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A deficiency in Mdm2 binding protein (MTBP) inhibits Myc-induced B cell proliferation and lymphomagenesis

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Abstract

Mdm2 binding protein (MTBP) has been implicated in cell cycle arrest and the Mdm2-p53 tumor suppressor pathway through its interaction with Mdm2. To determine the function of MTBP in tumorigenesis and its potential role in the Mdm2-p53 pathway, we crossed *Mtbp* deficient mice to Eμ-*myc* transgenic mice, in which overexpression of the oncogene c-Myc induces B cell lymphomas primarily through inactivation of the Mdm2-p53 pathway. We report that Myc-induced B cell lymphoma development in *Mtbp* heterozygous mice was profoundly delayed. Surprisingly, reduced levels of Mtbp did not lead to an increase in B cell apoptosis or affect Mdm2. Instead, an Mtbp deficiency inhibited Myc-induced proliferation and the upregulation of Myc target genes necessary for cell growth. Consistent with a role in proliferation, Mtbp expression was induced by Myc and other factors that promote cell cycle progression and was elevated in lymphomas from humans and mice. Therefore, Mtbp functioned independent of Mdm2 and was a limiting factor for the proliferative and transforming functions of Myc. Thus, Mtbp is a previously unrecognized regulator of Myc-induced tumorigenesis.

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Conflict of Interest

There are no competing financial interests with the research described.

Keywords

MTBP; Myc; Mdm2; p53; lymphoma

Introduction

MTBP, a 104 kDa protein with no known functional motifs, was identified through a yeast two-hybrid screen to bind to the E3 ubiquitin ligase Mdm2 (Boyd et al., 2000), a regulator of the p53 tumor suppressor (Marine & Lozano, 2009). MTBP overexpression inhibited cell cycle progression in a p53-independent, Mdm2-dependent manner following nocodazole treatment (Boyd et al., 2000). A subsequent study showed that MTBP regulates p53 through modulation of Mdm2 ubiquitin ligase activity (Brady et al., 2005). Specifically, overexpression of MTBP in tumor cell lines increased Mdm2 ubiquitin ligase activity increasing p53 degradation, and suppression of MTBP expression had the opposite effect. To determine the physiological role of MTBP, mice lacking *Mtbp* were generated (Iwakuma et al., 2008). *Mtbp* heterozygous mice were viable and did not have any obvious defects. However, loss of both alleles of *Mtbp* was embryonic lethal. In contrast to *Mdm2* deletion, the lethality of *Mtbp* deletion could not be rescued by loss of *p53* (Iwakuma et al., 2008), suggesting that Mtbp may not regulate Mdm2 and consequently p53 *in vivo*. Regardless of the Mtbp/Mdm2 relationship, decreased Mtbp expression has been linked to tumor metastasis (Iwakuma et al., 2008), and the chromosomal region where *MTBP* lies is frequently amplified in human colorectal cancer and multiple myeloma (Carrasco et al., 2006; Martin et al., 2007). Therefore, the function of MTBP in relationship to Mdm2 and p53 and in tumorigenesis is currently unclear.

Cell cycle and apoptosis are critical regulators of tumor development. Deletion of *E2F1*, a transcription factor essential for proliferation, inhibits c-Myc-induced B cell lymphomagenesis through upregulation of the cell cycle inhibitor p27 and suppression of cell cycle progression (Baudino et al., 2003). Moreover, reduced levels of ornithine decarboxylase (ODC), a transcriptional target gene of c-Myc required for polyamine synthesis, leads to decreased proliferation and inhibition of Myc-induced B cell lymphoma development (Nilsson et al., 2005). It is well established that Myc induces apoptosis in primary B cells, in part, by activating the ARF-Mdm2-p53 tumor suppressor pathway (Eischen et al., 1999). Myc activates ARF, which inhibits Mdm2 causing p53 activation and apoptosis. Inactivation of ARF or p53 or overexpression of Mdm2 are frequent events detected in B cell lymphomas that arise in mice overexpressing Myc (Eμ-*myc* transgenics) (Eischen et al., 1999). Moreover, mice that are deficient in *ARF* or *p53* or overexpress Mdm2 have an acceleration of lymphoma development due to a reduction in B cell apoptosis (Alt et al., 2003; Eischen et al., 1999; Schmitt et al., 1999; Wang et al., 2008). In contrast, *Mdm2* heterozygosity inhibits Myc-induced lymphomagenesis due to increased p53-dependent B cell apoptosis (Alt et al., 2003), which can be rescued with loss of one allele of *ARF* (Eischen et al., 2004). Therefore genes that influence Mdm2, as Mtbp is postulated to do, should have a significant effect on Myc-induced apoptosis and tumor development. However, our data show that loss of one allele of *Mtbp* did not impact apoptosis or function through Mdm2, yet lymphoma development in $Mtbp^{+/-}$Eμ-*myc*

transgenic mice was inhibited. *Mtbp* heterozygous cells had reduced rates of Myc-induced proliferation and decreased ability to upregulate Myc target genes necessary for cell growth. Our results indicate that Mtbp regulates Myc-induced lymphomagenesis not through Mdm2, but in cooperation with Myc.

Materials and Methods

Mice

Congenic C57Bl/6 Eμ-*myc* transgenic mice were from Drs. Alan Harris (Walter & Eliza Hall Institute, Melbourne, Australia) and Charles Sidman (University of Cincinnati, Cincinnati, OH), and $ARF^{-/-}$ mice were from Drs. Martine Roussel and Charles Sherr (St. Jude Children's Research Hospital, Memphis, TN). $Mtbp^{+/-}$ (C57Bl/6X129/Sv backcrossed onto C57Bl/6 at least five generations) mice were crossed to male Eμ-*myc* transgenics to generate F1's. F1's were crossed to generate F2's for analysis. F2's were also crossed to $ARF^{-/-}$ or $p53^{-/-}$ mice to generate mice deficient in *ARF* or *p53*. Mice were carefully followed, and at signs of disease were sacrificed. Statistical significance was determined by log-rank tests. All experiments with mice were approved by the Vanderbilt Institutional Animal Care and Use Committee (IACUC) and followed all federal and state rules and regulations.

Culture and infection of primary pre-B cells and lymphocyte phenotype analysis

Primary pre-B cell ($CD43^-$, $B220^+$, $CD19^+$, IgM^-) cultures were generated from bone marrow of 5–8 week old mice. Pre-B cells were infected with MSCV-MycER-IRES-GFP and GFP^+ cells were sorted by FACS. Phenotypic analyses of pre-B cells, whole spleens, bone marrow, and lymphomas were performed with fluorescently linked antibodies from Southern Biotechnology or BDPharMingen and flow cytometry. All procedures were previously described (Eischen et al., 1999).

Viability, apoptosis, proliferation, cell cycle, and chromosome analysis

Viability following explantation of bone marrow into IL-7 containing medium, after the addition of 1 μM 4-OHT (Sigma) to the culture medium of MycER expressing pre-B cells, or after IL-7 deprivation was determined at specific intervals by Trypan Blue Dye exclusion. Apoptosis and cell cycle was measured with propidium iodide (PI) staining and analysis on a FACScalibur. Quantification of fragmented (sub-G1) DNA was performed with CellQuest (BD Immunocytometry Systems). The percentage of cells in each phase of the cell cycle was determined by ModFIT (Verity House Software). Proliferation was determined by BrdU incorporation as previously described (Wang et al., 2008). MTS assays were performed at intervals as per manufacturer's protocol (Promega) following transfection of four replicates of siRNAs (on-target SMART pools for *Mtbp* or non-targeting control, Dharmacon) with Lipofectamine2000 (Invitrogen). Metaphases of splenocytes were analyzed for breaks and aneuploidy as previously described (Wang et al., 2008).

Western and Southern blotting

Murine pre-B cells, lymphomas, and spleens, and normal human lymph node, spleen, peripheral blood lymphocytes, and lymphoma cell lines were lysed as previously described

(Zindy et al., 1998). Antibodies specific for $p19^{ARF}$ (GeneTex), p53 (Ab-7, Calbiochem), Mdm2 (C-18, Santa Cruz), Myc (06–340, Upstate Biotechnology), murine Mtbp (Santa Cruz), human MTBP (PHL-1, Rockland), E2F1 (C20, Santa Cruz), p16 (M-156, Santa Cruz), p21 (SXM30, BD Biosciences), p27 (BD Biosciences), and β-actin (Sigma) were used to Western blot. HRP-linked secondary antibodies and ECL (GE Healthcare) or Supersignal (Pierce) to detect bound immunocomplexes were used. Southern blots for *ARF*, *p53*, and *Mtbp* were preformed as previously described (Eischen et al., 1999; Iwakuma et al., 2008).

Quantitative RT-PCR

Total RNA was isolated, cDNA was generated, and qRT-PCR with SybrGreen was performed as previously described (Wang et al., 2008). Primers for *Mtbp* and β-*actin* were previously described (Iwakuma et al., 2008; Wang et al., 2008).

Northern Blotting

Total RNA was prepared using RNAbee (tel-Test) according to manufacturer's instructions, separated by electrophoresis, transferred to hybond-N nylon membrane, and crosslinked by UV. Full-length human *MTBP* cDNA was labeled with ^{32}P (random primed labeling kit; Boehringer Mannheim), and hybridizations were performed in Rapid-hyb (GE Healthcare) according to manufacturer's instructions. For the cycloheximide experiments, cycloheximide (10μg/ml) was added 30 min. prior to the addition of 4-OHT (Eischen et al., 2001).

Results

Mtbp expression is regulated by mitogens, oncogenes, and cell cycle

To obtain a better understanding of Mtbp, we explored how Mtbp expression was regulated. The G1 cell cycle arrest induced by depleting serum from cultured $p53^{-/-}$ mouse embryo fibroblasts (MEFs) resulted in a dramatic decrease in Mtbp protein expression with a concomitant increase in the cell cycle inhibitor p27, which is upregulated in G1 (Fig 1A). Re-addition of serum back to serum starved MEFs restored Mtbp expression as cells left G1 and moved into S phase (Fig. 1B). Similar results were observed in wild-type or *ARF*-null MEFs (data not shown). Overexpression of cyclin D or cyclin E or of the oncogenes c-Myc or E2F1, which drive cells into S phase, led to an increase in MTBP protein expression in human carcinoma cells and murine fibroblasts (Fig. 1C & data not shown). In contrast, expression of the cell cycle inhibitors, $p21^{cip1}$ or $p16^{Ink4a}$, which arrest cells in G1, resulted in decreased MTBP protein expression (Fig. 1D). Therefore, pro-proliferative signals upregulated and anti-proliferative signals downregulated Mtbp protein expression in human and murine cells, and this occurred independent of ARF and p53. These results suggest a positive role for Mtbp in cell growth.

Mtbp transcription is induced by Myc

To address whether the increase in Mtbp protein levels from serum and oncogenes was due to changes in *Mtbp* transcripts, we evaluated RNA levels. *Mtbp*/*MTBP* mRNA, as measured by quantitative real-time PCR (qRT-PCR) or Northern blot, was significantly reduced by serum deprivation or following overexpression of p21 (Fig. 2A & 2B). Increased *MTBP*

mRNA was detected with the re-addition of serum to serum starved cells or overexpression of Myc or E2F1 (Fig. 2B). Two bands are visible in the E2F1 lane, which could indicate an alternative spliced form of *MTBP*, but at this time it is unclear. *Mtbp* mRNA expression was also induced *in vivo* by c-Myc. qRT-PCR showed significantly elevated *Mtbp* mRNA in spleens of $Mtbp^{+/+}$Eμ-*myc* transgenic mice, which overexpress c-Myc specifically in B cells, compared to levels in $Mtbp^{+/+}$ non-transgenic littermate spleens (Fig. 2C). There were also increased levels of *Mtbp* mRNA in $Mtbp^{+/-}$Eμ-*myc* spleens compared to levels in non-transgenic $Mtbp^{+/-}$ spleens. Interestingly, *Mtbp* levels in $Mtbp^{+/+}$Eμ-*myc* spleens were higher than those in $Mtbp^{+/-}$Eμ-*myc* spleens (Fig. 2C). To investigate whether *Mtbp* is directly induced by Myc, murine fibroblasts expressing a 4-hydroxytamoxifen (4-OHT) regulatable form of Myc (MycER)(Littlewood et al., 1995) were generated and treated with the protein synthesis inhibitor cycloheximide. Within 30 min. of MycER activation by 4-OHT, *Mtbp* mRNA expression increased (Fig. 2D), indicating that new protein synthesis was not required for Myc to induce *Mtbp* mRNA. There were modestly higher basal *Mtbp* levels in the MycER expressing cells in the absence of 4-OHT, likely due to the slight leakiness of the MycER construct. These results demonstrate that Myc may directly regulate *Mtbp* mRNA expression.

Mtbp regulates Myc-induced lymphomagenesis independent of Mdm2

It was reported that MTBP levels impact Mdm2 expression and function (Brady et al., 2005). Since increased Mdm2 levels accelerate and decreased Mdm2 levels inhibit lymphoma development in Eμ-*myc* transgenic mice due to disregulation of p53 (Alt et al., 2003; Wang et al., 2008), we generated $Mtbp^{+/-}$Eμ-*myc* mice to evaluate lymphoma development. $Mtbp^{+/-}$Eμ-*myc* mice had a profound delay in lymphoma development compared to wild-type Eμ-*myc* littermates (Fig. 3; p=0.0004, log-rank test). At one year of age, 40% of the $Mtbp^{+/-}$Eμ-*myc* transgenics were alive, whereas only 15% of the $Mtbp^{+/+}$Eμ-*myc* mice were alive. The 270 days mean survival of $Mtbp^{+/-}$Eμ-*myc* transgenics was twice that of the 135 days mean survival for $Mtbp^{+/+}$Eμ-*myc* mice. The lymphomas that developed in $Mtbp^{+/-}$Eμ-*myc* mice were typical pre-B/B cell lymphomas that arise in Eμ-*myc* mice (data not shown). Therefore, an Mtbp haploinsufficiency significantly hindered Myc-mediated B cell lymphomagenesis.

To determine whether a deficiency in *Mtbp* would also inhibit the rapid lymphoma development in $p53^{+/-}$Eμ-*myc* mice (Alt et al., 2003) and the contribution of p53 to Mtbp function, $p53^{+/-}Mtbp^{+/-}$Eμ-*myc* mice were generated. Deletion of one allele of *p53* in $Mtbp^{+/-}$Eμ-*myc* mice accelerated lymphomagenesis to rates analogous to those of $p53^{+/-}Mtbp^{+/+}$Eμ-*myc* transgenics (Fig. 3A); the mean survivals of both genotypes were 30 days. Thus, reduced levels of Mtbp suppressed Myc-induced lymphomagenesis, but the effect was abrogated by deletion of one *p53* allele. These data imply that p53 functions downstream of Mtbp or that loss of p53 dominates B cell transformation and supersedes any effect Mtbp haploinsufficiency has in Myc-induced lymphomagenesis.

The levels of p53 accessible to Mdm2 are regulated by ARF levels in Eμ-*myc* mice (Eischen et al., 2004). If Mtbp regulates Mdm2, then altering the amount of ARF present should impact the ability of Mtbp to regulate Mdm2; we generated $ARF^{+/-}Mtbp^{+/-}$Eμ-*myc*

transgenics to test this concept. In contrast to *ARF*$^{+/-}$*Mdm2*$^{+/-}$Eμ-*myc* mice (Eischen et al., 2004), loss of one allele of *ARF* did not restore lymphoma latency in *Mtbp*$^{+/-}$Eμ-*myc* mice to wild-type Eμ-*myc* rates (Fig. 3B). In fact, *ARF*$^{+/-}$*Mtbp*$^{+/-}$Eμ-*myc* mice rapidly developed lymphoma and had a similar mean survival to *ARF*$^{+/-}$*Mtbp*$^{+/+}$Eμ-*myc* littermates (60 days versus 62 days, respectively). Therefore, Mtbp levels did not impact the ability of ARF to regulate Mdm2. To assess this concept further, we evaluated loss of heterozygosity (LOH) of the wild-type allele of *ARF* in *ARF*$^{+/-}$*Mtbp*$^{+/-}$Eμ-*myc* lymphomas. *ARF* LOH was observed in 12 of 17 (71%) *ARF*$^{+/-}$ *Mtbp*$^{+/-}$Eμ-*myc* lymphomas analyzed, including three that had also deleted the knockout allele of *ARF* (Fig. 4A), a frequency analogous to that in *ARF*$^{+/-}$Eμ-*myc* lymphomas (77%, (Eischen et al., 1999)). If Mtbp regulates Mdm2, the expected frequency of *ARF* LOH in the *ARF*$^{+/-}$*Mtbp*$^{+/-}$Eμ-*myc* lymphomas should have been similar to the 44% observed in lymphomas from *ARF*$^{+/-}$ *Mdm2*$^{+/-}$Eμ-*myc* transgenics (Eischen et al., 2004). These genetic results indicate that Mtbp function is not influenced by ARF status, and that Mtbp does not appear to be functioning through Mdm2.

Another means to evaluate whether Mtbp levels are regulating Mdm2 function *in vivo* is to analyze *Mtbp*$^{+/-}$Eμ-*myc* lymphomas for p53 inactivation and Mdm2 levels. Of note, half of the *Mdm2*$^{+/-}$Eμ-*myc* lymphomas have mutated *p53* (Alt et al., 2003). As previously reported for Eμ-*myc* lymphomas (Eischen et al., 1999), p53 overexpression resulting from a mutation was present in a quarter of the lymphomas analyzed from *Mtbp*$^{+/+}$Eμ-*myc* littermates (data not shown). Similarly, 26% (7 of 27) of the lymphomas analyzed from *Mtbp*$^{+/-}$Eμ-*myc* transgenics contained mutant p53 (Fig. 4B). All mutations fell within the DNA-binding domain of p53, including one lymphoma (MT616) that had a nonsense mutation resulting in a truncated p53 that was not detected by Western blot. Only one of 27 *Mtbp*$^{+/-}$Eμ-*myc* lymphomas (MT163) had deleted *p53* (Fig. 4C), the same frequency as that observed in *Mtbp*$^{+/+}$Eμ-*myc* lymphomas. Biallelic *ARF* deletions were detected in 19% (5 of 27) of *Mtbp*$^{+/-}$Eμ-*myc* lymphomas (Fig. 4D), which is similar to the frequency of *ARF* deletions in wild-type Eμ-*myc* lymphomas (Eischen et al., 1999). At least one of the three isoforms of Mdm2 was overexpressed in 62% (17 of 27) of the *Mtbp*$^{+/-}$Eμ-*myc* lymphomas (Fig. 4B), which is analogous to the frequency in *Mtbp*$^{+/+}$Eμ-*myc* lymphomas. The levels of Mtbp protein in the lymphomas did not correlate to Mdm2 levels or p53 status. Moreover, Southern blots showed there was no LOH of the wild-type allele of *Mtbp* in *Mtbp*$^{+/-}$Eμ-*myc* lymphomas, and *Mtbp* was not deleted in *Mtbp*$^{+/+}$Eμ-*myc* lymphomas (Fig. 4E). Therefore, loss of one allele of *Mtbp* did not result in increased p53 activation, leading to an increased frequency of *p53* mutations or deletions in *Mtbp*$^{+/-}$Eμ-*myc* lymphomas, as would be expected if Mtbp regulated Mdm2. Moreover, Mdm2 levels were not influenced by Mtbp levels, and *ARF* deletions occurred at a normal frequency. Therefore, the data indicate Mtbp does not regulate Mdm2 or the ARF-Mdm2-p53 pathway during Myc-induced lymphomagenesis.

Loss of one allele of Mtbp does not alter Myc-induced apoptosis

Since our genetic results indicate that Mtbp does not function through Mdm2, but decreased levels of Mtbp did inhibit Myc-induced lymphomagenesis, we investigated the role of Mtbp in Myc functions. We first evaluated the effects of decreased Mtbp levels on the growth and survival of pre-B cells. Pre-B cells from *Mtbp*$^{+/-}$ and *Mtbp*$^{+/+}$ littermates emerged from

bone marrow cultures at a similar rate and had analogous doubling times and percentages of spontaneous apoptotic cells (Fig. 5A & data not shown). These results are in contrast to those obtained with *Mdm2*$^{+/-}$ bone marrow, which underwent apoptosis in culture (Alt et al., 2003). Western blots showed lower levels of Mtbp protein in *Mtbp*$^{+/-}$ pre-B cells in comparison to *Mtbp*$^{+/+}$ pre-B cells, whereas Myc and Mdm2 levels were equivalent in both genotypes (Fig. 5A). Since Mtbp expression is regulated by factors that induce growth (Fig. 1), and IL-7 is essential for pre-B cell growth and survival, we evaluated the susceptibility of *Mtbp*$^{+/-}$ pre-B cells to cytokine deprivation-induced apoptosis. *Mtbp*$^{+/-}$ pre-B cells underwent apoptosis from IL-7 deprivation at the same rate as *Mtbp*$^{+/+}$ pre-B cells (Fig. 5B). Therefore, pre-B cells with decreased levels of Mtbp grew and underwent spontaneous apoptosis at normal rates, and were equally as sensitive to apoptosis from growth factor deprivation as wild-type pre-B cells.

To determine whether reduced Mtbp levels impact Myc functions, we evaluated Myc-induced apoptosis. pre-B cells were infected with a bicistronic retrovirus encoding MycER and green fluorescent protein (GFP). Both wild-type and *Mtbp*$^{+/-}$ GFP^{+} pre-B cells underwent apoptosis at a similar rate upon MycER activation with 4-OHT (Fig. 5C). We also assessed Myc-induced apoptosis in cultures of pre-B cells from Eμ-*myc* mice. Bone marrow cultures from *Mtbp*$^{+/-}$Eμ-*myc* and *Mtbp*$^{+/+}$Eμ-*myc* littermates prior to any detectable disease consistently had similar numbers of dead or dying cells (data not shown). Additionally, overexpression of Myc results in DNA breaks, which is thought to contribute to apoptosis and chromosomal instability (Ray et al., 2006; Vafa et al., 2002; Wang et al., 2008). We evaluated metaphases from splenocytes from *Mtbp*$^{+/-}$Eμ-*myc* and *Mtbp*$^{+/+}$Eμ-*myc* littermates prior to detectable lymphoma. There were similar percentages of metaphases with DNA breaks (chromosome and chromatid) and aneuploidy (greater or less than 40 chromosomes) in both genotypes (Fig. 5D). Therefore, decreased Mtbp levels did not appear to influence Myc-induced apoptosis, DNA breakage, or chromosomal instability.

Loss of one allele of Mtbp inhibits Myc-driven proliferation

As an oncogene, increased levels of Myc force proliferation; consequently, we evaluated Myc-driven B cell growth. Bone marrows from *Mtbp*$^{+/-}$Eμ-*myc* and *Mtbp*$^{+/+}$Eμ-*myc* littermates prior to any detectable disease were placed into culture in IL-7 containing media, and viable cells were counted at intervals for 20 days. The *Mtbp*$^{+/-}$Eμ-*myc* pre-B cells emerged from the cultures at a slower rate than the *Mtbp*$^{+/+}$Eμ-*myc* pre-B cells (Fig. 6A). Once the pre-B cells dominated the cultures (7–10 days), the number of population doublings of the *Mtbp*$^{+/-}$Eμ-*myc* pre-B cells was less than that of pre-B cells from *Mtbp*$^{+/+}$Eμ-*myc* littermates. *Mtbp*$^{+/-}$Eμ-*myc* pre-B cells expressed lower levels of Mtbp protein and incorporated decreased amounts of BrdU compared to *Mtbp*$^{+/+}$Eμ-*myc* pre-B cells (Fig. 6B), illustrating fewer *Mtbp* haploinsufficient cells in S-phase. Therefore, the decreased rate of Myc-induced proliferation in *Mtbp*$^{+/-}$ B cells indicates that Mtbp is limiting when Myc is forcing proliferation.

Myc overexpression in the B cell compartment of wild-type Eμ-*myc* mice leads to increased numbers of pre-B cells, which escape the bone marrow and are detected in the spleens of these mice (Langdon et al., 1986). If decreased levels of Mtbp inhibit Myc-induced

proliferation, we postulated that B cell numbers would be altered in *Mtbp*$^{+/-}$Eμ-*myc* mice. Evaluation of *Mtbp*$^{+/-}$ and *Mtbp*$^{+/+}$ spleens from littermates showed normal numbers and percentages of total (B220^{+}/CD19^{+}) and mature (IgM^{+}/CD19^{+}) B cells (Fig. 6C). Although the percentage of total B cells in *Mtbp*$^{+/-}$Eμ-*myc* spleens was similar to that in *Mtbp*$^{+/+}$Eμ-*myc* littermates (data not shown), there were decreased numbers of total B cells in *Mtbp*$^{+/-}$Eμ-*myc* transgenic spleens. This difference in total B cell numbers was due to significantly reduced numbers of pre-B cells (IgM^{-}/CD19^{+}) in the spleens in *Mtbp*$^{+/-}$Eμ-*myc* mice, as immature/mature B cell (IgM^{+}/CD19^{+}) numbers were similar and not statistically different (Fig. 6C). Thus, B cell development was altered in *Mtbp*$^{+/-}$Eμ-*myc* mice and not in *Mtbp*$^{+/-}$ mice in the absence of the Myc transgene. Therefore, these results combined with the data above suggest that an insufficient amount of Mtbp is present in *Mtbp*$^{+/-}$Eμ-*myc* pre-B cells for Myc to effectively drive proliferation and expansion of this population.

To further investigate the decreased Myc-induced proliferation of Mtbp deficient cells, we evaluated the expression of four Myc transcriptional targets that are necessary for proliferation following Myc activation. MEFs from *Mtbp*$^{+/-}$ and *Mtbp*$^{+/+}$ littermates were infected with the bicistronic retrovirus encoding MycER and GFP. MEFs were chosen for these experiments, since in the presence of serum, they proliferate when MycER is activated instead of undergoing apoptosis. Equal levels of MycER and GFP were detected in both genotypes of MEFs, and both grew at similar rates (Fig. 6D & data not shown). Activation of MycER with 4-OHT in the GFP^{+} wild-type MEFs resulted in the induction of *ODC*, carbamoyl-phosphate synthetase 2/aspartate transcarbamylase/dihydroorotase (*CAD*), nucleophosmin (*NPM*), and nucleolin (*NCL*) in three hours (Fig. 6D). In contrast, in the GFP^{+} *Mtbp*$^{+/-}$ MEFs, these Myc target genes showed a significantly reduced induction of expression following MycER activation. Similar results were obtained up to eight hours post MycER activation (data not shown). Therefore, decreased expression of Mtbp resulted in a reduction of Myc-mediated transcription of genes necessary for proliferation.

As a separate independent measure of Mtbp's role in proliferation and to further test the contribution of p53 to Mtbp function, we knocked down *Mtbp* expression in *p53*$^{-/-}$ MEFs with *Mtbp*-specific siRNAs. Within 48 hours, MEFs transfected with a pool of four different *Mtbp*-specific siRNAs showed an approximately 90% reduction in Mtbp expression, a larger decrease in Mtbp to that in *Mtbp*$^{+/-}$ cells, compared to control (Fig. 6E). Control siRNA did not impact cell growth, whereas MEFs with the *Mtbp*-specific siRNAs showed a significant reduction in proliferation 96 hours post transfection. There was no detectable difference in apoptosis between the samples (data not shown). Therefore, loss of Mtbp expression suppressed proliferation in a p53-independent manner.

Mtbp is overexpressed in lymphomas

Our results suggest that Mtbp expression contributes to Myc functions necessary for growth and transformation, and is induced by Myc, and thus, we postulated Mtbp expression would be increased in lymphomas that overexpress Myc. *Mtbp* mRNA levels as determined by qRT-PCR were significantly elevated in Eμ-*myc* lymphomas compared to *Mtbp* levels in untransformed Eμ-*myc* splenocytes (Fig. 7A). Similarly, protein levels of Mtbp were also

increased in Eμ-*myc* lymphomas. There were also significantly elevated levels of *MTBP* mRNA and protein in human B cell lymphoma cell lines, as compared to three normal human lymphoid tissue controls (Fig. 7B). Additionally, there were increased levels of MTBP in human Burkitt lymphomas that have *MYC* translocations, as well as in the human diffuse large B cell lymphoma (DLBCL) cell lines, which frequently overexpress Myc. Therefore, B cell lymphomas express increased levels of MTBP, possibly due to its role in proliferation, and thus, may contribute to lymphomagenesis.

Discussion

A previous overexpression study has implicated Mdm2, and consequently p53, in mediating Mtbp functions (Boyd et al., 2000; Brady et al., 2005). However our unbiased genetic approach has revealed that Mdm2 appears to have little to do with Mtbp function. Utilizing the Eμ-*myc* transgenic mouse model, which has well-established pathways of tumor development, we have demonstrated that Mtbp is a novel regulator of tumor development and that decreased levels of Mtbp significantly inhibited lymphoma development in a manner different from that when Mdm2 or p53 levels are altered. Although both *Mtbp* and *Mdm2* heterozygous Eμ-*myc* mice had profound delays in tumor development, here we have made the novel finding that the extended tumor latency of both mice was caused by two distinct mechanisms. Specifically, cells from $Mtbp^{+/-}$ mice were impaired in their ability to respond to Myc overexpression, resulting in decreased proliferation and delayed tumor development. In contrast, $Mdm2^{+/-}$ B cells are hyper-responsive to Myc overexpression and undergo p53-dependent apoptosis causing a delay in tumorigenesis (Alt et al., 2003). If reduced levels of Mtbp lead to decreased levels of Mdm2 as was reported (Brady et al., 2005), then $Mtbp^{+/-}$Eμ-*myc* mice should have had increased B cell apoptosis and a high frequency of *p53* mutations in the lymphomas that arose. However, there was no significant increase in apoptosis of $Mtbp^{+/-}$ cells when Myc was overexpressed, and the frequency of *p53* mutations and deletions in $Mtbp^{+/-}$Eμ-*myc* lymphomas was similar to that of wild-type Eμ-*myc* lymphomas. In addition, loss of one allele of *ARF*, which restored lymphoma latency in $Mdm2^{+/-}$Eμ-*myc* mice to that of wild-type Eμ-*myc* mice (Eischen et al., 2004), only accelerated lymphoma latency in $Mtbp^{+/-}$Eμ-*myc* mice to the rate observed for $ARF^{+/-}$Eμ-*myc* mice. Notably, deletion of *p53*, which rescues the *Mdm2*-null lethality, did not rescue $Mtbp^{-/-}$ embryos (Iwakuma et al., 2008). Moreover, $Mtbp^{+/-}$ thymocytes had a normal p53 response following gamma irradiation (Iwakuma et al., 2008). Combined, the data demonstrate that Mtbp does not function through Mdm2, but it is clearly a regulator of Myc-induced tumorigenesis.

Determining the role of Mtbp in mediating the delay in lymphoma development was challenging, since it did not appear to function through Mdm2 or impact apoptosis. However, since Mtbp expression was increased by pro-proliferative signals from growth factors and oncogenes and decreased when cells were arrested, this suggested Mtbp may participate in proliferation. The decreased proliferation we detected following knockdown of *Mtbp* expression supported this concept. We also observed that under conditions of hyperproliferative signals from the oncogene Myc, decreased Mtbp expression had significant effects on Myc function. Specifically, cells from $Mtbp^{+/-}$ mice were impaired in their ability to respond to Myc overexpression, resulting in decreased proliferation and

transcriptional activation of Myc target genes necessary for cell growth. This was reflected in *Mtbp*$^{+/-}$Eμ-*myc* mice by a significantly reduced expansion of pre-B cells normally observed in Eμ-*myc* mice. Previously, B-cells with a decreased ability to proliferate from Myc overexpression have impaired Myc-induced lymphomagenesis. For example, deletion of *E2F1* or loss of one allele of *ODC* led to an inhibition of Myc-induced proliferation of MEFs or B cells, respectively (Baudino et al., 2003; Nilsson et al., 2005). Eμ-*myc* mice lacking *E2F1* or heterozygous for *ODC* had a delay in B cell lymphomagenesis that was attributed to reduced proliferation. We observed a decrease in the ability of Myc to upregulate *ODC* and other genes necessary for proliferation when *Mtbp* was heterozygous, which could explain the reduced rates of Myc-mediated proliferation in *Mtbp*$^{+/-}$ Eμ-*myc* B cells. Therefore, a decrease in Myc-induced proliferation should account for the significant delay in B cell lymphoma development in *Mtbp*$^{+/-}$Eμ-*myc* mice. However, we cannot rule out the possibility that Mtbp contributes to transformation in other ways as well. In addition, the biochemical mechanism by which Mtbp impacts Myc proliferative and transformation functions is unresolved. If Mtbp is a direct transcriptional target of Myc, as our data suggests, Mtbp could influence proliferation downstream of Myc. We have also detected Mtbp in the nucleus and associated with chromatin (CME unpublished data); thus, Mtbp could directly or indirectly affect Myc transcriptional functioning. Although additional studies are needed to define the biochemical mechanism of Mtbp function, our results do show that Mtbp is regulated by Myc and has a critical role in proliferation mediated by Myc.

Our data indicate that Mtbp regulates Myc-induced lymphoma development in a manner that is consistent with an oncogene rather than a tumor suppressor. Specifically, decreased expression of Mtbp inhibited Myc-induced proliferation and tumor development. *Mtbp* was not deleted in wild-type Eμ-*myc* lymphomas, nor was there LOH of *Mtbp* in *Mtbp*$^{+/-}$Eμ-*myc* lymphomas. In another study, *Mtbp*$^{+/-}$*p53*$^{+/-}$mice had a similar tumor latency as *p53*$^{+/-}$ mice (Iwakuma et al., 2008). Therefore Mtbp does not appear to be a classic tumor suppressor, as was originally proposed (Boyd et al., 2000). Instead, Mtbp levels were elevated in B cell lymphomas from mice and humans. These data are consistent with the observation that the region that encodes *MTBP* is frequently amplified in human colorectal cancers and multiple myeloma (Carrasco et al., 2006; Martin et al., 2007). Moreover, public microarray databases show increased *MTBP* mRNA in many human cancers as compared to normal tissues. Therefore, mounting data indicate Mtbp overexpression is selected for and likely contributes to tumorigenesis, and thus Mtbp may be oncogenic. However, there is also data suggesting that decreased levels of Mtbp contribute to tumor metastasis in *p53* heterozygous mice (Iwakuma et al., 2008). Although the biochemical function of Mtbp in metastasis in *p53*$^{+/-}$ mice is unresolved, the requirements of Mtbp in metastasis are likely to be different than that for Mtbp in Myc-induced proliferation and tumorigenesis. It will be important in the future to determine whether Mtbp is oncogenic in its own right or a critical partner of an oncogene such as Myc, and its precise role in metastasis.

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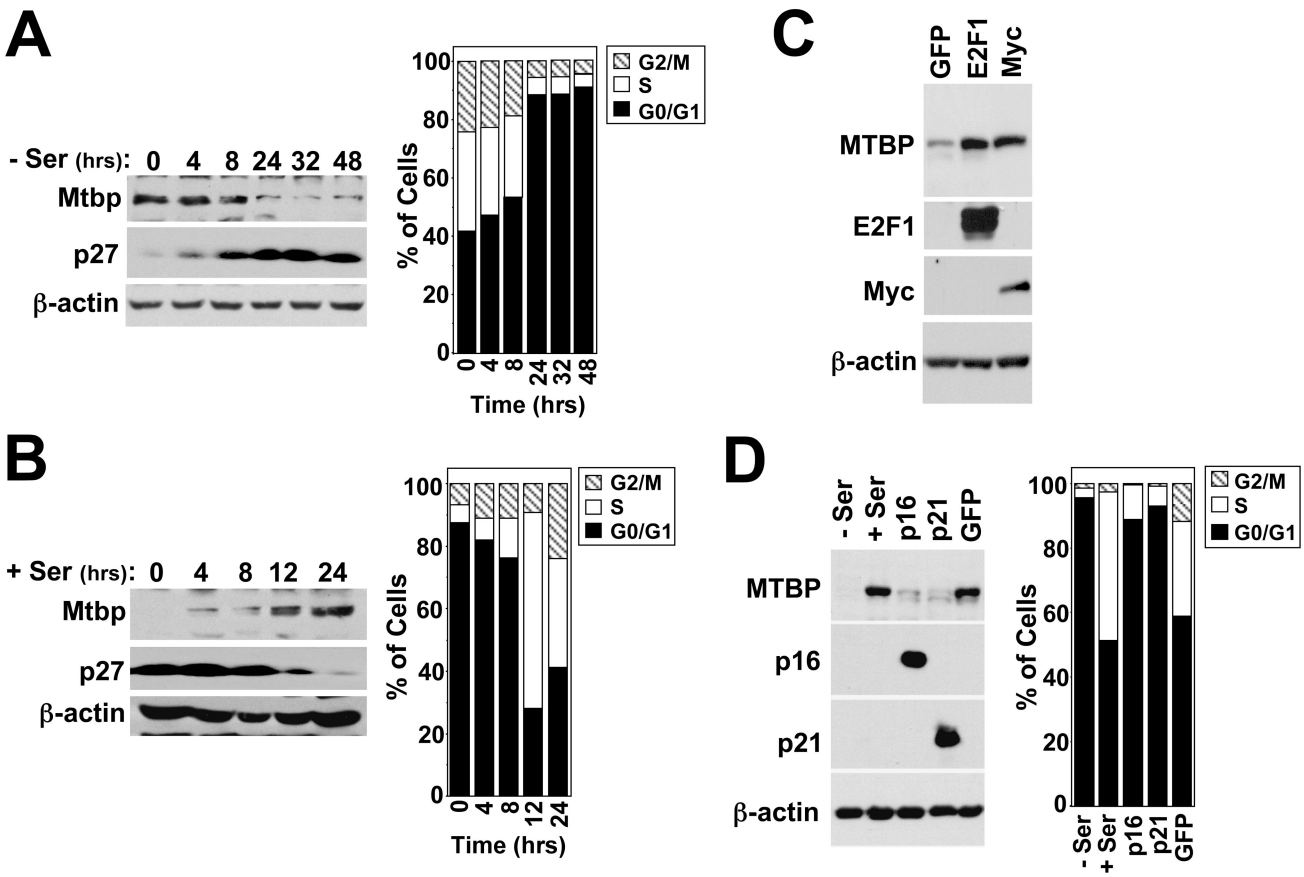

Figure 1.

Mtbp expression is regulated by proliferative signals. (A) Western blot and cell cycle analysis of *p53*$^{-/-}$ MEFs deprived of serum (−Ser) for the indicated hours. (B) Following 24 hours of serum deprivation, serum was added back to the culture medium of *p53*$^{-/-}$ MEFs for the indicated number of hours and Western blot and cell cycle analyses were performed. (C) Western blots of H1299 cells infected with recombinant adenoviruses (30 plaque-forming units/cell) encoding GFP alone, E2F1 and GFP, or c-Myc and GFP. (D) Western blot and cell cycle analyses of H1299 cells grown in medium with (+Ser) or without (−Ser) serum or with serum and infected with adenoviruses expressing GFP alone, p16 and GFP, or p21 and GFP.

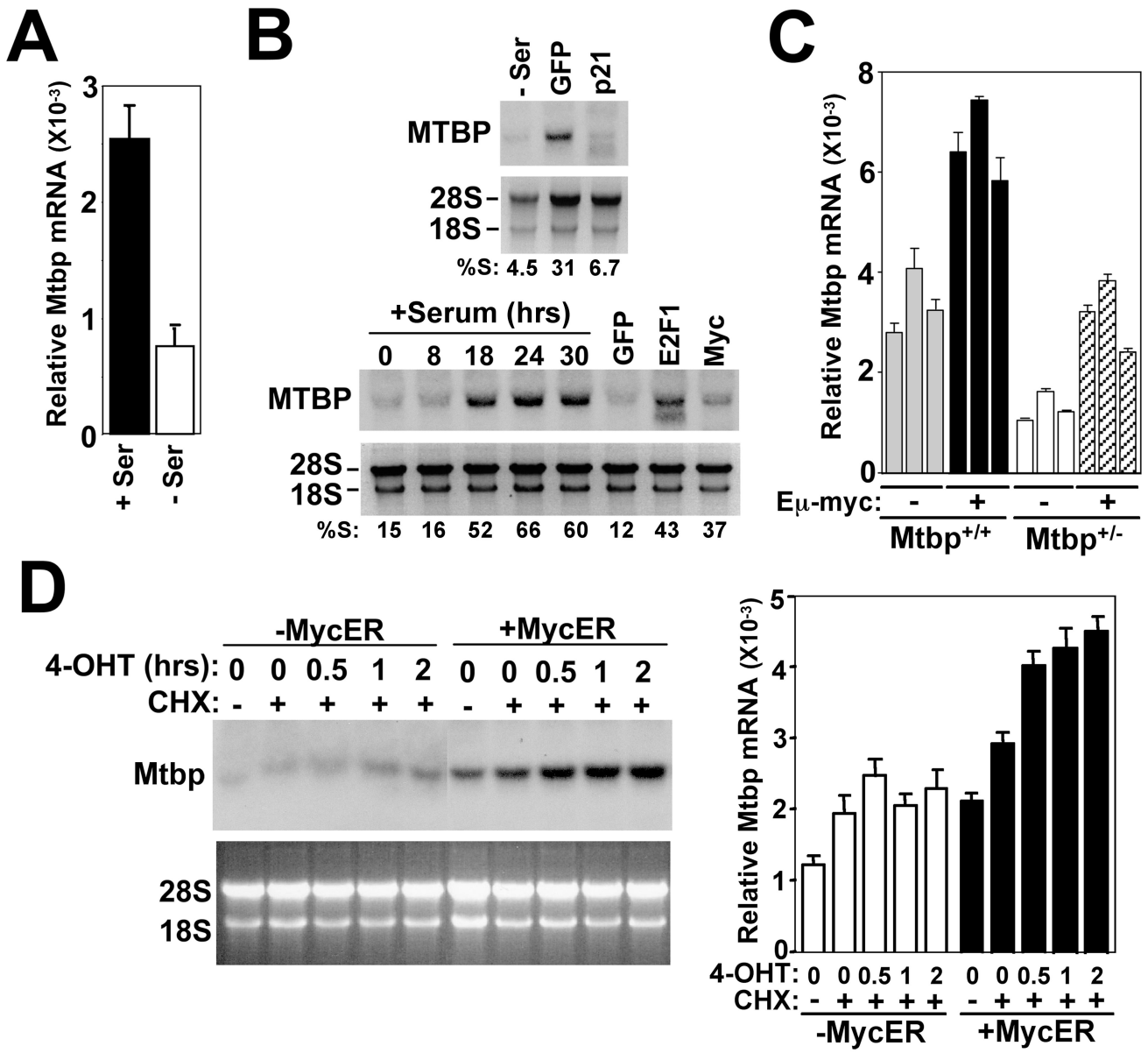

Figure 2.

Mtbp is transcriptionally regulated by Myc. (A) qRT-PCR analysis of *p53*$^{-/-}$ MEFs growing in (+Ser) or deprived of (−Ser) serum for 24 hours. (B) Following 24 hours of serum deprivation (−Ser), serum was added to H1299 cells and cells harvested at intervals (hours). Northern blots for *MTBP* mRNA from these samples and samples in Figure 1C and 1D were performed. 28S and 18S are also shown. The percentage of cells in S-phase (%S) in each sample is indicated. (C) qRT-PCR analysis of splenocytes from littermate matched mice that were *Mtbp*$^{+/+}$ or *Mtbp*$^{+/-}$ and either Eμ-*myc* negative (−) or Eμ-*myc* positive (+). (D) Murine fibroblasts infected with a bicistronic retrovirus encoding GFP alone (−MycER) or MycER and GFP (+MycER) were serum deprived for 24 hours and left untreated (−) or pre-treated (+) with cycloheximide (CHX) for 30 min; 4-OHT was then added for the

indicated times. Northern blot and qRT-PCR for *Mtbp* expression were performed. 28S and 18S are also shown. (A, C, & D) qRT-PCR data were generated in triplicate and normalized to β-*actin* levels.

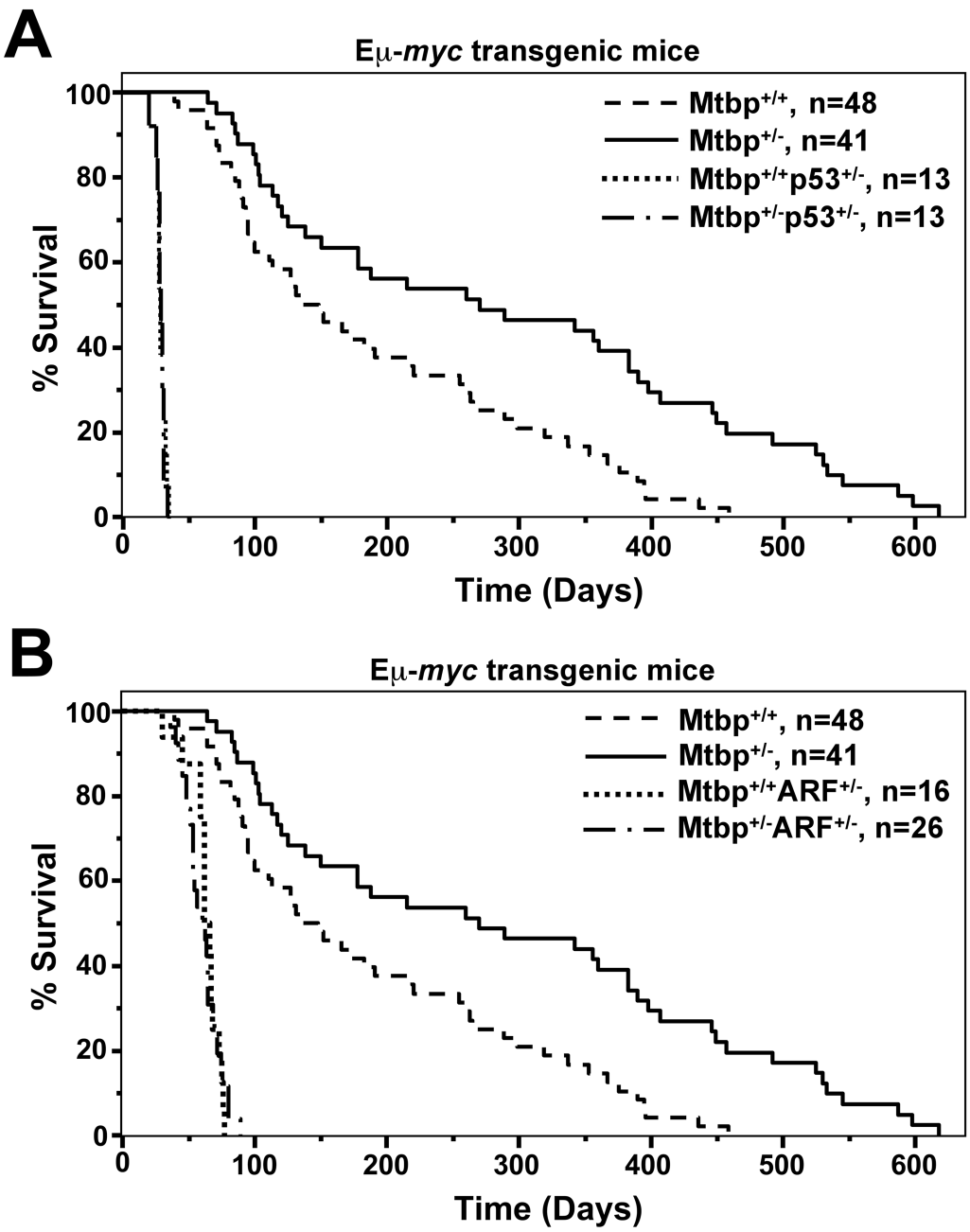

Figure 3.

Myc-induced lymphomagenesis is inhibited by *Mtbp* heterozygosity. (A, B) Kaplan-Meier survival curves of Eμ-*myc* transgenic mice of the indicated genotypes (p=0.0004 log-rank test, *Mtbp*$^{+/+}$Eμ-*myc* versus *Mtbp*$^{+/-}$Eμ-*myc*). The number of mice in each group is denoted by "n" values. Lymphoma was documented in all mice.

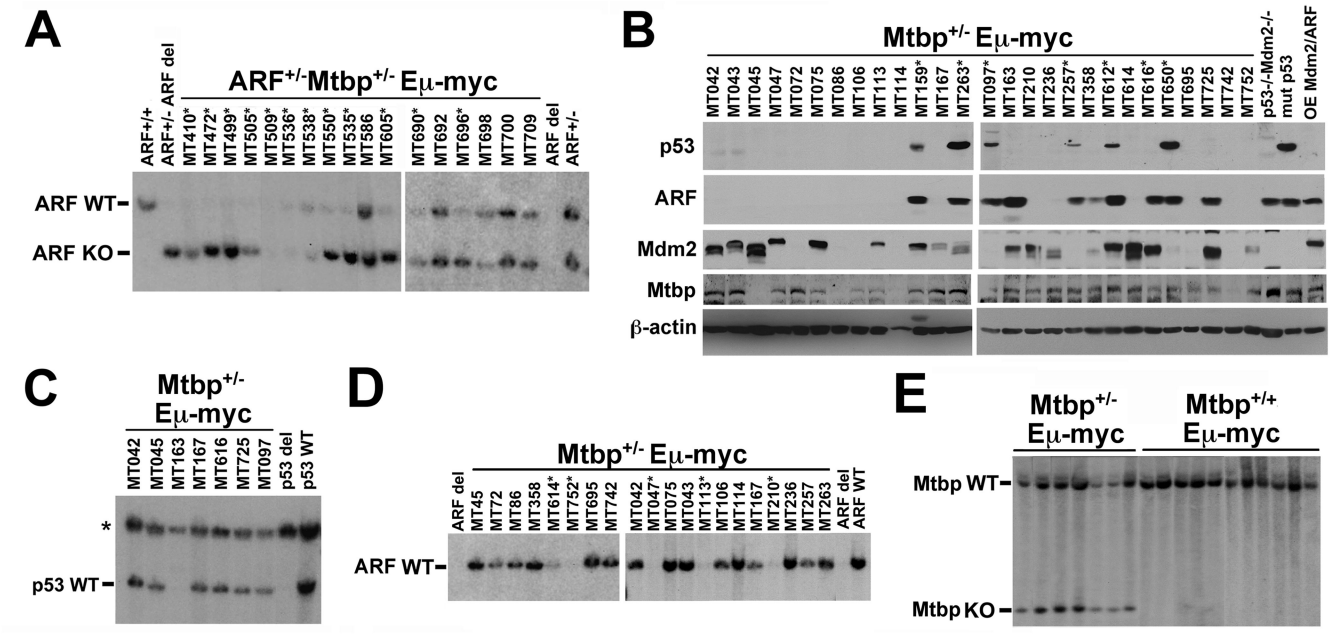

Figure 4.

Normal frequencies of p53, ARF, and Mdm2 alterations in *Mtbp*$^{+/-}$Eμ-*myc* lymphomas. (A) Southern blots for *ARF* in *ARF*$^{+/-}$*Mtbp*$^{+/-}$Eμ-*myc* transgenic lymphomas. Location of the *ARF* wild-type (WT) and knockout (KO) alleles are indicated. Asterisks denote lymphomas that have deleted *ARF*. (B) Levels of the indicated proteins in whole cell extracts of *Mtbp*$^{+/-}$Eμ-*myc* lymphomas were assessed by Western blotting. Lysates from *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs, a lymphoma expressing mutant p53 (mut p53), and a lymphoma overexpressing Mdm2 and ARF (OE Mdm2/ARF) were controls. Asterisks indicate lymphomas with mutant p53. p53 in MT616 is not detectable due to a nonsense mutation. (C) Southern blot for *p53* in *Mtbp*$^{+/-}$Eμ-*myc* lymphomas. The asterisk denotes the location of the pseudogene. (D) Southern blot for *ARF* in *Mtbp*$^{+/-}$Eμ-*myc* lymphomas. Asterisks indicate lymphomas that have deleted *ARF*. (E) Southern blot for *Mtbp* in Eμ-*myc* lymphomas. The location of *Mtbp* wild-type (WT) and knockout (KO) alleles are indicated.

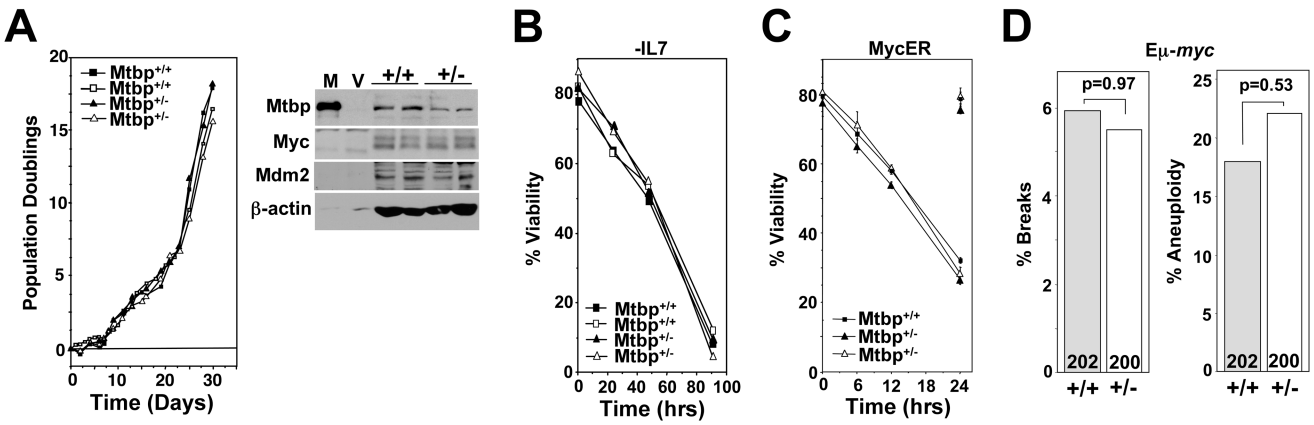

Figure 5.

Normal rates of Myc-induced apoptosis and chromosome instability in *Mtbp* heterozygous B cells. (A) Bone marrow from two $Mtbp^{+/+}$ (squares, +/+) and two $Mtbp^{+/-}$ (triangles, +/−) littermates was placed into IL-7 containing medium on day 0. Viable cells were counted at intervals. Data is representative of three independent experiments. Whole cell lysates from pre-B cells that emerged from the cultures were Western blotted. Protein lysates from 293T cells transfected with a vector encoding Mtbp (M) or empty vector (V) were controls (1/6 of the amount of protein of the other samples). (B) Littermate matched pre-B cells of the indicated genotypes were deprived of IL-7, and viability determined at intervals with Trypan Blue Dye exclusion. (C) 4-OHT was added at time 0 to MycER expressing pre-B cells (littermate matched) to activate MycER, and their viability was determined at intervals thereafter by Trypan Blue Dye exclusion. Apoptosis was confirmed by analysis of subdiploid DNA content after staining with PI. Symbols in upper right corner are 24 hour vehicle (ethanol) control treated MycER expressing pre-B cells. (D) Metaphases from splenocytes from $Mtbp^{+/-}$ Eμ-*myc* transgenic (+/−) and $Mtbp^{+/+}$Eμ-*myc* littermates (+/+) prior to any detectable lymphoma. The number of total metaphases analyzed is indicated at the bottom of each bar. Breaks were chromosome or chromatid. Aneuploidy is defined as any metaphase having more or less than 40 chromosomes. Chi-squared test was used to determine significance.

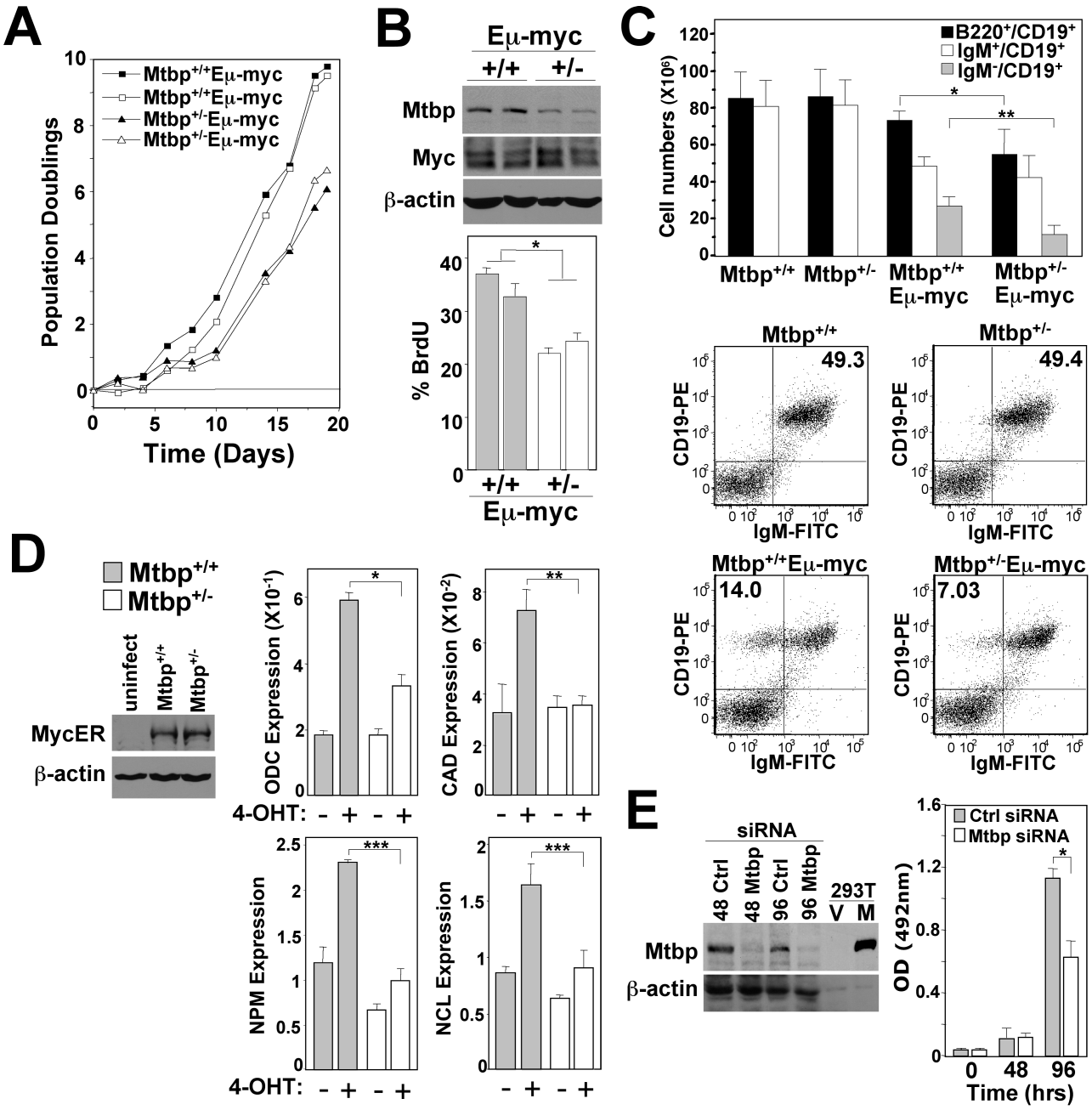

Figure 6.

Mtbp heterozygous pre-B cells have reduced Myc-driven proliferation. (A) Bone marrow from two *Mtbp*$^{+/+}$Eμ-*myc* (squares) and two *Mtbp*$^{+/-}$Eμ-*myc* (triangles) littermates prior to any detectable lymphoma was placed into IL-7 containing medium (day 0). Viable cells were counted at intervals. Data is representative of three independent experiments from separate litters of mice. (B) Western blots for the indicated proteins and the percentage of cells with BrdU incorporation in pre-B cells from two *Mtbp*$^{+/-}$Eμ-*myc* transgenics (+/−) and two *Mtbp*$^{+/+}$Eμ-*myc* transgenic littermates (+/+) was determined. Differences in BrdU

incorporation between the two genotypes were significant (p=0.02, t-test). (C) Total numbers of B cells ($B220^+/CD19^+$) or B cell subsets in spleens of the indicated genotypes. Each bar represents four-six mice of each genotype. Asterisks denote statistically significant differences (*p=0.02; **p=0.0006, t-test). Representative dot plots of CD19-PE versus IgM-FITC gated on total lymphocytes from spleens. (D) Relative mRNA levels of the indicated gene transcripts were determined by qRT-PCR in $Mtbp^{+/-}$ and $Mtbp^{+/+}$ MycER expressing MEFs following MycER activation with 4-OHT (*p=0.0005, **p=0.003, ***p=0.002; t-test). All data were generated in triplicate and normalized to β-*actin* levels. The levels of MycER were determined by Western blot. (E) $p53^{-/-}$ MEFs were transfected with control siRNA (Ctrl) or a pool of Mtbp-specific siRNAs. MTS assays were performed at intervals (hours) (*p=0.0003, t-test) and protein lysates were Western blotted. Protein lysates from 293T cells transfected with a vector encoding Mtbp (M) or empty vector (V) were controls (1/6 of the amount of protein of the other samples).

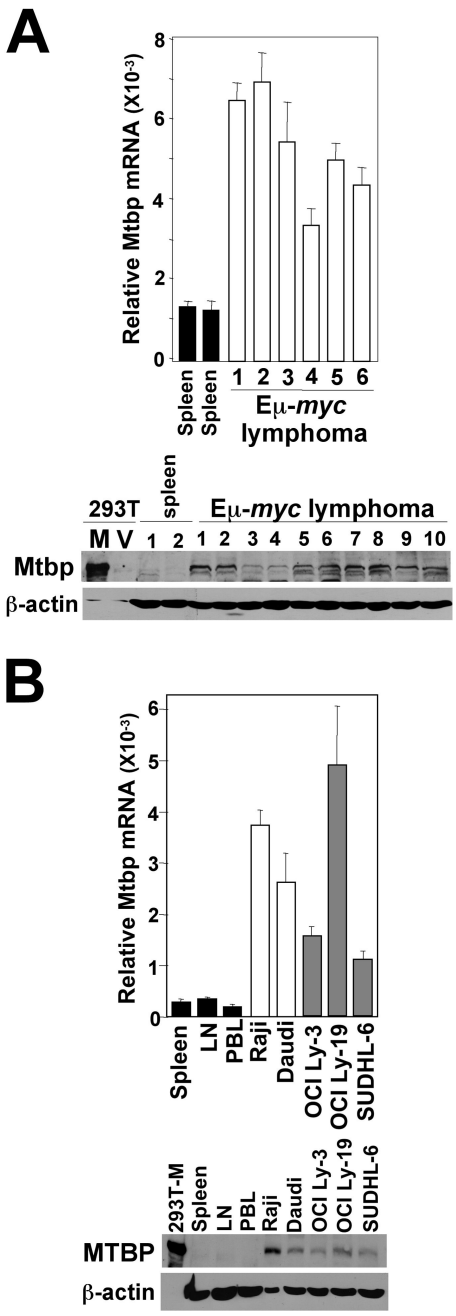

Figure 7.

Increased Mtbp expression in murine and human lymphomas. (A, B) qRT-PCR and Western blots were performed with splenocytes (prior to detectable disease) and B cell lymphomas from wild-type Eμ-*myc* transgenic mice (A) and human B cell lymphoma lines (Burkitt lymphomas, white bars; DLBCL, grey bars) and normal human lymphatic tissues (spleen; lymph node, LN; peripheral blood lymphocytes, PBL, black bars) (B). Protein lysates from 293T cells transfected with an Mtbp expressing vector (M) or empty vector (V) were

controls (1/6 of the amount of protein of the other samples). qRT-PCR data were generated in triplicate and normalized to β-*actin* levels.