *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.

Keywords

MTBP; MYC; oncogene; transformation; TIP48/TIP49; breast 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,

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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, while 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, since 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). Additionally, 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.

MATERIALS AND METHODS

Cell Culture, vectors, transfection, and infection

NIH3T3, HEK293T, H1299, HCC1806, MDA-MD-231, and Raji cells were cultured as described by the American Type Culture Collection (Manassas, VA). HCC1806, MDA-MD-231, immortalized human mammary epithelial (HMLE) cells, rat fibroblasts, and human retinal epithelial cells were provided by Drs. Jennifer Pietenpol, John Sedivy, or David Cortez (17–19). Mouse embryonic fibroblasts (MEFs) 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, Madison, WI), Lipofectamine 2000 (Invitrogen, Grand Island, NY) or calcium-phosphate, respectively. Cells were infected with retroviruses, as previously described (20).

Proliferation, cell cycle, apoptosis and transformation assays

To measure proliferation, 1000–5000 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 (see below). 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, Indianapolis, IN) were injected subcutaneously (flanks) with NIH3T3 fibroblasts. 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, St. Louis, MO), anti-HA (F7, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mtbp (K20, Santa Cruz), or isotype control antibodies as previously described (25). Antibodies against Flag (M2, Sigma), HA (F7, Santa Cruz or Roche, 1158381600), Mtbp (B5, Santa Cruz), TIP48 (36569, Ab-Cam, Cambridge, MA for Fig 6A or from Dr. Michael Cole for Fig 2B), TIP49 (from Dr. Michael Cole), Myc (C33, Santa Cruz Biotechnology or 06-340, Upstate Biotechnology, Lake Placid, NY), cleaved Caspase 3 (D175, Cell Signaling Technology, Danvers, MA), and β-actin (AC15, Sigma) were used to Western blot. For Figure 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 immunoaffinity 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 supplement).

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 ^{35}S-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 mM Tris-HCl at pH 8.0, 200 mM KCl, 2.5 mM $MgCl_2$, 1% triton X-100 and 5% glycerol, 0.1 mM 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 488 (A11058 and A21206, Invitrogen) were used.

Chromatin immunoprecipitation (ChIP)

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 ChIP endogenous MTBP and MYC. ChIP protocol from Upstate Biotechnology was followed except Raji cells were crosslinked for 1–2 hrs. DNA was sheared into ~500 bp pieces with sonication (VirSonic 600, Gardener, NY). 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 (RT-PCR)

NIH3T3 cells infected with a MSCV retrovirus encoding MycER (28) and transfected with non-targeting control siRNA or *Mtbp* siRNA (SMARTpool ON-TARGETplus, Thermo-Scientific, Pittsburgh, PA) or vectors encoding Mtbp or Mtbp mutants were treated with 1 μM 4-hydroxytamoxifen (4-OHT; Sigma) or ethanol vehicle control for 0, 6, or 8 hours. Total RNA was isolated, cDNA was generated, and qRT-PCR for Myc target genes was performed as previously described (15). For ChIP, qRT-PCR was performed 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-April 2013 (breast) and November 2013 (colon and lung adenocarcinoma). For Kaplan-Meier survival curves, normalized RNA-Seq data (version 2, level 3) was 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.cbiopor2tal.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.

Statistical evaluation

Student's t-test (Figs 1A–D, 3B–E, 4A–E, 5A–B, 6C–G, S1, S3A, S6–8), Fisher's exact test (Table S1), and log-rank tests (Figs 5C, S4, Table 1) were used to compare data.

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 (Tables 1 and S1). To evaluate whether Mtbp overexpression contributes to cancer, the biological 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; Fig S1), indicating a conserved function of Mtbp. Additionally, 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 to 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

Since 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 MTBP (104 kDa), the MYC transcriptional cofactors TIP49 (49 kDa) and TIP48 (48 kDa), and HSP70 (70 kDa; Fig 2A and 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 two 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. Additionally, the results indicate Mtbp associates indirectly with Myc by binding directly to Tip48/Tip49.

Mtbp associates with Myc at promoters

Through interactions with two Myc transcriptional cofactors and Myc itself, 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. Chromatin immunoprecipitation (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 two proteins occupy the same sites concurrently. Thus, Mtbp and Myc interact together at Myc-targeted promoters.

Mtbp enhances the oncogenic activity of Myc

Since 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 eight 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, due to 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. While 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 (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 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 (Figs 4C and S3B). Since 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 cell 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 to 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 (Figs 4E and 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 to 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 to 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 human colon and lung (adenocarcinoma) cancer patients (Fig S4). Additionally, evaluation of TCGA copy number alterations in 20 different human cancers revealed that among those that had amplified *MYC*, *MTBP* was frequently co-amplified (Table S1; 30, 31). This occurred even though *MTBP* and *MYC* are 7.2 megabases apart at 8q24.12 and 8q24.21, respectively (Fig S5). Notably, in 200 of 913 (21.9%) breast carcinomas with amplified *MYC*, 85% co-amplified *MTBP*. 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, since sequence analysis revealed no potential functional motifs to guide mutation generation, we divided Mtbp (aa 1–894) into thirds. The central (aa 299–596) and C-terminal (aa 597–894) Mtbp mutants were detectable by Western blot, 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. Additionally, 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 transcription of pro-proliferative genes (Fig 6D). Similar results were observed when *Mtbp* was knocked down with siRNA (Fig S6). The C-terminal Mtbp mutant also inhibited Myc-driven cellular proliferation (Fig 6E) and soft agar colony formation (Fig 6F), whereas the central domain Mtbp mutant had no significant effect. Similarly, *MTBP* knockdown in human or murine cells reduced proliferation (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 to the central domain mutant or empty lentivirus control (Figs 6G and S8). Thus, the C-terminal Mtbp mutant appears 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 (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 hexomer/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 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 (Fig S9), a complex critical for Myc-induced transformation (10).

Mtbp alone was less oncogenic than Myc, an established powerful oncogene (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 (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 supports the conclusion that Mtbp directly facilitates Myc-induced transcription. Additionally, Myc controls the expression of many of its

regulators. We previously reported that Mtbp appears 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 feed-forward regulatory loop with Myc (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 Tables 1 and S1; (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 to those with low levels of *MTBP* and high levels of *MYC*. A similar trend was also observed for colon and lung caner patients. Additionally, 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 appears to be pro-proliferative and a positive contributor to tumorigenesis in the majority of cell types evaluated.

Our studies also likely have broader implications. While 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 pre-replication 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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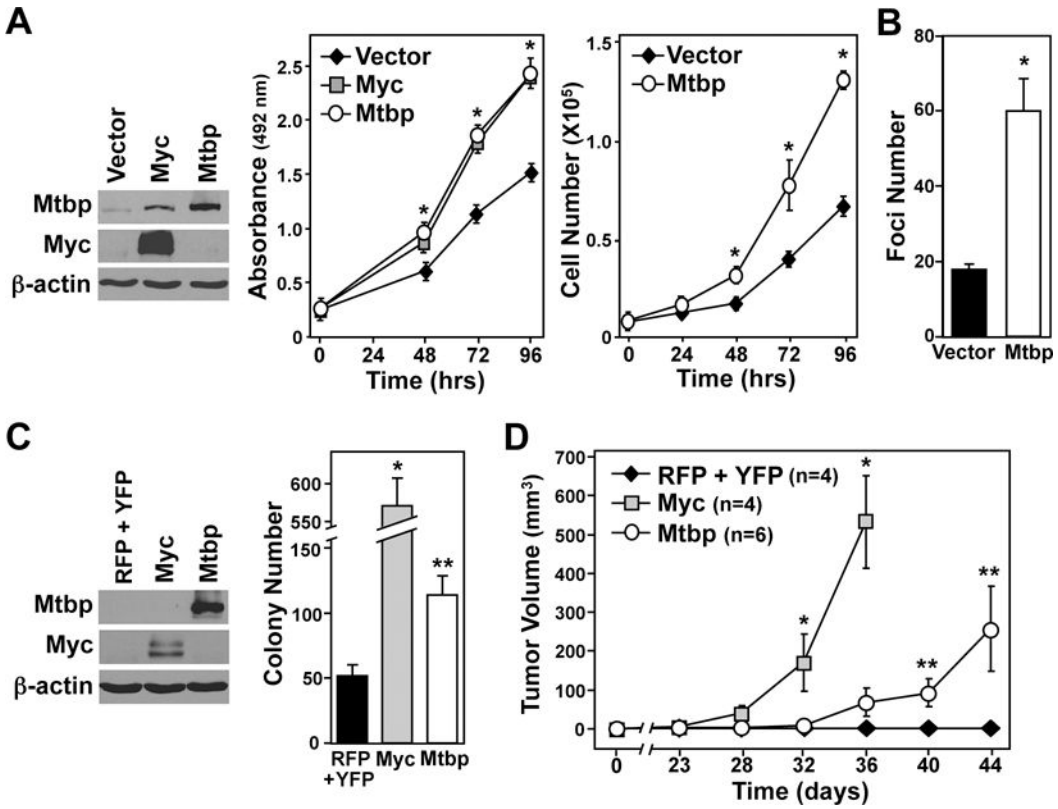

Figure 1. Mtbp is oncogenic

(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 hr 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=0.0011). (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 (*<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's t-tests.

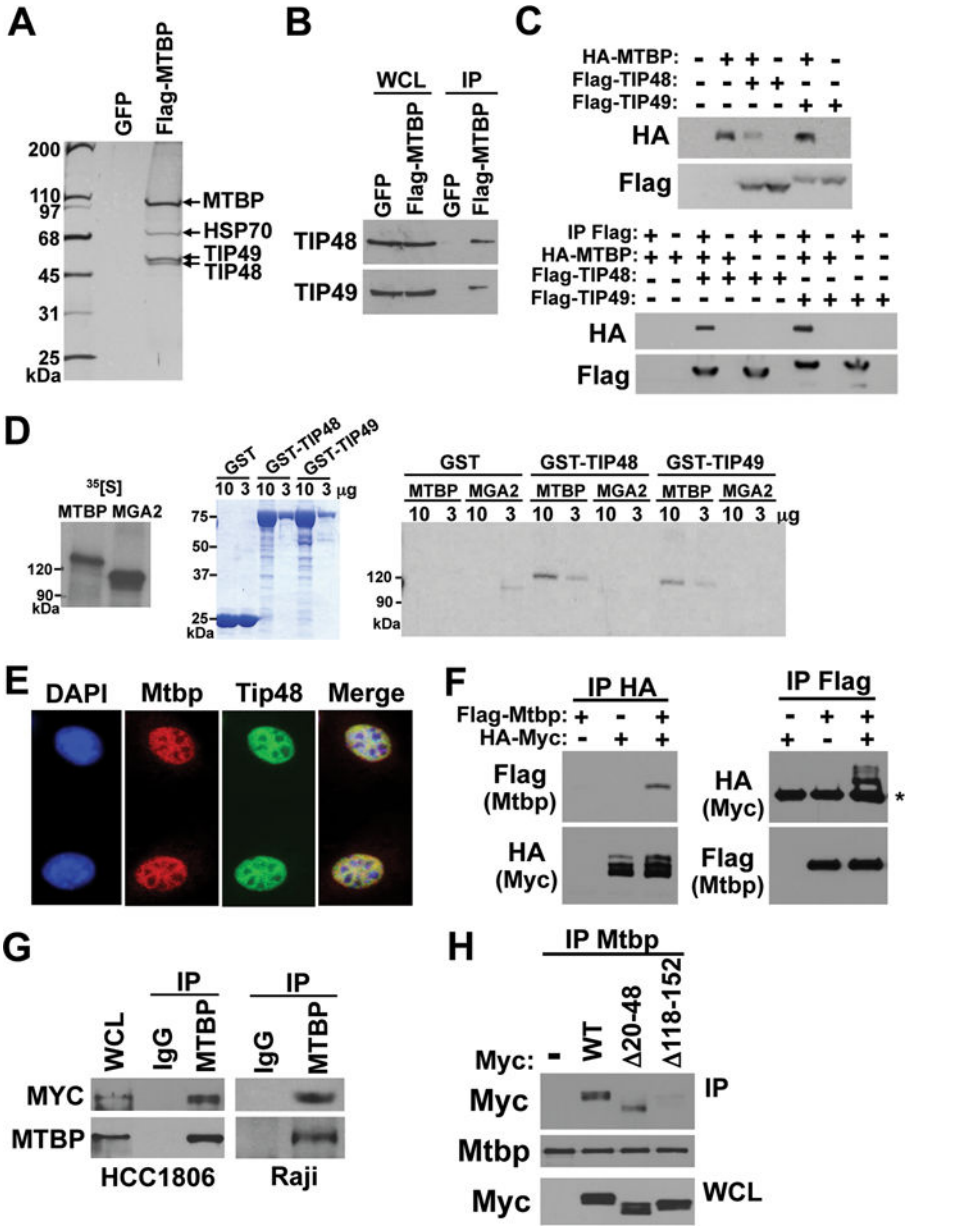

Figure 2. Mtbp binds to Tip48, Tip49, and Myc

(A) Anti-Flag immunoprecipitation of whole cell protein lysates from H1299 cells expressing Flag-MTBP or GFP control were separated by SDS-PAGE, and proteins silver stained. Arrows denote proteins excised and identified by mass spectrometry (peptides identified in Fig S2). Molecular weights (kDa) indicated. (B) Western blots for endogenous TIP48 and TIP49 in whole cell lysates (WCL) and anti-Flag immunoprecipitations (IP) using lysates from (A). (C) Whole cell lysates of H1299 cells expressing HA-MTBP, Flag-TIP48, and/or Flag-TIP49 were Western blotted (top) and subjected to anti-Flag (+) or isotype control (−) IP and then Western blotted (bottom). (D) Input ^{35}S-Met labeled *in vitro* translated MTBP and control protein MGA2 (left) were incubated with 10 or 3 µg of GST, GST-TIP48, or GST-TIP49 (Coomassie Blue stained SDS-PAGE, center). Bound ^{35}S-Met-labeled MTBP and MGA2 were visualized by fluorography (right). (E) Immunofluorescence

of Mtbp (red), Tip48 (green), and a merged image (yellow) in mouse embryonic fibroblasts. DAPI staining of DNA marked the nucleus (blue). (F, H) 293T cells were transfected with vectors encoding the indicated proteins (wild-type, WT; Δ20–48 or Δ118–152 Myc mutants; empty vector, -). Whole cell lysates (WCL) were immunoprecipitated (IP) as indicated, and Western blots were performed for the proteins to the left of each panel. Asterisk denotes location of immunoglobulin heavy chain. (G) Immunoprecipitations for endogenous MTBP in whole cell protein lysates (WCL) from HCC1806 and Raji cells with anti-MTBP or isotype (IgG) control were Western blotted.

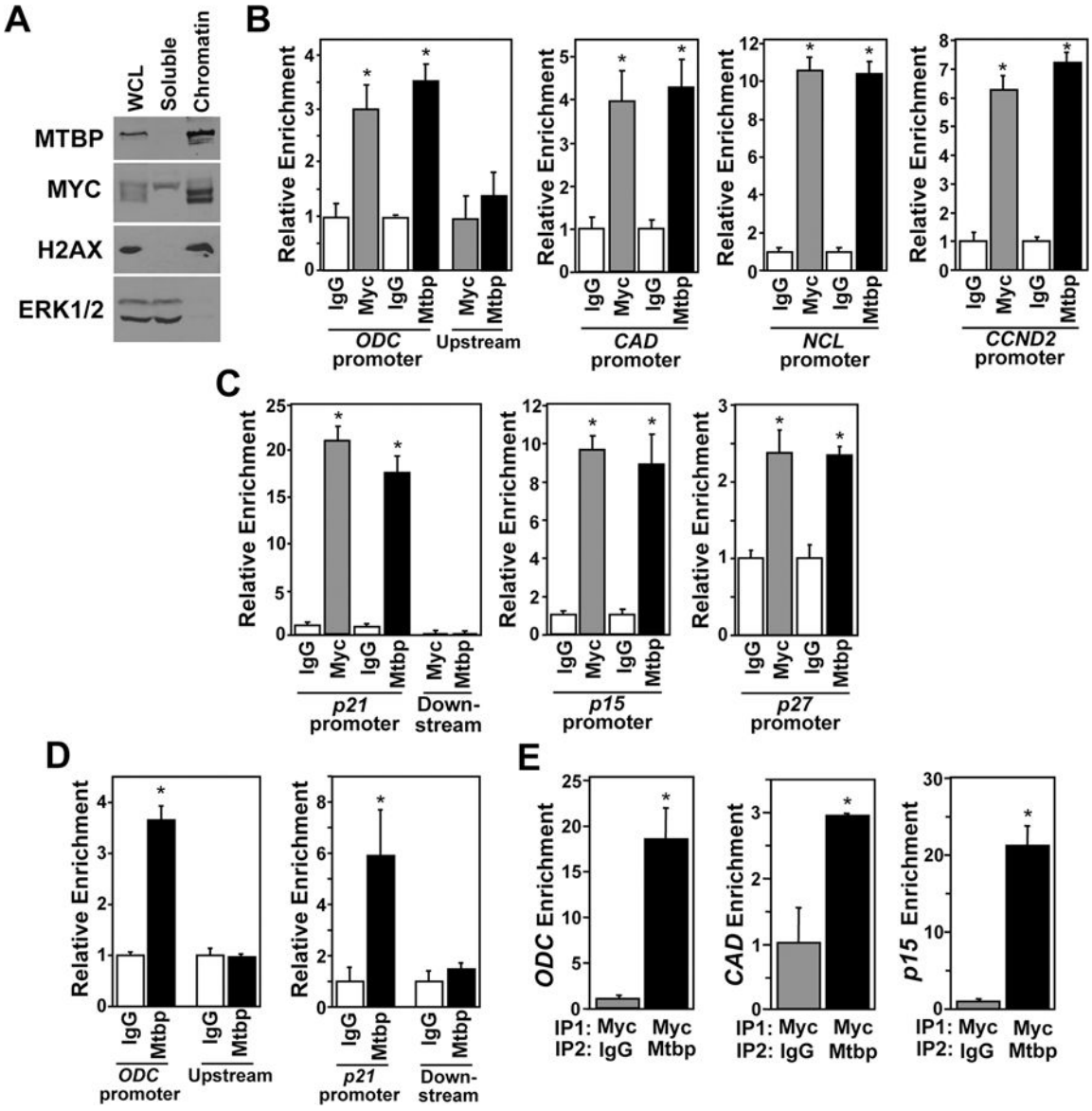

Figure 3. Mtbp associates with Myc at Myc bound promoters

(A) Whole cell lysate (WCL), soluble protein fraction, and chromatin-associated protein fraction from 293T cells were Western blotted. (B, C) 293T cells were transfected with vectors encoding Myc or Mtbp. Following ChIP with anti-Myc, anti-Mtbp, or isotype controls (IgG), qRT-PCR for the indicated promoter regions Myc binds or upstream or downstream regions Myc does not bind was performed. (D) qRT-PCR for the indicated promoter regions was performed after Raji cells were subjected to ChIP with anti-Mtbp or isotype controls (IgG). (E) 293T cells were transfected with vectors encoding Myc or Mtbp. After sequential ChIP for Myc followed by Mtbp or IgG control, qRT-PCR for the indicated promoter regions was performed. For B–E, values are relative to their respective IgG control and input DNA. Error bars represent standard deviation; *p≤0.01 compared to appropriate IgG for B, C, and E; *p<0.02 for D. P values determined by student's t-tests.

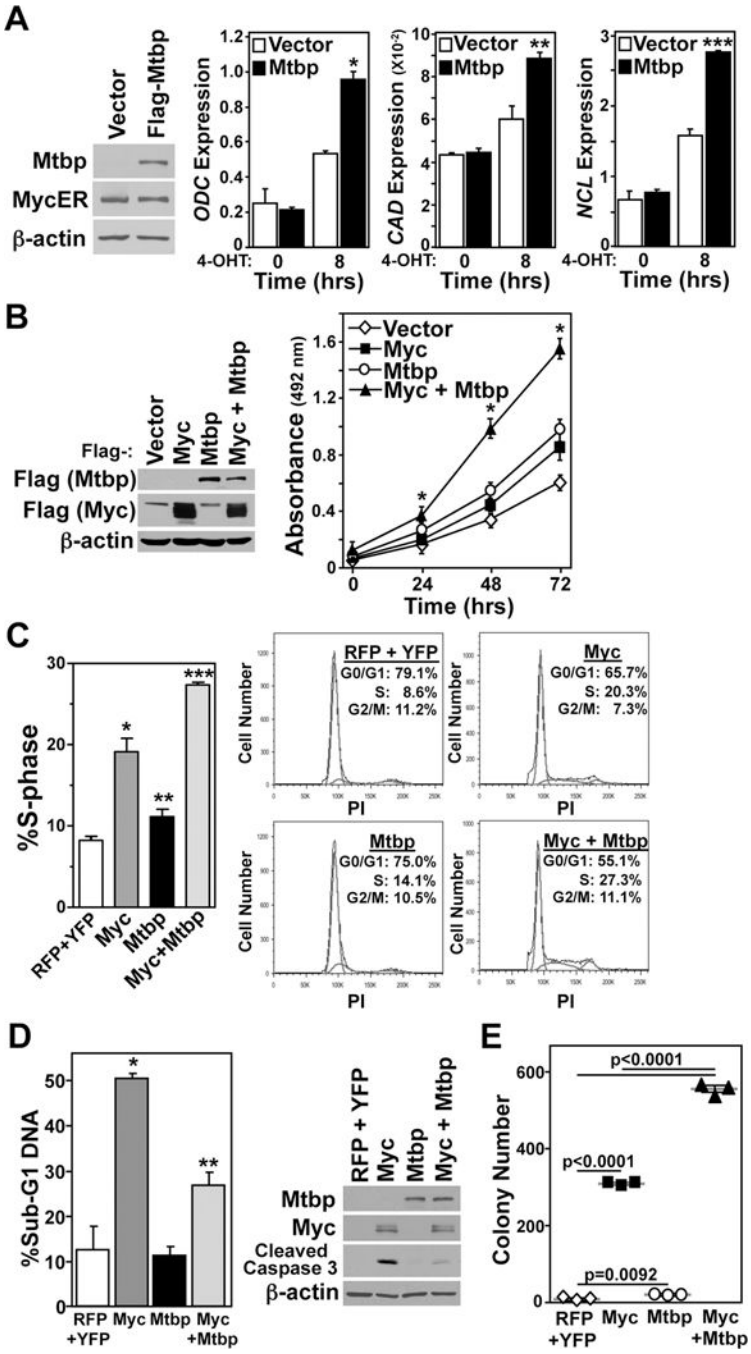

Figure 4. Mtbp overexpression promotes Myc oncogenic activity

(A) MycER expressing NIH3T3 were transfected with an empty vector or a vector encoding Flag-Mtbp. Western blots of whole cell lysates were performed. Additionally, MycER was activated in the fibroblasts with 4-OHT for 0 or 8 hrs. 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 whole cell lysates were performed. MTS assays were performed at 24 hr intervals (*p<0.001 Myc vs. Myc + Mtbp).

(C–E) NIH3T3 cells infected with two bicistronic retroviruses encoding the indicated proteins were analyzed. (C, 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 (grey line; *p=0.012 RFP+YFP vs. Myc, **p=0.0296 RFP+YFP vs. Mtbp, ***p=0.0191 Myc + Mtbp vs. Myc); 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 RFP + YFP vs. Myc, **p=0.0018 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’s t-tests.

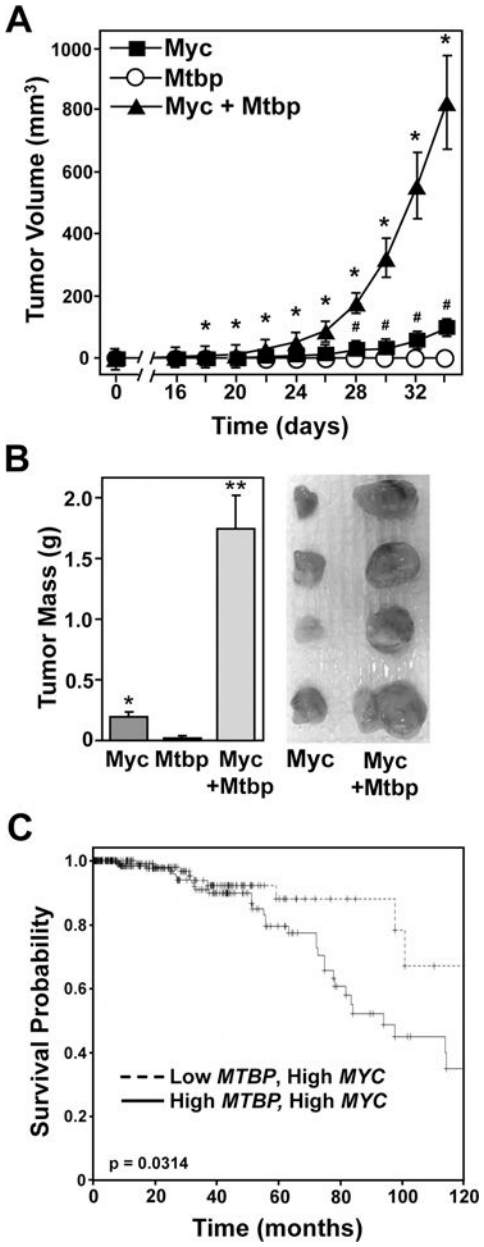

Figure 5. Increased Mtbp expression cooperates with Myc overexpression *in vivo* and correlates with poor breast cancer prognosis

(A) Equal numbers of NIH3T3 cells expressing Mtbp, Myc, or both were injected subcutaneously into the flanks of nude mice (n = 16/group), and tumor volume was measured at intervals (#p<0.001 Myc vs. Mtbp, *p<0.0001 Myc vs. Myc + Mtbp). (B) After 34 days, tumors were extracted, weighed (*p=0.0073 Mtbp vs. Myc, **p<0.0001 Myc vs. Myc + Mtbp), and photographed. Photo of representative tumors shown. Error bars represent standard error of the mean; p values determined by student's t-tests (A, B). (C) Kaplan Meier survival curves from RNA-Seq mRNA expression data from the TCGA database of breast cancers with high *MYC* mRNA expression divided into low (n = 171) or high (n = 250) *MTBP mRNA* expression. Log-rank test determined p-value.

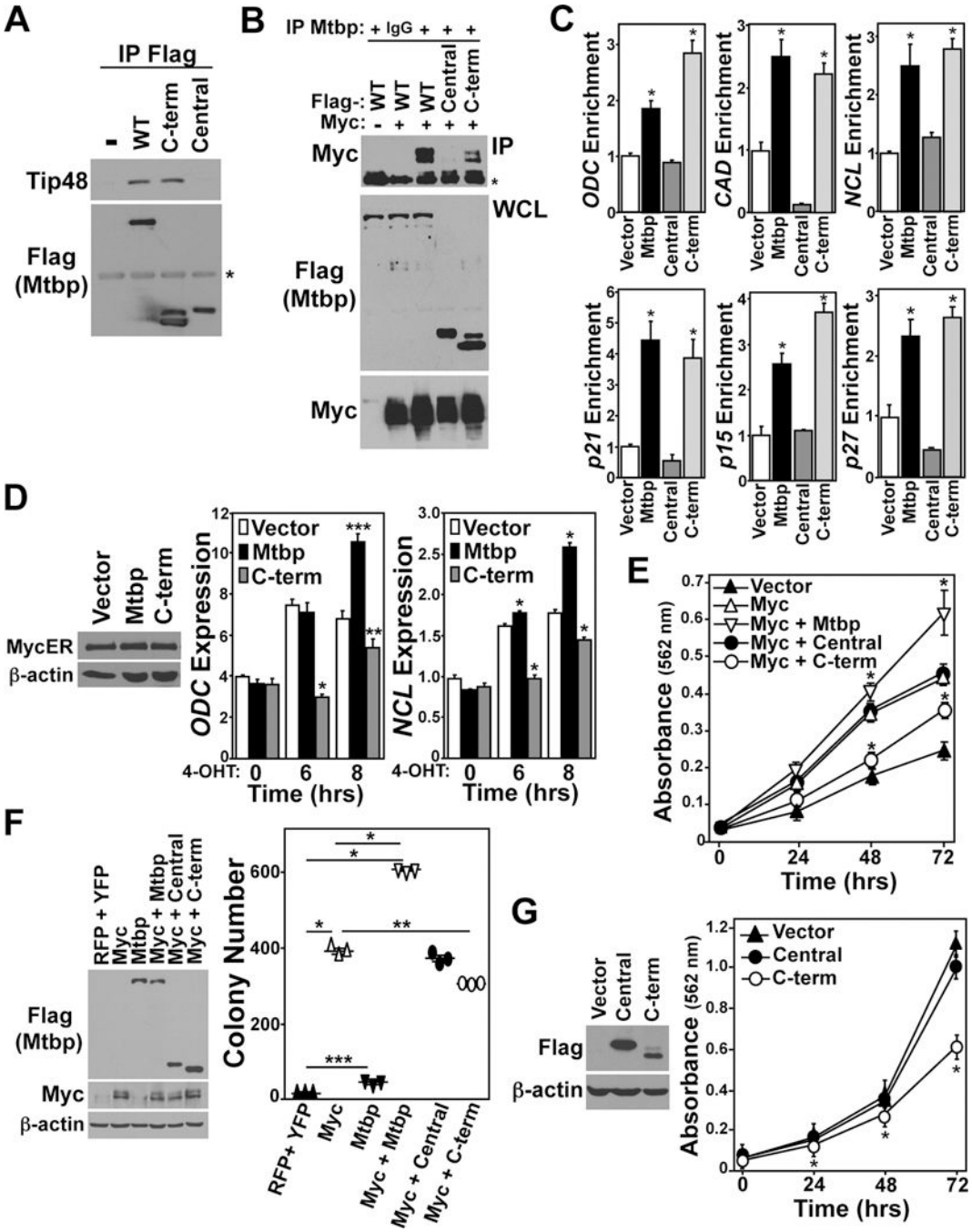

Figure 6. Mtbp C-terminus functions as an inhibitor of Myc

(A–C) 293T cells were transfected with vectors encoding Flag-tagged wild-type (WT) Mtbp, the C-terminal (597–894) or central domain (299–597) Mtbp mutants, or empty vector control (–) and for (B) a vector that did (+) or did not (−) encode Myc. (A) Anti-Flag immunoprecipitation of whole cell lysates were Western blotted for Flag and endogenous Tip48. Asterisk denotes location of immunoglobulin heavy chain. (B) Whole cell lysates (WCL) were Western blotted for Flag and Myc (below) and subjected to anti-Mtbp or IgG control IP and then Western blotted for Myc (top). (C) Following ChIP with anti-Flag, qRT-

PCR was performed for the indicated promoter regions. Values are relative to their respective vector control and input DNA (*p≤0.01 compared to vector). (D) MycER expressing NIH3T3 cells were transfected with an empty vector or a vector encoding full-length Mtbp or the C-terminal Mtbp mutant. Western blots were performed. To activate MycER, 4-OHT was added to the cultures for the indicated time. qRT-PCR was performed in triplicate for the indicated Myc target genes and is expressed relative to *β-actin* levels (*p<0.001 vector vs. C-term or Mtbp, ***p=0.006 vector vs. Mtbp, **p=0.05 vector vs. C-term). (E) NIH3T3 cells transfected with empty vector or vectors encoding the indicated proteins were subjected to MTT assay at 24 hr intervals. (p<0.001 for Myc vs. Myc + Mtbp and Myc + C-term vs. Myc or Myc + Mtbp). (F) NIH3T3 cells infected with bicistronic retroviruses encoding the proteins indicated were subjected to soft agar colony assay. Colony number quantified after 10 days (*p<0.0001, **p=0.0004, ***p=0.0006). (G) HCC1806 cells expressing YFP alone (vector) or YFP with Flag-tagged central or C-terminal Mtbp mutants were Western blotted (left). Cells were also subjected to MTT assays at 24 hr intervals (*p<0.05 Vector vs. C-term). Error bars are standard deviation and p values determined by student's t-tests.

Table 1

MTBP mRNA is overexpressed in many human cancers

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

MTBP mRNA expression data in cancer (C) compared to normal (N) tissue from Oncomine; p-values determined by log-rank test