THE FUNCTION OF MTBP IN PROLIFERATION,

TUMORIGENESIS AND TUMOR CELL MAINTENANCE

By

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Dedicated to my wonderful wife Cassie and our baby Charlotte Joy,

you are and always will be my greatest source of joy.

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4-OHT	4-hydroxytamoxifen
Ac	acetyl group
ACTN4	alpha actinin 4
ATCC	American Type Culture Collection
ATP	adenosinetriphosphate
BCL2	B-cell CLL/lymphoma 2
bHLH	basic helix-loop-helix
BRCA1	breast cancer 1
Brd4	bromodomain containing 4
BrdU	bromodeoxyuridine
C/EBP-β	CCAAT/enhancer binding protein beta
CAD	carbamoyl-phosphate synthetase 2
CCND2	cyclin D2
CDK	cyclin dependent kinase
cDNA	complimentary DNA
ChIP	chromatin immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
E2F1	E2F transcription factor 1
Eμ	immunoglobulin heavy chain enhancer

EGFR	epithelial growth factor receptor
ER	estrogen receptor
ERK	mitogen-activated protein kinase 1
FASN	fatty acid synthetase
FBW7	F-box and WD repeat domain containing 7
G1	growth phase 1
G2	growth phase 2
GCN5	general control non-derepressible 5
GFP	green fluorescent protein
GST	glutathione S-transferases
HAT	histone acetyl transferase
HDAC	histone deacetylase
HER1	human epidermal growth factor receptor 1
HER2	human epidermal growth factor receptor 2
hTERT	human telomerase reverse transcriptase
IGFR	insulin-like growth factor 1 receptor
IgG	immunoglobulin G
INI1	SWI/SNF related matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
INO80	INO80 complex subunit
IP	immunoprecipitation
kDa	kilodalton
LN	lymphnode
LZ	leucine zipper

LOH	loss of heterozygosity
M	mitosis
Mad2	mitotic arrest-deficient 2
Max	MYC associated factor X
MC29	myelocytomatosis virus 29
Mdm2	murine double minute 2
Me	methyl group
MEF	mouse embryonic fibroblast
miRNA	micro RNA
Miz1	zinc finger and BTB domain containing 17
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSCV	murine stem cell virus
MTBP	Mdm2 Binding Protein
mTOR	mammalian target of rapamycin
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-*(*4,5-Dimethyl-2-thiazolyl*)*-2,5-diphenyl-2H-tetrazolium bromide
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NCL	nucleolin
NFY	nuclear transcription factor Y
NLS	nuclear localization sequence
NPM	nucleophosmin
ODC	ornithine decarboxylase

P	phosphate
p15	cyclin-dependent kinase inhibitor 2B
p16	cyclin-dependent kinase inhibitor 2A
p21	cyclin-dependent kinase inhibitor 1A
p27	cyclin-dependent kinase inhibitor 1B
p53	tumor protein p53
p300	E1A binding protein p300
PARP	poly-(adenosine diphosphate-ribose) polymerase
PBL	peripheral blood lymphocytes
PCAF	lysine acetyltransferase 2B
PCR	polymerase chain reaction
PI	propidium iodide
POL	polymerase
PR	progesterone receptor
P-TEFb	positive transcription elongation factor b
qRT-PCR	quantitative real time polymerase chain reaction
Ras	rat sarcoma viral oncogene homolog
ROS	reactive oxygen species
RFP	red fluorescent protein
RNA	ribonucleic acid
rRNA	ribosomal RNA
S	synthesis
SCCHN	squamous cell carcinoma of the head and neck

SCF	skp2 cullin F-box
Ser	serine
Seq	sequencing
shRNA	short hairpin ribonucleic acid
siRNA	short interfering ribonucleic acid
SIRT1	sirtuin 1
Skp2	S-phase kinase-associated protein 2
SP1	Sp1 transcription factor
Src	v-src avian sarcoma viral oncogene homolog
TAD	transcriptional activation domain
TCF	T cell transcription factor
TCGA	The Cancer Genome Atlas
Thr	threonine
Tip48	48 kDa TATA box-binding protein-interacting protein
Tip49	49 kDa TATA box-binding protein-interacting protein
Tip60	KAT5, K (lysine) acetyltransferase 5
TNBC	triple negative breast cancer
TRRAP	transformation/transcription domain-associated protein
Veh	vehicle
WBM	WDR5 binding mutant
WCL	whole cell lysate
YFP	yellow fluorescent protein

CHAPTER I

INTRODUCTION

Introduction to Cancer

According to the American Cancer Society, 1.7 million new cancer diagnoses and 585,000 deaths are expected for 2014 in the United States (ACS, 2014). This will cost approximately $212 billion in medical expenses or lost productivity (ACS, 2014). Thus, cancer research is a critical scientific effort.

Modern cancer research has greatly increased our molecular understanding of cancer (Hanahan & Weinberg, 2000). In broad terms, cancer is promoted by oncogenes that provide growth signals and prevent apoptosis, allowing for limitless rapid proliferation and necessary supportive metabolic changes. Tumor suppressors, in contrast, are disabled by cancer as they prevent DNA replication and cell division in states of DNA damage and oncogenic signaling, often initiating apoptosis. Continuing to identify and understand how these factors are regulated is critical to impacting cancer development, progression and survival at a patient level, particularly with novel therapeutics.

Early cancer therapeutics were genotoxic, cytotoxic and anti-metabolic agents, but these indiscriminately kill proliferating cells. Modern efforts have birthed targeted cancer therapies that attempt to inhibit oncogenic signaling utilized by cancers, offering increased efficacy and decreased toxicity. While many targeted agents are highly celebrated, they are often beneficial only in select cases and cancers frequently develop resistance to them by mutating or bypassing the drug target or its pathway (Sodir & Evan,

2011). Thus, there is a need to identify and study oncogenes that are essential, functionally non-redundant, and widely compromised in cancer, so targeted therapies that are broadly applicable and less susceptible to resistance can be designed.

MYC

Discovery

The v-*myc* gene was identified in the avian retrovirus MC29 as the causative agent of chicken myelocytomatosis, reflected in its name (Sheiness *et al.*, 1978). A homolog of v-*myc* termed c-*MYC* (referred to as *MYC*) was soon identified in normal cells (Sheiness & Bishop, 1979; Sheiness *et al.*, 1980; Westin *et al.*, 1982). Shortly thereafter, *MYC* mRNA was observed to be elevated in human cancer cells (Eva *et al.*, 1982) and the *MYC* gene was discovered to be translocated in Burkitt's lymphoma, resulting in increased MYC expression in the malignant cells (Dalla-Favera *et al.*, 1982; Taub *et al.*, 1982). These and other early breakthroughs provided evidence that MYC is oncogenic (Meyer & Penn, 2008; Tansey, 2014). Moreover, they inspired over three decades of research that has implicated MYC in many aspects of both normal and cancer cell biology.

Transcription Factor and Biologic Activity

Myc is a highly conserved multifunctional basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factor (Meyer & Penn, 2008; Tansey, 2014). Although it was recently shown that Myc may act as a global transcriptional amplifier (Lin *et al.*, 2012; Nie *et al.*, 2012), this model is controversial and unresolved (Walz *et al.*, 2013). Myc is

more well known to function by binding DNA where it transcriptionally activates or represses the expression of a large, but specific, subset of genes, referred to as Myc target genes (Blackwell *et al.*, 1990; Cleveland *et al.*, 1988; Cowling & Cole, 2006; Herkert & Eilers, 2010; Kato *et al.*, 1990; Li *et al.*, 1994; Prendergast & Ziff, 1991). Data suggest Myc controls expression of 10-15% of the genome and mediates transcription by all three RNA polymerases (Arabi *et al.*, 2005; Dang *et al.*, 2006; Gomez-Roman *et al.*, 2003; Grandori *et al.*, 2005; O'Connell *et al.*, 2003; Patel *et al.*, 2004; Zeller *et al.*, 2006), regulating mRNAs, rRNAs, tRNAs and miRNAs.

For transcriptional activation, the highly conserved carboxy-terminal bHLH-LZ domain of Myc binds Max and Myc-Max heterodimers bind DNA at E-box sequences (CACGTG; Figure 1; Amati *et al.*, 1993; Blackwell *et al.*, 1990; Blackwood & Eisenman, 1991). Here, Myc recruits transcriptional machinery and a variety of cofactors to promote transcription. For example, Myc recruits complexes with histone acetyltransferase (HAT) activity that acetylate lysine resides on histones, relaxing DNA-histone interactions and allowing for binding of transcription machinery (Cowling & Cole, 2006). Moreover, after transcription initiation Myc can also recruit the positive transcription elongation factor b (P-TEFb) to sites with proximal promoter paused RNA polymerase II, promoting phosphorylation of the polymerase and subsequent transcriptional elongation (Eberhardy & Farnham, 2001; Eberhardy & Farnham, 2002). In contrast, during Myc-mediated transcriptional repression, Myc-Max heterodimers can associate with and inhibit other transcriptional activators such as Miz1, NFY, C/EBPβ and SP1 (Feng *et al.*, 2002; Gartel *et al.*, 2001; Herkert & Eilers, 2010; Mao *et al.*, 2003; Peukert *et al.*, 1997; Steinmann *et al.*, 2009; Yang *et al.*, 1993). After binding, Myc

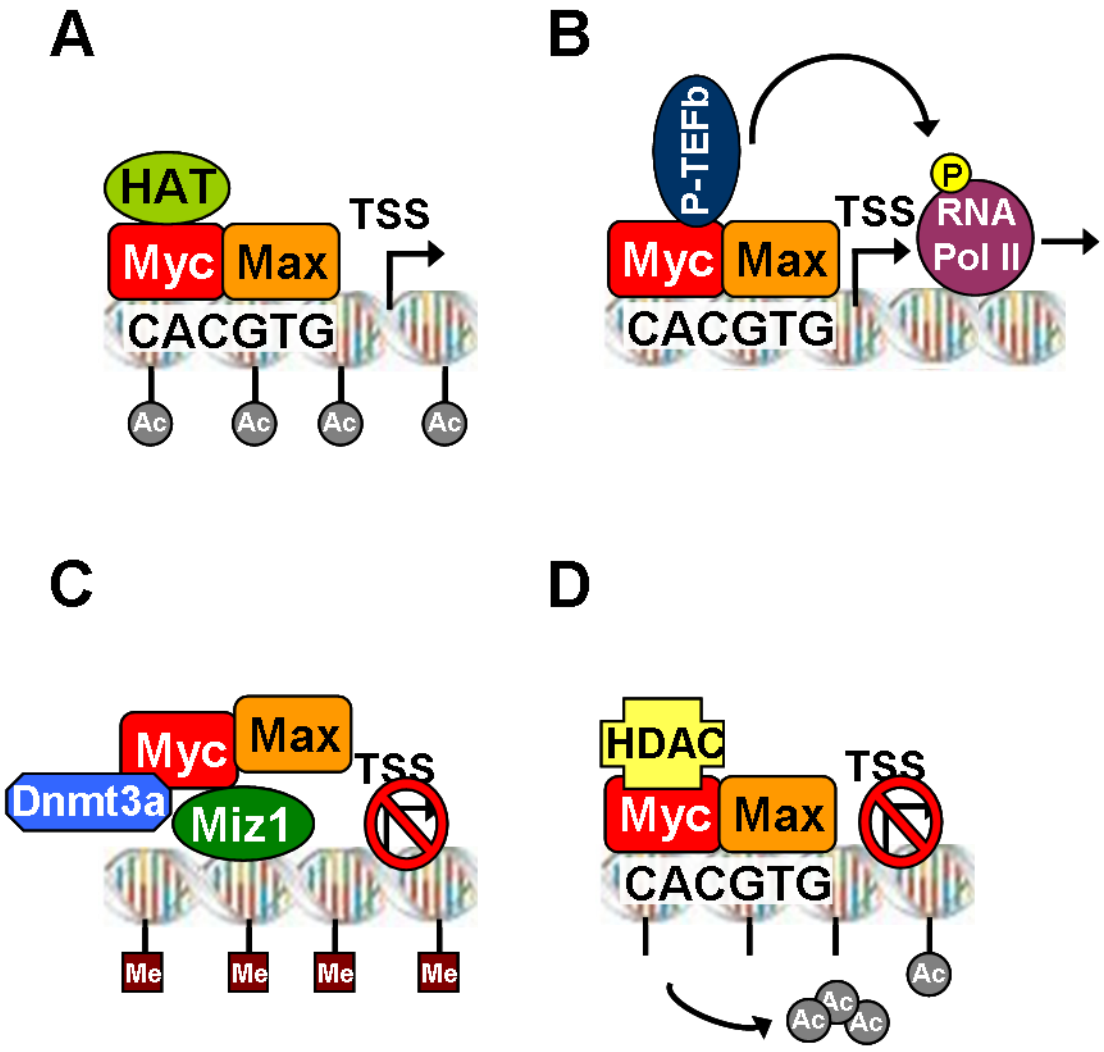

Figure 1 – Myc mediates transcriptional activation and repression. (A&B) In transcriptional activation, Myc binds E-box sequences (CACGTG) with its partner Max through a highly conserved bHLH-LZ domain and recruits cofactors to help activate transcription, such as (A) histone acetyltransferases (HATs) that relax chromatin or (B) P-TEFb that phosphorylates paused RNA polymerase II (Pol II) and promotes transcriptional elongation. (C&D) For transcriptional repression, (C) Myc binds a variety of other transcription factors like Miz1, displaces activating cofactors and recruits transcriptional repressors such as the DNA methyltransferase Dnmt3a. (D) Myc can also directly recruit histone deacetylases (HDACs) to DNA that constrict chromatin and repress transcription. (TSS, transcriptional start site; Ac, acetyl group; P, phosphate; Me, methyl group)

displaces transcriptional co-activators and recruits transcriptional repressors like the DNA methyltransferase Dnmt3a to repress transcription (Brenner *et al.*, 2005). Myc has also been demonstrated to repress gene transcription directly by recruiting histone deacetylases (HDACs) that tighten DNA-histone interactions and prevent transcription (Jiang *et al.*, 2007; Kurland & Tansey, 2008; Sun *et al.*, 2013).

At the individual gene level, transcriptional changes mediated by Myc activity are relatively weak when compared to other transcription factors, usually on the order of 2 to 3 fold (Dang *et al.*, 2006; Kretzner *et al.*, 1992; Levens, 2002; Patel *et al.*, 2004). However, genome-wide Myc coordinates a large transcriptional program that controls several critical cellular processes (Figure 2). For example, Myc has long been associated with cellular proliferation (Obaya *et al.*, 1999; Roussel *et al.*, 1991). Myc expression promotes cell cycle progression and is essential for efficient transition from G0/G1 to S phase (de Alboran *et al.*, 2001). It transcriptionally activates expression of cell cycle machinery like Cyclin D1, Cyclin D2, Cyclin E1, Cyclin A2, and Cyclin dependent kinase (Cdk) 4, while repressing transcription of prominent cell cycle inhibitors like p15, p21, and p27 (Beier *et al.*, 2000; Gartel *et al.*, 2001; Meyer & Penn, 2008; Obaya *et al.*, 2002; Roussel *et al.*, 1991; Staller *et al.*, 2001). Moreover, in some situations, Myc can transcriptionally activate telomerase (hTERT), enabling maintenance of telomeres and indefinite proliferative capacity (Blasco & Hahn, 2003; Greenberg *et al.*, 1999; Wu *et al.*, 1999). This is particularly important in stem cells where, in many tissue types, elevated Myc expression is needed to maintain proliferative self-renewal and to inhibit differentiation (Coppola & Cole, 1986; Dmitrovsky *et al.*, 1986; Langdon *et al.*, 1986; Prochownik & Kukowska, 1986; Wilson *et al.*, 2004).

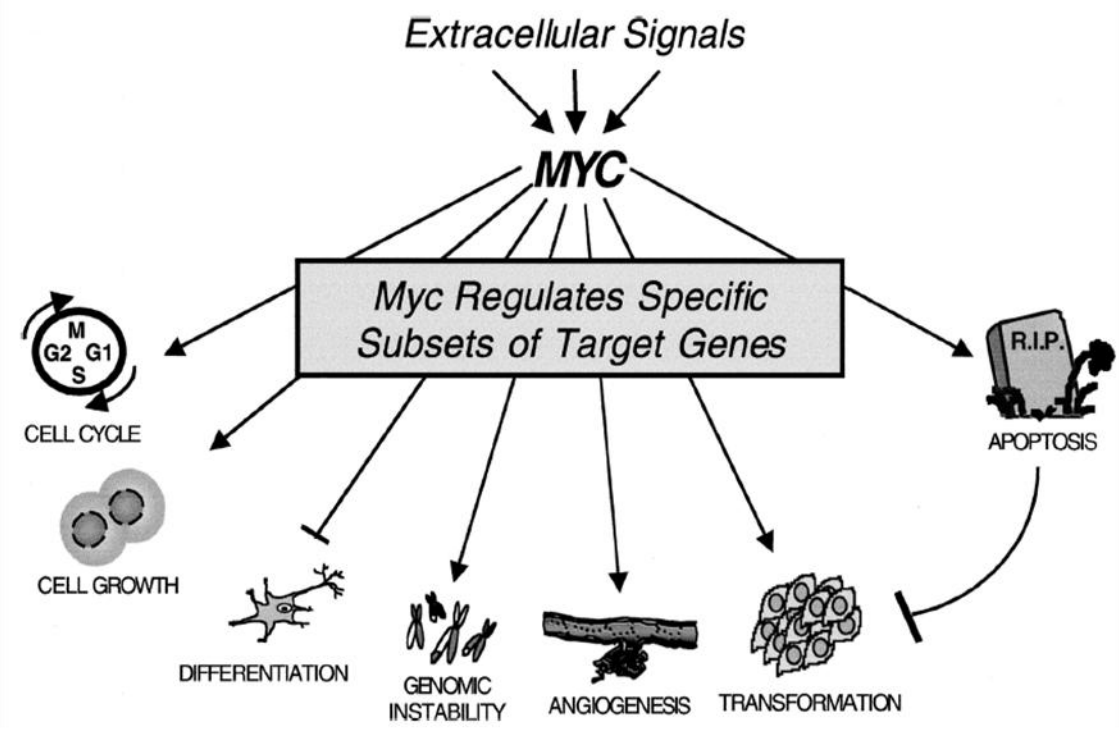

Figure 2. Myc regulates critical cellular functions. Myc responds to changes in the microenvironment and cellular signaling to regulate expression of many target genes. Through this function as a transcription factor, Myc affects a variety of biological activities. Myc has been shown to regulate cell cycle progression and proliferation, cell growth, differentiation, genomic stability, and angiogenesis. Importantly, Myc can also trigger apoptosis providing a mechanism to hinder the expansion of a clone harboring overactive Myc. Myc appears to orchestrate distinct downstream genetic programs to control each of these processes. (Adapted from Oster *et al.*, 2002.)

In order to sustain high rates of proliferation, Myc controls transcription of genes that tailor cellular metabolism specifically for growth and proliferation (Dang, 2013). First, Myc transcriptionally activates several nuclear encoded mitochondrial genes important for mitochondrial growth and division, providing sufficient mitochondria to maintain increased energy production and to support two daughter cells following mitosis (Kim *et al.*, 2008; Li *et al.*, 2005; Zhang *et al.*, 2007). Myc also transitions cells from fatty acid metabolism, commonly observed in quiescent cells, to glycolysis and glutaminolysis by increasing glucose and glutamine uptake as well as transcriptionally activating genes encoding relevant enzymes (Gao *et al.*, 2009; Hu *et al.*; Kim *et al.*, 2004; Morrish *et al.*, 2009; Osthus *et al.*, 2000; Wang *et al.*, 2011; Wise *et al.*, 2008). These pathways, in addition to producing needed ATP, also provide substrates required by proliferating cells to build macromolecules, including amino acids, nucleotides and fatty acids (Cantor & Sabatini, 2012; Chaneton & Gottlieb, 2012; Dang, 2013). In addition to providing energy and macromolecular building blocks, Myc transcriptionally activates genes involved in the assembly of complex macromolecules. For example, Myc is a master regulator of protein translation. It transcriptionally activates expression of ribosomal proteins, rRNA, tRNA, and nucleolar proteins involved in ribosomal assembly, like nucleophosmin (*Npm*) and nucleolin (*Ncl*) that control ribosomal assembly (Iritani & Eisenman, 1999; van Riggelen *et al.*, 2010). Furthermore, it regulates other basic metabolic processes like fatty acid production, urea cycling, and pyrimidine nucleotide synthesis through transcriptional regulation of fatty acid synthetase (*Fasn*), ornithine decarboxylase (*Odc*) and carbamoyl-phosphate synthetase 2 (also known as *Cad*), respectively (Loven *et al.*, 2012; Miltenberger *et al.*, 1995; Wagner *et al.*, 1993).

Collectively, these Myc metabolic and pro-proliferative target genes represent some of the most highly conserved Myc target genes, highlighting Myc's central role in regulating proliferation (Dang, 2013).

Given the collective biological functions of Myc, it is not surprising that Myc overexpression and dysregulation promotes cellular transformation and tumor growth (Meyer & Penn, 2008). Fortunately, apoptosis poses a significant barrier to Myc-induced transformation. In untransformed cells, overactive Myc induces apoptosis by indirectly suppressing anti-apoptotic factors like Bcl2 and activating pro-apoptotic factors like the tumor suppressor p53 (Askew *et al.*, 1991; Eischen *et al.*, 2001a; Eischen *et al.*, 2001b; Evan *et al.*, 1992; Hermeking & Eick, 1994; Shi *et al.*, 1992; Wagner *et al.*, 1994). These pro-apoptotic tumor suppressor pathways are inactivated in cancers. In addition to apoptosis, Myc provides other cellular stresses. Myc is thought to induce genome instability (Felsher & Bishop, 1999; Yin *et al.*, 1999) and has been shown to induce replicative stress and premature cellular senescence (Factor *et al.*, 1997). Collectively, these stresses are evident in studies where ectopic Myc expression has been shown to deplete stem cell populations, resulting in decreased tissue regeneration and wound healing (Factor *et al.*, 1997; Waikel *et al.*, 2001). This was even observed in the hematopoietic system where Myc expression is elevated physiologically to maintain stem cells (Wilson *et al.*, 2004).

Clearly, Myc has a vast and critically important role in the coordination of proliferation, differentiation, cellular metabolism, and apoptosis.

Myc Regulation

Since Myc is a master transcriptional regulator, it is not surprising that multiple pathways have evolved to ensure Myc itself is tightly regulated at the transcriptional, post-transcriptional and post-translational level (Meyer & Penn, 2008). It is well documented that serum starvation and anti-proliferative signals downregulate Myc expression (Campisi *et al.*, 1984; Dean *et al.*, 1986; Gonda & Metcalf, 1984; Lachman & Skoultchi, 1984), while many growth factors and downstream signaling pathways activate Myc expression at the transcriptional level (Kelly *et al.*, 1983), including the estrogen receptor (ER), epithelial growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), Wnt, Ras, Sonic Hedgehog and Notch (Cheng *et al.*, 2006; He *et al.*, 1998; Meyer & Penn, 2008; Oliver *et al.*, 2003; Palomero *et al.*, 2006; Weng *et al.*, 2006). Moreover, *Myc* mRNA is short lived (Brewer & Ross, 1988; Dani *et al.*, 1984; Hann & Eisenman, 1984; Jones & Cole, 1987), but can be stabilized in response to growth signaling (Bernstein *et al.*, 1992; Hann, 2006; Vervoorts *et al.*, 2006).

While these layers of regulation ensure *Myc* expression is tightly controlled, conserved amino acid sequences known as Myc Boxes (MB) I, II, IIIa, IIIb and IV provide an additional level of regulation by mediating associations between Myc and cofactors implicated in Myc protein stability and activity (Figure 3; Tansey, 2014). The precise order and regulation of cofactor association/dissociation with Myc remains unclear, but several important interactions have been characterized. For example, MBI is the site of conserved sequential phosphorylation events at Ser62 and Thr58 that enhance Myc transcriptional activity, but also facilitate ubiquitination by the SCF^{Fbw7} ubiquitin ligase and subsequent proteasomal degradation (Lutterbach & Hann, 1994;

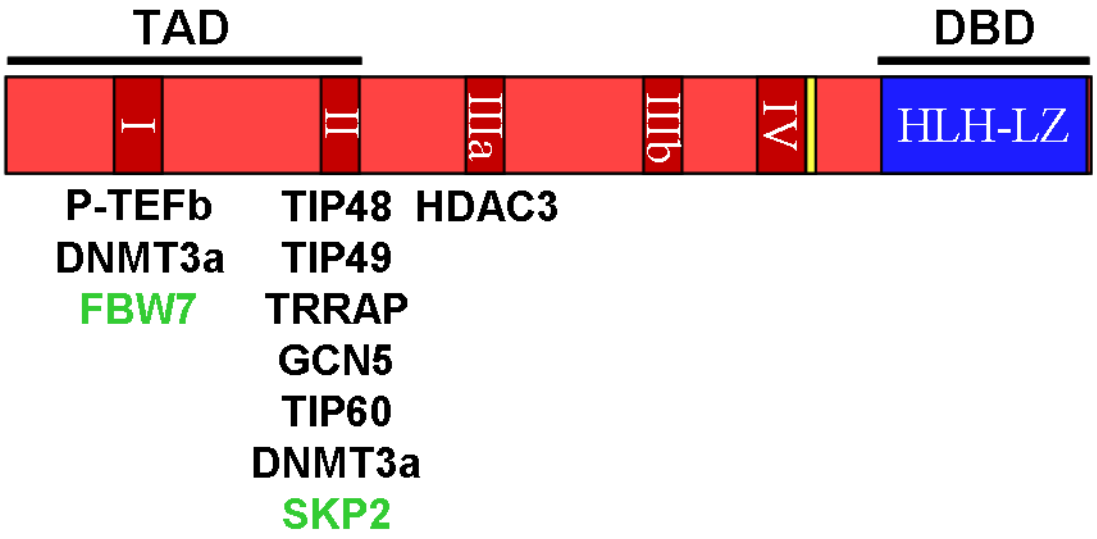

Figure 3 – Conserved elements in Myc mediate association with cofactors. Diagram denoting conserved Myc amino acid sequences. The location of the canonical nuclear localization sequence (NLS) is marked in yellow. The C-terminal basic helix-loop-helix leucine zipper (bHLH-LZ, blue) forms the DNA Binding Domain (DBD) when Myc is associated with its partner Max. The Myc Boxes I, II, IIIa, IIIb, and IV are labeled and marked in dark red. MBI and II make up the transcriptional activation domain (TAD). Select cofactors important for Myc protein stability (green) or transcriptional function (black) are listed below the region mediating their association with Myc.

Welcker *et al.*, 2004a; Welcker *et al.*, 2004b; Welcker *et al.*, 2003). The ubiquitin ligase SCF^{Skp2} similarly controls Myc activity and stability through an association at MBII (Kim *et al.*, 2003; von der Lehr *et al.*, 2003). Other interactions at MBII are mediated by the essential cofactor TRRAP. It recruits regulatory cofactors, like Tip60 and Gcn5 that acetylate histones at Myc DNA binding sites, stabilizing Myc binding and/or promoting Myc transcriptional activity (Frank *et al.*, 2003; McMahon *et al.*, 1998; McMahon *et al.*, 2000). Still other cofactors like Tip48 and Tip49, which are thought to bind Myc without TRRAP, are reported to have ATPase activity, although it is unclear how this activity influences Myc function (Grigoletto *et al.*, 2011; Wood *et al.*, 2000). Together, MBI and MBII comprise the transcriptional activation domain (TAD), which is necessary for Myc-mediated transformation (Stone *et al.*, 1987) and can activate gene transcription when fused to a DNA binding protein (Kato *et al.*, 1990).

Much less is understood about MBIIIa, MBIIIb and MBIV. While the function of MBIIIb has not been evaluated (Tansey, 2014), MBIIIa is known to associate with HDAC3 and contribute to Myc-mediated transcriptional repression (Kurland & Tansey, 2008). MBIV is thought to stabilize Myc binding to DNA, but the mechanism for this function remains unclear (Cowling *et al.*, 2006). It may also be the site of ubiquitination by Hetch9 that increases the ability of Myc to repress Miz1-mediated transcription (Adhikary *et al.*, 2005).

While their conservation alone suggests all the MB regions contribute to Myc function, arguably the most important is MBII (Meyer & Penn, 2008; Tansey, 2014). Mutants of Myc lacking MBII are mostly deficient in Myc transcriptional and downstream biologic activities, such as Myc-induced cellular transformation, *in vitro* and

in vivo (Atchley & Fitch, 1995; Bush *et al.*, 1998; Grandori *et al.*, 2000; Stone *et al.*, 1987). Thus, cofactors associating with Myc through MBII are highly significant to its function. This is supported by evidence that catalytically inactive mutants of many of these Myc cofactors reduce Myc activity (Frank *et al.*, 2003; McMahon *et al.*, 1998; Wood *et al.*, 2000). Collectively, these observations demonstrate Myc transcriptional cofactors are critical and functionally relevant regulators of Myc.

While significant advances have been made in the understanding of Myc transcriptional cofactors, many questions remain. Notably, are there still yet undiscovered cofactors, essential for Myc function? Do novel cofactors work with existing cofactors? Can modulation of these critical cofactors genetically or with small molecules attenuate Myc activity? In the chapters that follow, I will show data that address these questions. Specifically, chapter 2 details the identification of a novel Myc transcriptional cofactor that regulates Myc oncogenic activity. Moreover, chapter 3 demonstrates that shRNA-mediated knockdown of this novel Myc cofactor inhibited the growth of breast cancer cells.

Myc in Cancer

Soon after its discovery, Myc was shown to transform cells and promote tumor development (Meyer & Penn, 2008), as evidenced by several *in vivo* models systems like the Eμ-*myc* mouse in which Myc is overexpressed specifically in B cells and drives formation of B cell lymphoma (Adams *et al.*, 1985). The breadth of this is reflected by estimates that MYC is overexpressed and/or overactive in the vast majority of human cancers (Eilers & Eisenman, 2008; Meyer & Penn, 2008; Tansey, 2014; Vita &

Henriksson, 2006). As such, a variety of mechanisms by which MYC expression can be dysregulated have been identified. Genetically, *MYC* can undergo chromosomal translocations, retroviral insertional mutagenesis at its promoter/enhancer, and genetic amplifications (Alitalo *et al.*, 1983; Boxer & Dang, 2001; Collins & Groudine, 1982; Dalla-Favera *et al.*, 1982; Hayward *et al.*, 1981; Neel *et al.*, 1982; Taub *et al.*, 1982). *MYC* can also be mutated, resulting in decreased mRNA and/or protein turnover (Albert *et al.*, 1994; Bahram *et al.*, 2000; Bhatia *et al.*, 1994; Noubissi *et al.*, 2006; Pulverer *et al.*, 1994; Rabbitts *et al.*, 1983; Schiavi *et al.*, 1992; Yano *et al.*, 1993). However, in many cases MYC is dysregulated by unrelenting signals from any number of compromised upstream oncogenic pathways that rely on the MYC transcriptional program to mediate their effect (Sodir & Evan, 2011; Wierstra & Alves, 2008). Normally, these genetic or oncogenic signaling insults that dysregulate MYC would induce apoptosis (Evan *et al.*, 1992), but developing cancers attenuate apoptosis through a variety of mechanisms, such as inactivating the p53 tumor suppressor pathway or overexpressing anti-apoptotic proteins like BCL2 (Eischen *et al.*, 1999; Eischen *et al.*, 2001b; Hermeking & Eick, 1994). Thus, regardless of whether MYC is a driving oncogene, it represents a centrally important oncogenic factor in many human cancers under diverse genetic circumstances.

Given the function of MYC and the wide distribution of its dysregulation in human cancer, MYC has long been hypothesized to be a potent therapeutic target (Sodir & Evan, 2011). Several models have shown that reducing MYC expression and/or activity in MYC-driven cancers, even briefly, causes regression through a variety of mechanisms, including apoptosis, senescence, folding of tumor vasculature and terminal

differentiation (Felsher, 2010; Giuriato *et al.*, 2006; Pelengaris *et al.*, 2002; Shachaf *et al.*, 2004; Wu *et al.*, 2007). Fortunately, transient or moderate inhibition of MYC appears to be sufficient to cause cancers to regress (Jain *et al.*, 2002; Shachaf *et al.*, 2008). These studies (and similar studies with other oncogenes) reveal the cancers become dependent on or addicted to oncogenes (Weinstein, 2002; Weinstein & Joe, 2008). However, Myc inhibition has been shown to cause regression of a mutant Ras driven lung cancer, reportedly without signs of resistance (Soucek *et al.*, 2008; Soucek *et al.*, 2013). These studies highlight the critical importance of MYC in the survival and maintenance of a wide variety of cancers, even when MYC is not a direct oncogenic driver.

While these experimental results are promising, targeting MYC therapeutically has proven extremely challenging. For example, several small molecules have been developed that attempt to disrupt the interaction between MYC and its essential partner MAX, but they have limited activity (Berg, 2010). Other strategies tried to reduce *MYC* transcription by altering the 3-dimensional structure of the *MYC* promoter (Brown *et al.*, 2011; Nasiri *et al.*, 2014). MYC protein stability has also been proposed as a target by promoting ubiquitin-mediated proteasomal degradation (Frezza *et al.*, 2011). In more recent studies, the bromodomain protein BRD4, which is thought to bind acetylated histone lysines at gene enhancer regions, was shown to regulate *MYC* transcription. Disrupting binding of BRD4 to histones with the inhibitor JQ1 reduced *MYC* transcription and ultimately MYC protein expression, inhibiting cancer cell growth; however, this only occurs in a narrow subset of cancers (Alderton, 2011; Delmore *et al.*, 2011; Mertz *et al.*, 2011; Zuber *et al.*, 2011). To date, there is no method to effectively inhibit MYC activity directly in a broad range of cancers. As an alternative and indirect

method to target MYC activity, studies have shown inactivating or removing MYC transcriptional cofactors (as discussed above) reduces MYC-mediated proliferation, cellular transformation and cancer cells survival (Frank *et al.*, 2003; McMahon *et al.*, 1998; Wood *et al.*, 2000). Thus, identifying and investigating vital MYC transcriptional cofactors critically important in tumorigenesis and established cancers represents a critical area of investigation. My thesis work significantly contributes to this area of research in chapters 2 and 3 by identifying a novel MYC transcriptional cofactor and showing its expression is important for tumorigenesis and cancer cell survival.

MTBP

Discovery and Early Controversy

The 104 kDa Mdm2 Binding Protein (Mtbp) was discovered in a yeast-two hybrid screen as binding to Mdm2, but had no known functional domains when this thesis project was initiated (Boyd *et al.*, 2000a). Mdm2 is a negative regulator of the tumor suppressor p53, which induces cell cycle arrest and apoptosis in response to stress, inhibiting cancer development (Vogelstein *et al.*, 2000). With this in mind, Boyd *et al.* further reported that, similar to p53, Mtbp induced a G1 cell-cycle arrest independent of p53 that was reversed by Mdm2 overexpression (Boyd *et al.*, 2000a). A follow-up report though suggested Mtbp stabilized Mdm2 and promoted Mdm2-mediated degradation of p53 (Brady *et al.*, 2005). Unfortunately, subsequent *in vivo* genetic investigations did not support either of these initial reports.

Deletion of *Mdm2* is embryonic lethal, but it is rescued by deletion of *p53*, demonstrating the lethality is due to unchecked p53 activity (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995). Deletion of *Mtbp* was also discovered to be embryonic lethal, but it was not rescued by *p53* deletion (Iwakuma *et al.*, 2008). This provided the first evidence that Mtbp does not regulate Mdm2, as previously reported (Boyd *et al.*, 2000a; Brady *et al.*, 2005).

The connection between Mtbp and Mdm2 was further challenged by examining tumorigenesis, *in vivo*. Decreased Mdm2 expression delays tumor onset due to increased p53-mediated apoptosis, resulting in higher rates of p53 mutation and/or deletion in tumors that do develop (Alt *et al.*, 2003; Mendrysa *et al.*, 2006). In contrast to Mdm2, Mtbp haploinsufficiency did not significantly change the rate of tumor development in wild-type or $p53^{+/-}$ mice, nor did it alter the incidence of *p53* mutations/deletions (Iwakuma *et al.*, 2008). In a separate analysis, *Mtbp* heterozygosity, like *Mdm2* heterozygosity, delayed Myc-driven B cell lymphoma development in the Eμ-*myc* mouse model, which overexpresses Myc specifically in B cells; however, the delay was determined not to be caused by increased p53 activity, and Mtbp status did not alter the incidence of p53 mutations/deletions detected in lymphomas (Odvody *et al.*, 2010). Taken together, these data indicate Mtbp does not regulate Mdm2 *in vivo* during the complex process of tumorigenesis.

In the tumors analyzed from the above studies as well as in studies with wild-type or $Mtbp^{+/-}$ thymocytes, Mtbp expression was never shown to correlate with Mdm2 or p53 expression (Iwakuma *et al.*, 2008; Odvody *et al.*, 2010), as previously demonstrated (Boyd *et al.*, 2000a; Brady *et al.*, 2005). Thus, at the initiation of this thesis project, the

preponderance of evidence indicated Mtbp does not regulate Mdm2, leaving the central question of this thesis project: What is the function of Mtbp?

MTBP in Proliferation

Instead of supporting a model where Mtbp regulated Mdm2, available data suggested Mtbp may function in proliferation. Mtbp protein expression was shown to be responsive to the proliferative status of cells and to the expression of cell cycle regulators (Odvody *et al.*, 2010). Arresting cells in G1 by serum starvation or overexpression of the cell cycle inhibitors p16 or p21 significantly decreased Mtbp expression, whereas stimulation with serum or overexpression of the pro-proliferative oncogenes Myc or E2F1 rapidly increased Mtbp expression, corresponding with active cell cycle progression (Odvody *et al.*, 2010). Moreover, *Mtbp* was shown to be a direct transcriptional activation target of Myc (Odvody *et al.*, 2010), indicating *Mtbp* may be one of many Myc transcriptional target genes activated to induce cellular proliferation (Dang *et al.*, 2006; Meyer & Penn, 2008; O'Connell *et al.*, 2003; Patel *et al.*, 2004; Zeller *et al.*, 2006).

Additionally, some studies indicated reducing Mtbp expression in highly proliferative cells decreases proliferation. For example, Myc overexpressing $Mtbp^{+/-}$ pre-B cells grew slower than $Mtbp^{+/+}$ control cells with no significant difference in apoptosis or Myc expression (Odvody *et al.*, 2010). Knockdown of Mtbp using siRNA was shown to decrease growth in *p53*-null mouse embryonic fibroblasts (MEFs; (Odvody *et al.*, 2010). This finding has since been recapitulated using siRNA in HeLa cells (Agarwal *et al.*, 2011; Boos *et al.*, 2013). In contrast, modulation of Mtbp/MTBP expression did not

affect proliferation of wild-type pre-B cells from mice or the growth of osteosarcoma cell lines (Agarwal *et al.*, 2013; Iwakuma *et al.*, 2008; Odvody *et al.*, 2010). These differences may reflect unappreciated cell-specific effects or simply variability in the timing of experiments. It is also possible cells driven to higher rates of proliferation may have a greater requirement to maintain Mtbp expression, as suggested by the difference in the impact of *Mtbp* gene status in pre-B cells with and without Myc overexpression. Regardless, at the outset of this thesis project, it was clear additional investigation utilizing several cell/tissue types into the potential pro-proliferative function of Mtbp was required and I investigate this in chapter 2.

While the link between Mtbp and proliferation remained unclear, a few potential mechanistic insights were available. *Mtbp* heterozygosity was shown to limit the ability of Myc to promote transcriptional activation of its pro-proliferative target genes, suggesting Mtbp may be a limiting factor in Myc-mediated pro-proliferative transcription (Odvody *et al.*, 2010). Additionally, two studies published following the initiation of this thesis work showed siRNA-mediated knockdown increased the proportion of cells in late S or G2/M phases of the cell cycle, attributing the change to reduced DNA replication and irregular chromosome segregation, respectively (Agarwal *et al.*, 2011; Boos *et al.*, 2013). However, these two studies only utilized HeLa cells to ascertain the physiologic function of Mtbp. My thesis work presented in both chapter 2 and chapter 3 examines the impact of Mtbp/MTBP overexpression or knockdown on proliferation and cell cycle distribution in multiple transformed and non-transformed cell types.

MTBP in Cancer

Elevated and unregulated cellular proliferation is a hallmark of cancer (Hanahan & Weinberg, 2000). While the available data implicate Mtbp in cellular proliferation, its role in cancer tumorigenesis, progression and survival remained unresolved. The earliest *in vivo* study reported $Mtbp^{+/-}$ and $p53^{+/-}Mtbp^{+/-}$ mice did not differ significantly in the rate of tumor-free survival compared to wild-type or $p53^{+/-}Mtbp^{+/+}$ control mice, respectively (Iwakuma *et al.*, 2008). Yet, a second *in vivo* study demonstrated *Mtbp* heterozygosity significantly limited the ability of the oncogene Myc to drive lymphoma development (Odvody *et al.*, 2010). Here, decreased Mtbp expression inhibited Myc-driven pro-proliferative transcriptional activity and downstream proliferation. Thus, even with these two reports, the question remains whether Mtbp expression can influence cancer development. I directly address this question in chapter 2.

Beyond tumorigenesis, very little data are available that addresses the expression or function of MTBP in mature cancers. One study showed loss of MTBP expression evaluated by immunohistochemistry in a very specific subset of squamous cell carcinomas of the head and neck (SCCHN) correlated with decreased patient survival (Vlatkovic *et al.*, 2011). This could possibly reflect two studies demonstrating MTBP may suppress metastasis (Iwakuma & Agarwal, 2012). *Mtbp* heterozygous mice developed more metastatic tumors, *in vivo*, and siRNA-mediated knockdown or overexpression of Mtbp in osteosarcoma cells increased or decreased invasion, respectively, *in vitro*, (Iwakuma *et al.*, 2008). Moreover, Mtbp was recently shown to associate with the cytoplasmic pro-metastatic factor alpha-actinin-4 (ACTN4) and to

suppress an ACTN4-mediated increase in osteosarcoma cell migration when expressed in the cytoplasm (Agarwal *et al.*, 2013).

A limitation to this model is the predominately nuclear distribution of Mtbp (see chapter 2 and Agarwal *et al.*, 2013; Agarwal *et al.*, 2011; Vlatkovic *et al.*, 2011). Moreover, in contrast to the role report on SCCHN and cancer cell metastasis, other available data, albeit limited, suggest MTBP expression is maintained and even selected for during tumorigenesis. For example, *MTBP* was reported to be amplified in colorectal carcinoma and multiple myeloma (Carrasco *et al.*, 2006; Martin *et al.*, 2007), as well as several human cancer cell lines (Barretina *et al.*, 2012). Additionally, in all mouse models assessing tumorigenesis, loss of heterozygosity (LOH) for *Mtbp* has never been reported (Iwakuma *et al.*, 2008; Odvody *et al.*, 2010). These data suggest Mtbp expression must be maintained and even perhaps increased during cancer development. Moreover, cancer cell proliferation and metastasis have been shown to have a dichotomous interaction, further complicating whether Mtbp is a direct metastasis suppressor (Liu *et al.*, 2012). Regardless, the expression status and function of Mtbp in mature cancers clearly remains unresolved. In chapter 2, I evaluate MTBP expression in a variety of human cancers and extend this analysis in chapter 3 to examine MTBP function and influence on patient prognosis, with the goal of determining whether MTBP expression is a benefit or hindrance to mature cancers.

CHAPTER II

ONCOGENIC PROTEIN MTBP INTERACTS WITH MYC TO PROMOTE TUMORIGENESIS

Work from this chapter was published in by Grieb *et al.* in *Cancer Research*.

Background and Significance

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 (Eilers & Eisenman, 2008; Horiuchi *et al.*, 2012; Meyer & Penn, 2008; Xu *et al.*, 2010). Although recently Myc was shown to act as a global transcriptional amplifier (Lin *et al.*, 2012; Nie *et al.*, 2012), it is known to function by binding promoters where it transcriptionally activates or represses many genes that control critical cellular processes (Meyer & Penn, 2008; Walz *et al.*, 2013; Zeller *et al.*, 2003). 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*) and, carbamoyl-phosphate synthetase 2/aspartate transcarbamylase/dihydroorotase (*CAD*), and cyclin D2 (*CCND2*) that are needed for ribosomal assembly, polyamine generation, and pyrimidine synthesis, and cell cycle progression respectively (Meyer & Penn, 2008; Zeller *et al.*, 2003). As such, overexpression of Myc induces proliferation and transformation; cells initially inhibit these processes through activation of apoptosis or senescence (Eilers & Eisenman, 2008; Meyer & Penn, 2008). Myc-mediated transcription

is regulated, in part, by transcriptional cofactors. For example, the nuclear ATPases Tip48 (Pontin/RUVBL2) and Tip49 (Reptin/RUVBL1), that form hexamers or dodecamers, bind Myc and are necessary for Myc-mediated *in vitro* transformation (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Grigoletto *et al.*, 2011; Wood *et al.*, 2000). 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 (Boyd *et al.*, 2000a). However, subsequent data demonstrated Mtbp does not regulate Mdm2 *in vivo (Iwakuma et al., 2008; Odvody et al., 2010)*. Instead, data suggested Mtbp may function in proliferation, as Mtbp expression increased in response to pro-proliferative factors and siRNA-mediated knockdown of Mtbp reduced proliferation regardless of p53 status (Agarwal *et al.*, 2011; Odvody *et al.*, 2010). Additionally, we reported *Mtbp* heterozygosity limited the ability of Myc to promote proliferation and activate transcription of pro-proliferative target genes. Furthermore, in a mouse model, *Mtbp* heterozygosity delayed Myc-driven lymphomagenesis (Odvody *et al.*, 2010). 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 novel regulator of MYC.

Materials and Methods

Cell Culture, transfection, infection, vectors and shRNA

NIH3T3, HEK293T, H1299, HCC1806, and MDA-MD-231 cells were cultured as described by the American Type Culture Collection (Manassas, VA). HCC1806, MDA-MD-231, HMLE and human retinal epithelial cells were provided by Drs. Jennifer Pietenpol and David Cortez (Cortez, 2003; Elenbaas *et al.*, 2001). Mouse embryonic fibroblasts were isolated and cultured as previously described (Prouty *et al.*, 1993). All cell lines were recently obtained from ATCC or authenticated by STR profiling or similar method. 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 (Prouty *et al.*, 1993). Vectors encoding Flag-tagged full-length Myc and Myc deletion mutants (Δ20-48 and Δ118-152) were provided by Dr. Michael Cole (Wood *et al.*, 2000). The MSCV-MycER-IRES-GFP bicistronic retrovirus was previously published (Alt *et al.*, 2003; Zindy *et al.*, 1998). A pCMV-Tag2B vector encoding Flag-tagged full length Mtbp was provided by Dr. Tomoo Iwakuma. Flag-Mtbp mutants encoding amino acids 1-298, 299-596, or 597-894 were generated by PCR using the pCMV-Tag2B-Flag-Mtbp vector and cloned back into the pCMV-Tag2B vector. Flag-Mtbp and Mtbp mutants were cloned into the MSCV-IRES-YFP bicistronic retrovirus and Flag-Myc was cloned into the MSCV-IRES-RFP bicistronic retrovirus. Human MTBP, TIP48 and TIP49 were cloned by RT-PCR, tagged with HA or Flag, and cloned into the pCEP vector; plasmids were sequenced to verify wild-type sequence for each. Glutathione S-transferase-tagged

TIP48 and TIP49 were subcloned into the pGEX vector for protein production. Human MTBP and yeast protein MGA2 were cloned into pYES-BBV for *in vitro* transcription and translation. Murine *Mtbp* siRNA was purchased (SMARTpool ON-TARGETplus, Thermo-Scientific, Pittsburgh, PA). Human *MTBP* shRNA (shRNA1 GGAGAGTGTTCTAGCTATT or shRNA2 GAAACACAGTATTACCGAG) and non-targeting control (GACTTACGAGATCAGAAAG) were used in pSuper constitutive expression constructs (Oligoengine, Seattle, WA).

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 (subG1 DNA content) were evaluated under low serum culture conditions by flow cytometry, following DNA staining with propidium iodide. Cleaved caspase 3 expression was evaluated by Western blot. Colony growth after culturing cells for 7 days at low density was evaluated as described (Zindy *et al.*, 1998). Soft agar assays were performed as described (Wang *et al.*, 2006).

Mice

Female athymic nude mice (Harlan, Indianapolis, IN) were injected subcutaneously in their 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 (Bouska *et al.*, 2008; Boyd *et al.*, 2000a; Prouty *et al.*, 1993; Wang *et al.*, 2006; Zhang *et al.*, 2011). 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 (Braden *et al.*, 2006). 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.

Identification of MTBP binding proteins by mass spectrometry

Whole cell extracts from H1299 cells infected with retroviruses expressing Flag-tagged MTBP or GFP control were prepared and immunoprecipitated with anti-Flag immunoaffinity matrix (M2, Sigma) as described previously (Bouska *et al.*, 2008). Immunoprecipitates were eluted with Flag peptides, resolved by SDS-PAGE, and stained with silver as previously described (Alt *et al.*, 2005). Silver-stained protein bands were excised and subjected to in-gel trypsin digestion. Peptides were analyzed by LC-MS-MS

LC-MS-MS analysis of the peptides was performed using a Thermo LTQ ion trap mass spectrometer equipped with a Thermo MicroAS autosampler, Thermo Surveyor HPLC pump, and nanospray source. Utilizing a "vented column" setup (https://gygi.med.harvard.edu/index.html/node/10), the peptides were first trapped on a 100 μm x 4 cm reversed phase column (Jupiter C_{18}, 5 μm, 300 Å, Phenomonex, Torrance, CA), and then resolved using an aqueous to acentonitrile gradient on a 15 cm column analytical column pack directly into a pulled capillary emitter tip. A flow split was employed to allow for approximately 700 nl/min of flow across the columns. Peptide MS/MS spectra were acquired in a data dependent manner utilizing dynamic exclusion to minimize acquisition of redundant spectra. These spectra were queried against the protein database using Sequest (http://pubs.acs.org/doi/abs/10.1021/ac00104a020) and the resulting identifications filtered and collated into protein identifications using CHiPs.

***In vitro* binding assay**

In vitro binding assays were performed as previously described (Boyd *et al.*, 2000a), using MTBP and control MGA2 translated in rabbit reticulocyte lysate in the presence of ^{35}S-methionine (TNT T7 Reticulocyte System, Promega; L4610) 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 coverslips were processed and analyzed as reported (Wang *et al.*, 2006). Anti-Mtbp (K20, Santa Cruz), anti-Tip48 (Ab-Cam 36509), and/or isotype controls, followed by Alexa Fluor 594 and 488 (A11058, A21206, Invitrogen) were used.

Chromatin immunoprecipitation (ChIP)

HCC1806, Raji, or MDA-MB-231 were used to ChIP endogenous MTBP and MYC. HEK293T, HREC or HMLE cells were made to express Flag-Mtbp, Flag-Mtbp mutants, Flag-Myc, HA-Myc, or empty vector control as indicated by transfection (HEK293T). ChIP protocol from Upstate Biotechnology was followed. 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, SDS was removed from buffers. Sequential ChIP for Myc (anti-HA, F7, Santa Cruz) and then Mtbp (anti-Flag), was performed as previously described (Jiang *et al.*, 2004), except using formaldehyde as a cross-linking agent and sonication to shear DNA. Quantitative PCR of precipitated DNA described below.

Quantitative PCR

NIH3T3 cells infected with a MSCV retrovirus encoding MycER (Littlewood *et al.*, 1995) and transfected with non-targeting control siRNA or *Mtbp* siRNA (SMARTpool ON-TARGETplus, Thermo-Scientific, Pittsburgh, PA, USA), or expression constructs 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 (Odvody *et al.*, 2010). For ChIP DNA, quantitative real-time PCR was performed with primers specific for Myc-binding sites in promoter regions or up/downstream regions; values are relative to the respective vector or IgG control and input DNA. Primers used for to evaluate gene expression were CAD-F AACTGCGTAGGCTTCGACCATACA, CAD-R AATCAATGCGGGTGAGCTCGTAGA, ODC-F GCATGTGGGTGATTGGAT GCTGTT, ODC-R TTGCCACATTGGCCGTGACATTAC, NCL-F ACTGGAAAGACC AGCACTTGGAGT, NCL-R CCCTTTAGGTTTGCCATGTGGGTT. Primers used for ChIP were CAD-F AGTCTCTGCTGCTGCCGCCAA, CAD-R GAGAGGCGCATCAC AGAGTGGGATAA, NCL-F TTTTGCGACGCGTACGAG, NCL-R ACTAGGGCCGA TACCGCC, ODC-F ATTTCCCTTTTCCGCTCTCG, ODC-R TGAACGGCAGAGCCT GTAGC, ODC Upstream-F TTTCAGCCAGTCCAACCACC, ODC Upstream-R CTCA CCTAAGTTCTGGGACCAA, Cyclin D2-F – CATCAGGGCGCTGGTCTCT, Cyclin D2-R TGGCGTTTCTTCACCTCCT, p15-F TCCTAGGAAGGAGAGAGTGC, p15-R CGCTGGCCAGACCCTCATC, p21-F – ACCGGCTGGCCTGCTGGAACT, p21-R – TCTGCCGCCGCTCTCTCACCT, p21 Downstream-F ATGTTAGGCAAGTTACTTAA CTTA, p21 Downstream-R CTCTTGGTAACTTCACACCAAGTT, p27-F AGCAGGTT TGTTGGCAGCA, p27-R GAAAATGATTGACACGGCGAG.

Patient Data

Breast 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. 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. Log-rank tests were used to compare survival between 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 of their differences were obtained from Oncomine (www.oncomine.org) June 2013.

Statistical evaluation

Student's t-test (Figures 5A-D, 6, 10B-E, 11, 13-14, 17C, 18-21, and Table 1), log rank test (Figure 15) and a Fisher's exact test (Table 2) were used to compare data.

Results

MTBP is overexpressed in cancer

An Mtbp haploinsufficiency suppressed pre-B cell proliferation *in vitro* and inhibited Myc-driven B cell lymphoma development *in vivo*, yet Mtbp was shown to be a Myc transcriptional activation target (Odvody *et al.*, 2010). These observations suggested Mtbp is pro-proliferative and its expression may be increased in lymphomas where Myc is dysregulated. To first test this concept, *Mtbp* mRNA and protein expression were evaluated in primary lymphomas derived from Eμ-*myc* mice, in which Myc is overexpressed specifically in B cells (Adams *et al.*, 1985). *Mtbp* mRNA and protein expression were both significantly elevated in Eμ-*myc* lymphomas compared to

untransformed Eμ-*myc* splenocytes (Figure 4A). Moreover, we evaluated MTBP expression in human Burkitt lymphoma and diffuse large B cell lymphoma cells lines, which have *MYC* translocations and frequently overexpress MYC. In both cell types, *MTBP* mRNA and MTBP protein were significantly increased compared to normal human lymphoid tissue controls (Figure 4B). Therefore, both murine and human B cell lymphomas express increased levels of MTBP. In a broader search, analysis of public mRNA expression and copy number data showed *MTBP* is overexpressed and/or amplified in many human cancers (Tables 1 and 2). These data suggest MTBP expression is selected for during tumorigenesis.

Mtbp has oncogenic activity

To evaluate whether Mtbp overexpression contributes to cancer, I investigated the biological effects of Mtbp overexpression. Similar to Myc, Mtbp overexpression significantly enhanced proliferation in NIH3T3 fibroblasts (Figure 5A). This pro-proliferative effect of Mtbp was evident in fibroblasts and epithelial cells and in cells from different species (human, mouse, and rat; Figure 6), indicating a conserved function of Mtbp. Additionally, elevated Mtbp levels increased foci formation of cells cultured at low density (Figure 5B). Mtbp expression also significantly augmented soft agar colony formation, although the increase was moderate compared to that induced by the powerful oncogene Myc (Figure 5C).

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 day 36 that continued to

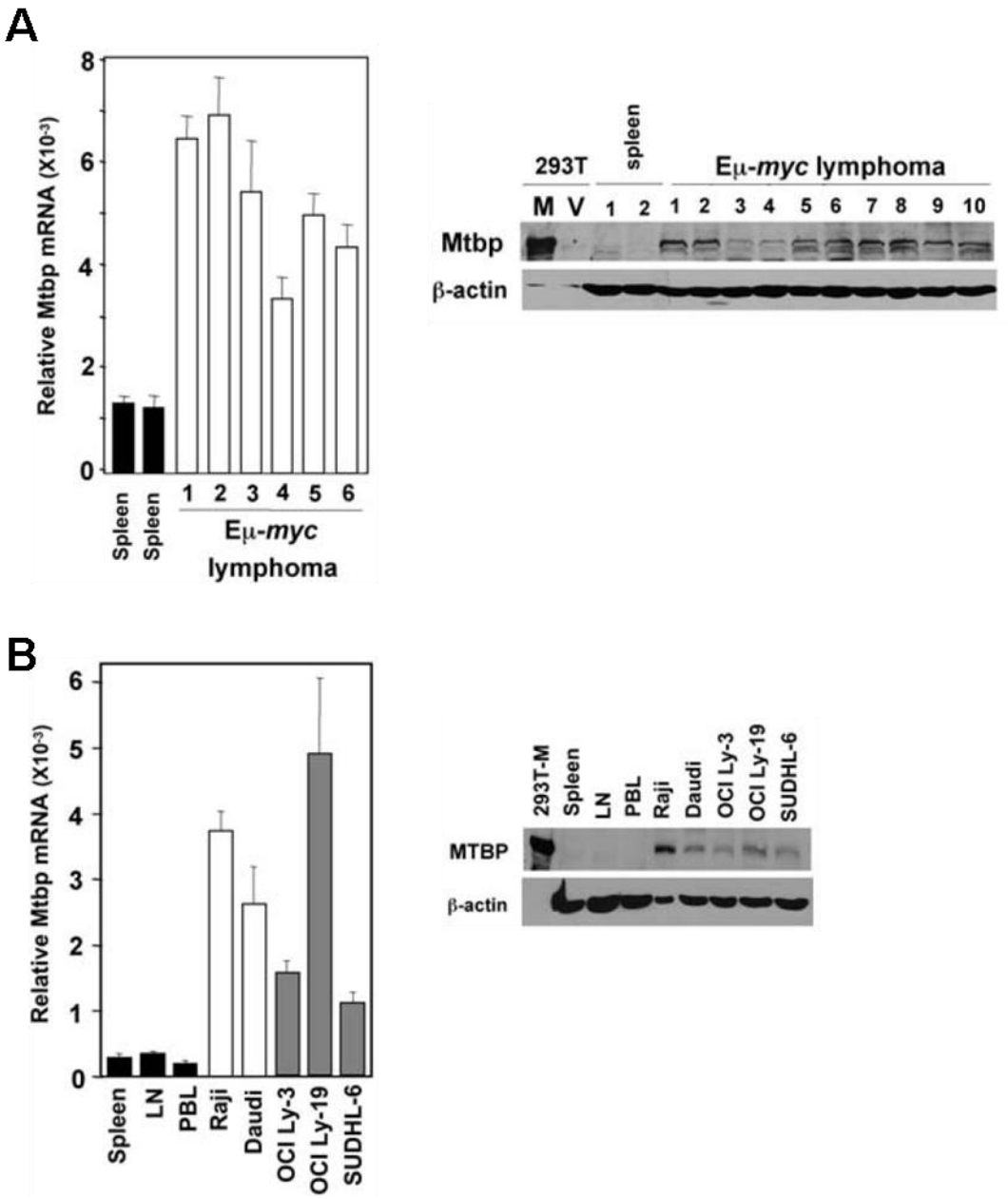

Figure 4. Mtbp/MTBP overexpressed in murine and human lymphomas. (A, B) qRT–PCR and Western blotting was performed with splenocytes (before detectable disease) and B-cell lymphomas from wild-type Eμ-*myc* transgenic mice (A) and human B-cell lymphoma lines (Burkitt lymphomas, white bars; diffuse large B cell lymphoma, gray 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. (This data was published by Odvody *et al.*, 2010. I performed the western blot in B and acknowledge Maria Pia Arrate and Tiffany Vincent for the remaining data.)

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	Gluck *et al.*, 2012 Finak *et al.*, 2008 Richardson *et al.*, 2006
Cervical carcinoma *(N=8, C=20)*	2.12	1.8E-06	Pyeon *et al.*, 2007
Colorectal carcinoma *(N=97, C=273)*	1.60 - 3.71	4.6E-04 to 1.2E-19	Kaiser *et al.*, 2007 Skrzypczak *et al.*, 2010
Gastric adenocarcinoma *(N=50, C=91)*	1.43 - 2.25	3.1E-04 to 1.9E-10	D'Errico *et al.*, 2009
Glioblastoma *(N=23, C=81)*	2.25	4.8E-04	Sun *et al.*, 2006
Lung adenocarcinoma *(N=65, C=45)*	1.54	2E-07	Hou *et al.*, 2010
Lung large cell carcinoma *(N=65, C=19)*	2.28	1.9E-06	Hou *et al.*, 2010
Lung squamous cell carcinoma *(N=65, C=27)*	1.62	1.9E-08	Hou *et al.*, 2010
Prostate carcinoma *(N=8, C=13)*	1.65	9.1E-04	Arredouani *et al.*, 2009
Squamous cell carcinoma *(N=4, C=11)*	1.97	9.5E-04	Riker *et al.*, 2008

MTBP mRNA expression data in cancer (C) compared to normal (N) tissue from Oncomine; p-values determined by Student's t-test

Table 2. *MTBP* is commonly co-amplified with *MYC* in multiple human cancers

Cancer[a]	Sample #	*MTBP* amp[b]	*MYC* amp[b]	*MYC* and *MTBP* co-amp[b]	*MTBP* amp in *MYC* amp[b,c]	Significance[d]
Ovarian Serous Cystadenocarcinoma	569	32.2% (183)	42.0% (239)	31.1% (177)	74.1% (177/239)	$p < 0.0001$
Breast Invasive Carcinoma	913	18.7% (171)	21.9% (200)	18.6% (170)	85.0% (170/200)	$p < 0.0001$
Hepatocellular Carcinoma	97	19.6% (19)	20.6% (20)	18.6% (18)	90.0% (18/20)	$p < 0.0001$
Prostate Adenocarcinoma	187	8.6% (16)	10.2% (19)	8.6% (16)	84.2% (16/19)	$p < 0.0001$
Head & Neck Squamous Cell Carcinoma	306	8.2% (25)	11.8% (36)	7.8% (24)	66.7% (24/36)	$p < 0.0001$
Stomach Adenocarcinoma	306	6.9% (21)	10.5% (32)	6.9% (21)	65.6% (21/32)	$p < 0.0001$
Glioma, Lower Grade	220	6.8% (15)	7.3% (16)	6.8% (15)	93.8% (15/16)	$p < 0.0001$
Lung Adenocarcinoma	230	7.0% (16)	9.1% (21)	6.1% (14)	66.7% (14/21)	$p < 0.0001$
Urothelial Carcinoma	150	6.7% (10)	9.3% (14)	6.0% (9)	64.3% (9/14)	$p < 0.0001$
Cutaneous Melanoma	244	5.3% (13)	5.7% (14)	5.3% (13)	92.9% (13/14)	$p < 0.0001$
Colon and Rectum Adenocarcinoma	575	4.7% (27)	6.3% (36)	4.5% (26)	72.2% (26/36)	$p < 0.0001$
Adenoid Cystic Carcinoma	60	3.3% (2)	5.0% (3)	3.3% (2)	66.7% (2/3)	$p = 0.0017$
Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma	126	4.0% (5)	7.9% (10)	3.2% (4)	40.0% (4/10)	$p = 0.0001$
Renal Chromophobe Carcinoma	66	3.0% (2)	3.0% (2)	3.0% (2)	100% (2/2)	$p < 0.0001$
Soft Tissue Sarcoma	207	3.4% (7)	3.4% (7)	2.9% (6)	85.7% (6/7)	$p < 0.0001$
Uterine Corpus Endometrioid Carcinoma	363	3.0% (11)	8.0% (29)	2.8% (10)	34.5% (10/29)	$p < 0.0001$
Glioblastoma Multiforme	497	1.8% (9)	2.0% (10)	1.4% (7)	70.0% (7/10)	$p < 0.0001$
Renal Clear Cell Carcinoma	436	0.9% (4)	0.9% (4)	0.9% (4)	100% (4/4)	$p < 0.0001$
Lung Squamous Cell Carcinoma	179	0.6% (1)	4.5% (8)	0.6% (1)	12.5% (1/8)	$p = 0.0447$
Acute Myeloid Leukemia	191	0.5% (1)	0.5% (1)	0.5% (1)	100% (1/1)	$p = 0.0052$

[a]Gene copy number gains in *MTBP* and/or *MYC* in human cancers in TCGA from the cBioPortal for Cancer Genomics obtained June 2013.
[b]Percentage of tumors with the indicated amplification and in parentheses, the number of samples
[c]The percentage of tumors with *MTBP* amplified in cancers that had amplified *MYC*.
[d]Significance for *MYC* and *MTBP* co-amplification calculated using a two-tailed Fisher's exact test.

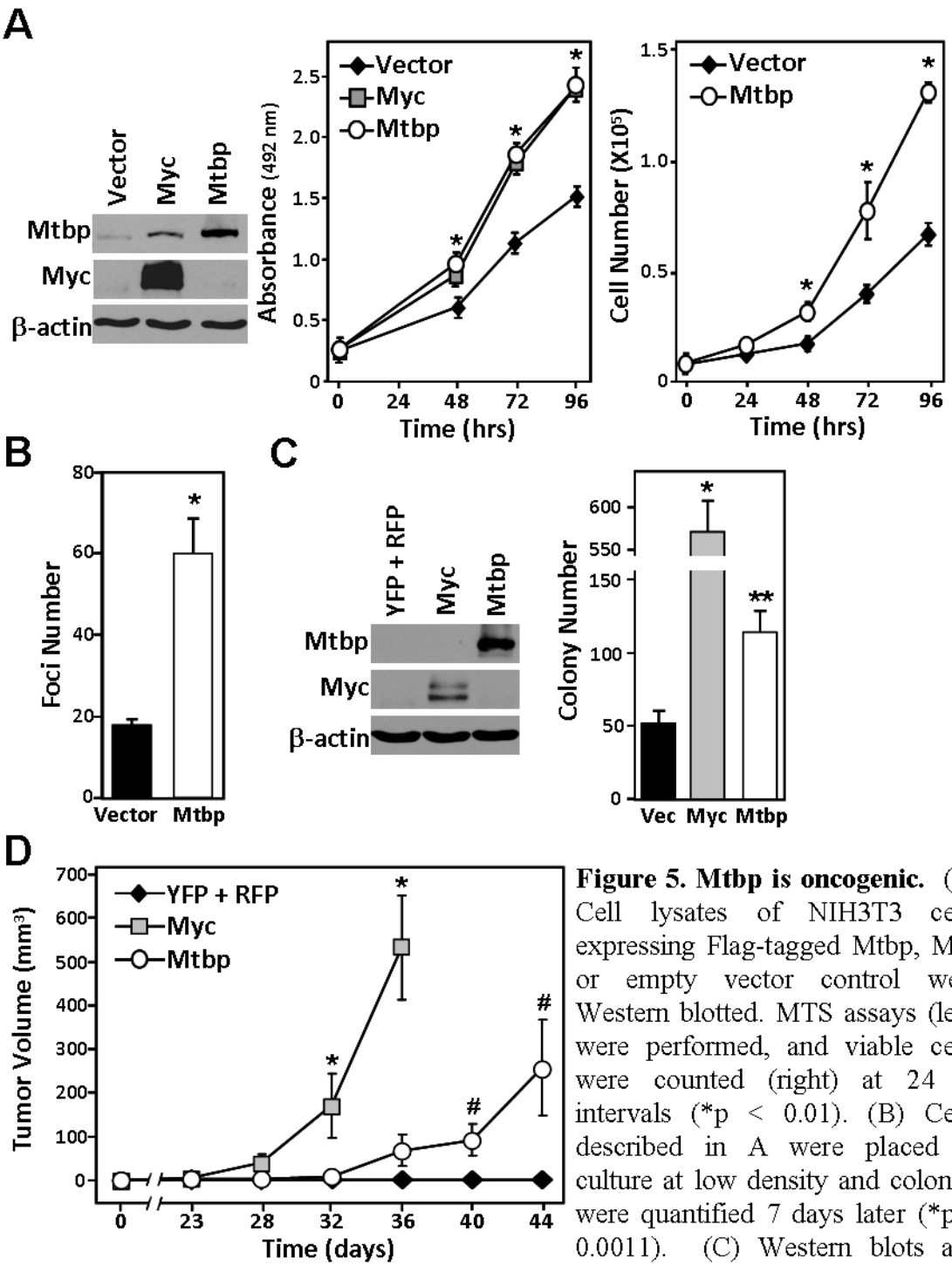

Figure 5. Mtbp is oncogenic. (A) Cell lysates of NIH3T3 cells expressing Flag-tagged Mtbp, Myc or empty vector control were Western blotted. MTS assays (left) were performed, and viable cells were counted (right) at 24 hr intervals (*$p < 0.01$). (B) Cells described in A were placed in culture at low density and colonies were quantified 7 days later (*$p = 0.0011$). (C) Western blots and soft agar assays of NIH3T3 cells expressing YFP and RFP, Flag-Mtbp and YFP or Flag-Myc and RFP; colonies quantified after 21 days (*$p = 0.0036$). (D) Cells described in C were injected subcutaneously into nude mice (n = 4 for YFP+RFP, Myc and 6 for Mtbp) at day 0, and tumor volume was measured at intervals (*$p<0.03$ YFP+RFP vs. Myc, #$p<0.05$ YFP+RFP vs. Mtbp). Error bars are standard deviation in A-C and standard error of the mean in D.

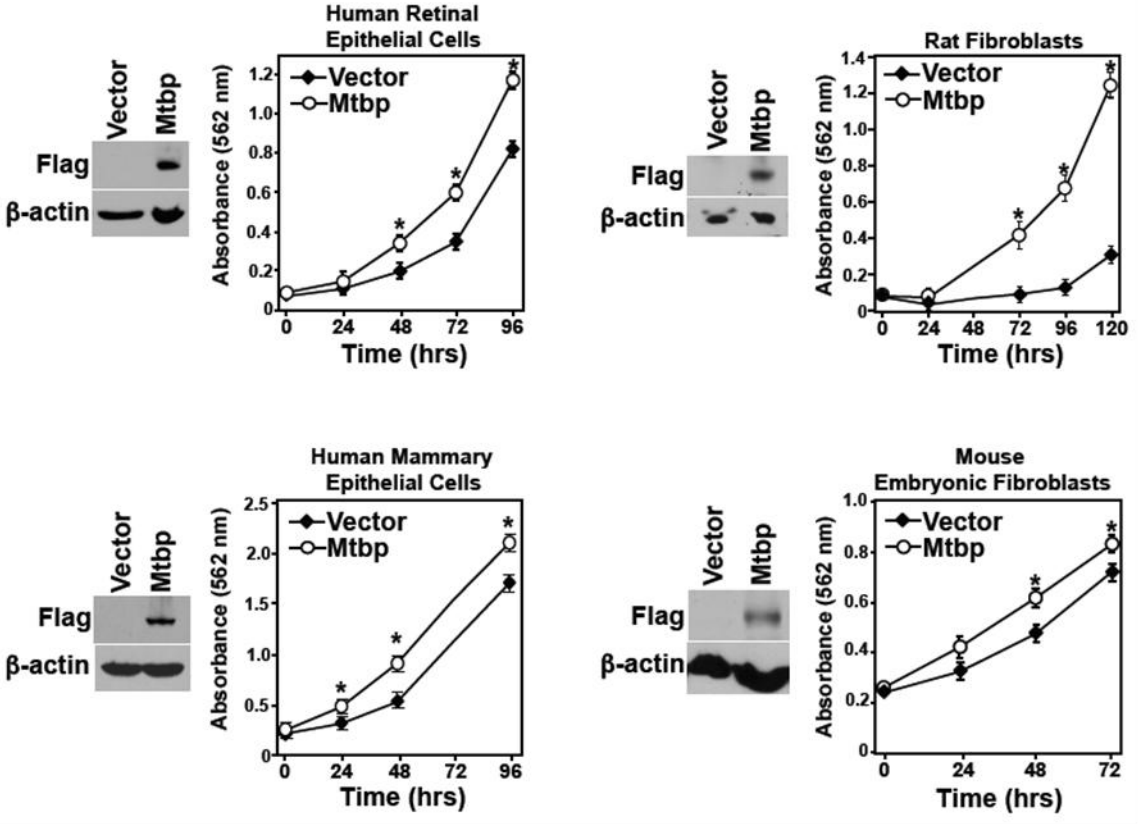

Figure 6. **Mtbp induces proliferation.** Flag-tagged Mtbp or vector control was overexpressed in rat fibroblasts, human retinal epithelial cells, wild-type mouse embryonic fibroblasts and immortalized non-tumorigenic human mammary epithelial cells. Whole cell lysates were Western blotted for Flag or as a control, β-actin. Proliferation was monitored by MTT at the indicated intervals (*$p < 0.001$). Error bars represent standard deviation and.

grow, whereas none of the negative controls had developed tumors by day 44 (Figure 5D). 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, 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; Figure 7A and Figure 8). HSP70 was not investigated further as it is known to bind overexpressed proteins (Diehl *et al.*, 2003). Immunoprecipitation of the same lysates confirmed endogenous TIP48 and TIP49 co-immunoprecipitated with MTBP (Figure 7B). Immunoprecipitations with tagged proteins further demonstrated the MTBP and TIP48/TIP49 interaction (Figure 7C). Binding assays revealed *in vitro* translated MTBP, but not the control MGA2 yeast transcription factor, bound to both GST-tagged TIP48 and TIP49 (Figure 7D). Mtbp was also localized to the nucleus and shared an overlapping nuclear distribution with Tip48 (Figure 7E). Therefore, TIP48/TIP49 are novel MTBP binding proteins and directly bind MTBP.

Given Tip48/Tip49 are Myc transcriptional cofactors and directly bind Myc at MBII (Wood *et al.*, 2000), we tested whether Mtbp interacted with Myc. Flag-tagged Mtbp and HA-tagged Myc co-immunoprecipitated one another (Figure 9A). We detected

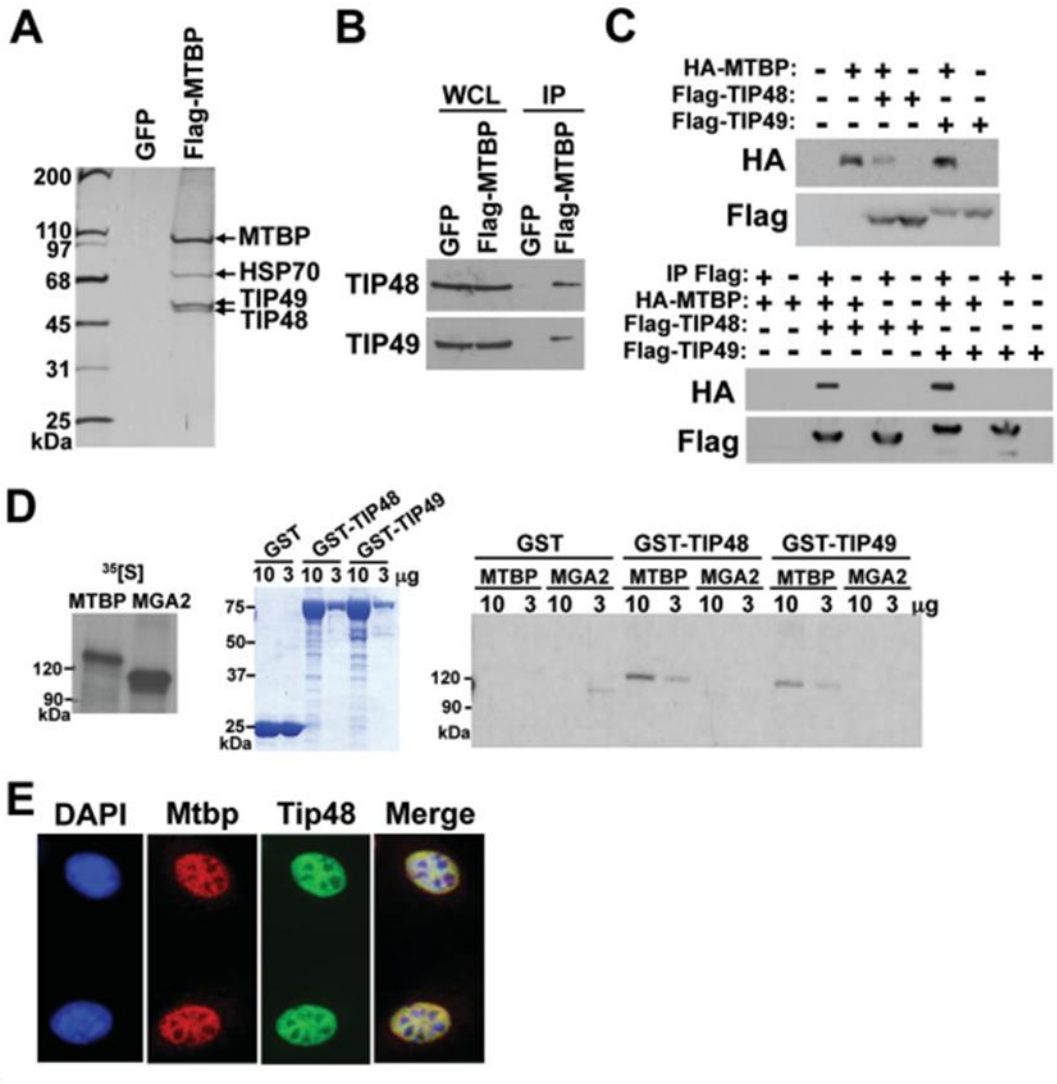

Figure 7. **Mtbp binds Tip48 and Tip49.** (A) Anti-Flag immunoprecipitation of whole cell protein lysates from H1299 cells expressing Flag-MTBP or GFP control were separated by SDS-PAGE, and proteins silver stained. Arrows denote proteins excised and identified by mass spectrometry (peptides identified in Figure 8). 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 mg 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).

Figure 8. **TIP48 and TIP49 identified by mass spectrometry.** CHiPS data of TIP48 and TIP49 amino acid coverage. Amino acid sequences identified by the mass spectrometry analysis of the silver-stained protein bands in Figure 7 are indicated in red.

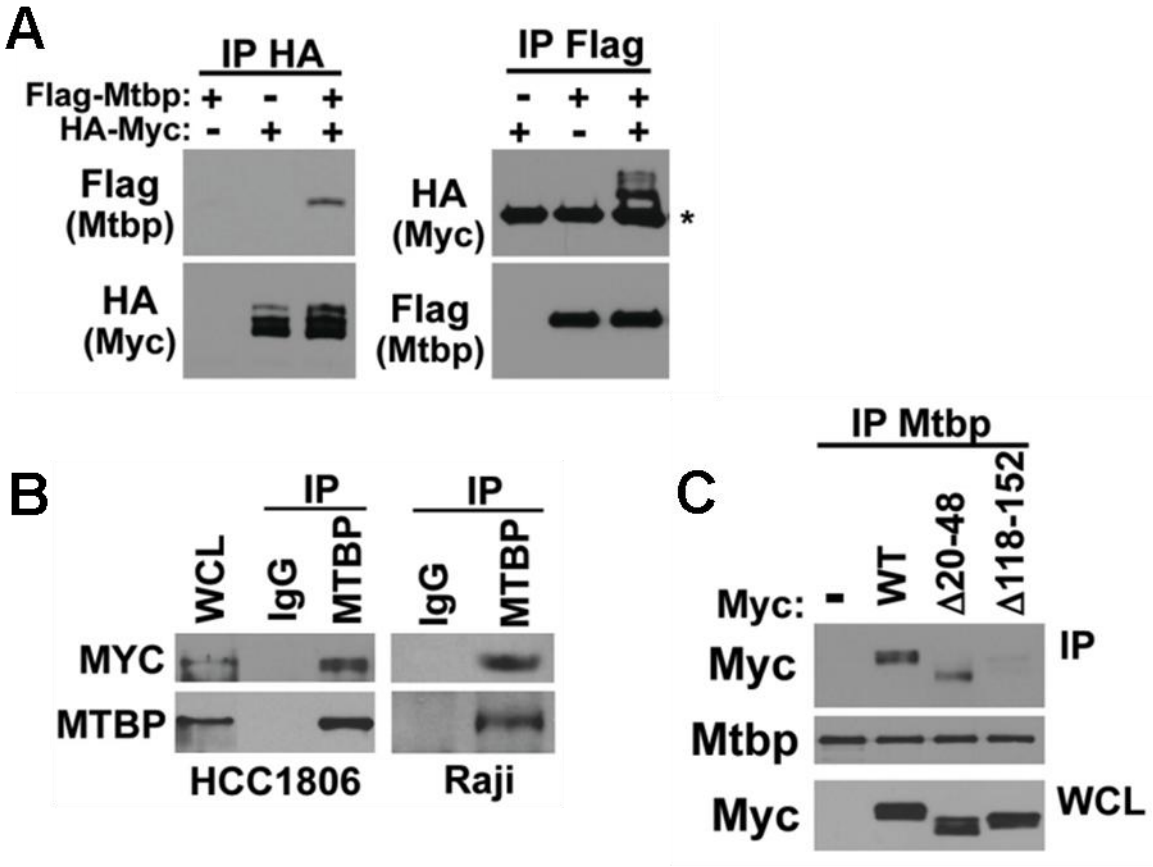

Figure 9. M**tbp associates with Myc through Myc Box II.** (A&C) 293T cells were transfected with vectors encoding the indicated proteins (wild-type, WT; Δ20-48 or Δ118-152 Myc mutants; empty vector, -). Whole cell lysates (WCL) were immunoprecipitated (IP) as indicated, and Western blots were performed for the proteins to the left of each panel. Asterisk denotes location of immunoglobulin heavy chain. (B) Immunoprecipitations for endogenous MTBP in whole cell protein lysates from HCC1806 and Raji cells with anti-MTBP or isotype (IgG) control were Western blotted. WCL on left represents 2.5% of protein used for IP.

endogenous association between MTBP and MYC in two human cancer cell lines driven by MYC and harboring amplified *MYC* (HCC1806 human breast carcinoma and Raji Burkitt lymphoma cells; Figure 9B). 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; Figure 9C), 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 (Wood *et al.*, 2000) and critical for Myc transcriptional and oncogenic activity (Meyers and Penn Review), is required for Mtbp association. Additionally, the results collectively 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 (Braden *et al.*, 2006). 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 (Figure 10A). We then tested whether Mtbp associated with promoter regions bound and transcriptionally regulated by Myc. 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 *CCND2*, *ODC*, *NCL*, and *CAD* (genes Myc transcriptionally activates) were enriched, but not upstream or downstream elements (Figure 10B and data not shown). Mtbp also immunoprecipitated the promoter regions of *p15, p21* and *p27*,

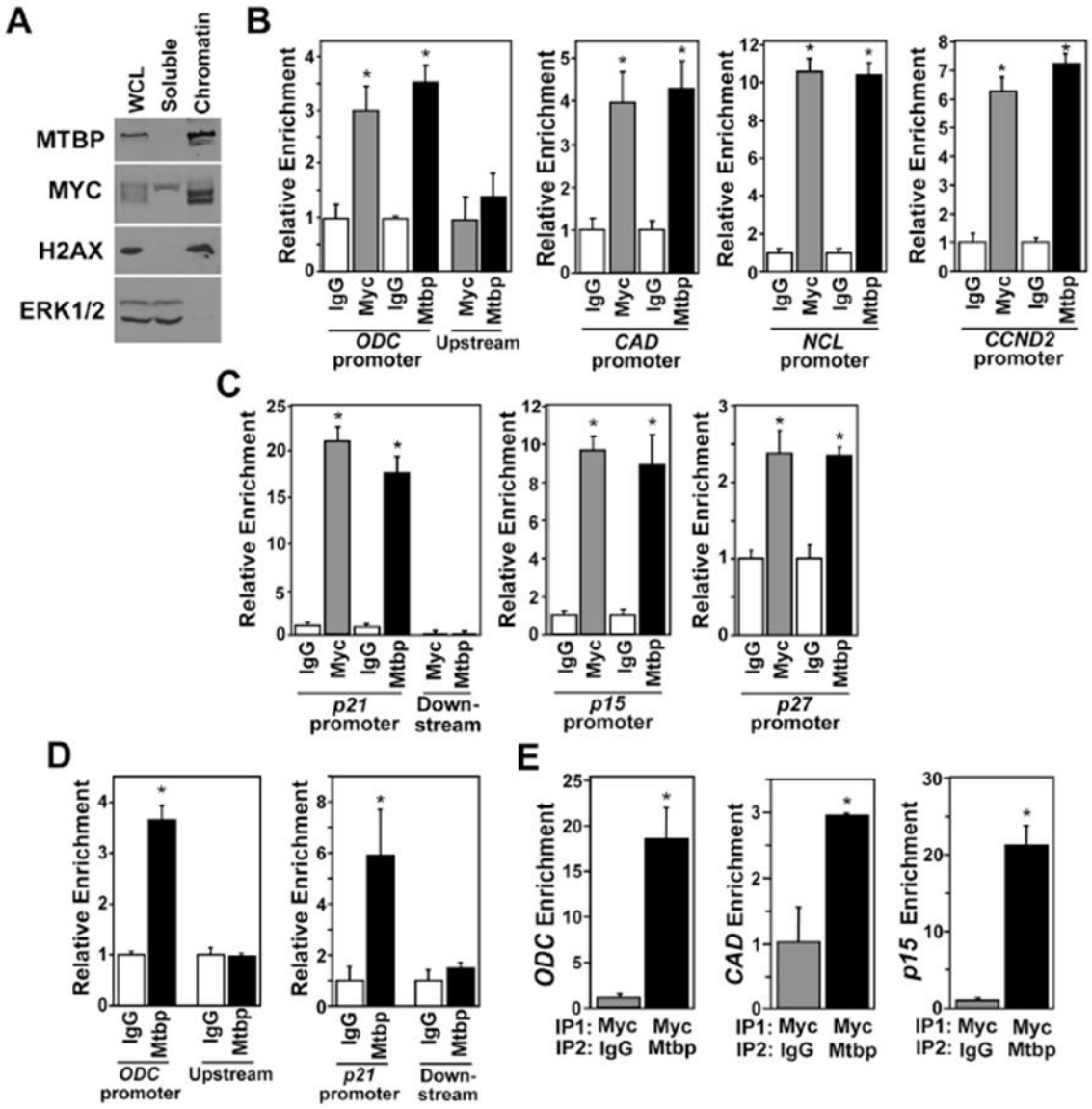

Figure 10. **Mtbp associates with Myc at Myc bound promoters.** (A) Whole cell lysate (WCL), soluble protein fraction, and chromatin-associated protein fraction from 293T cells were Western blotted. (B, C) 293T cells were transfected with vectors encoding Myc or Mtbp. Following ChIP with anti-Myc, anti-Mtbp, 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 and 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; *$p \leq 0.01$ compared to appropriate IgG.

genes transcriptionally repressed by Myc (Figure 10C). Similarly, endogenous MTBP was present at Myc regulated promoters in Raji cells (Figure 10D). Notably, sequential ChIP of Myc first followed by Mtbp showed enrichment at both Myc transcriptionally activated and repressed promoter regions (Figure 10E), 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, I evaluated the effects of Mtbp overexpression on Myc-induced transcription. Mtbp was overexpressed in NIH3T3 cells expressing a 4-hydroxytamoxifen (4-OHT) regulatable form of Myc, MycER (Littlewood). 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 (Figure 11A), 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 (Odvody *et al.*, 2010) 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 (Figure 11B), a cooperative effect also observed in immortalized human mammary

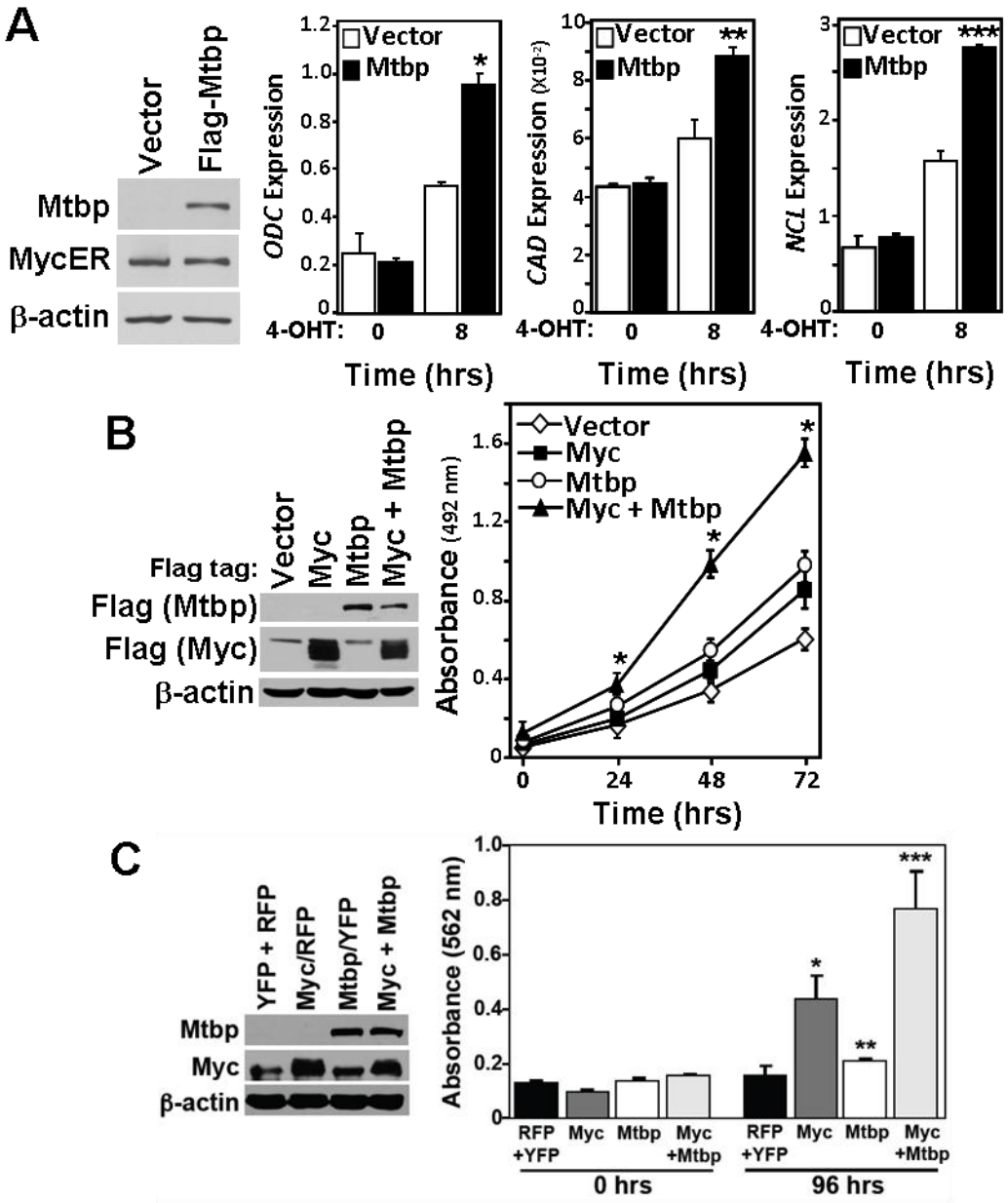

Figure 11. Mtbp overexpression promotes Myc-mediated transcription and proliferation. (A) MycER expressing NIH3T3 cells were transfected with an empty vector or a vector encoding Flag-Mtbp. Western blots of whole cell lysates were performed. Additionally, MycER was activated in the fibroblasts grown in full serum 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) Immortalized non-tumorigenic human mammary epithelial (HMLE) cells were transduced with lentivirus encoding YFP, RFP, Mtbp and YFP, or Myc and RFP, as indicated, and were Western blotted. Proliferation was monitored by MTT.

epithelial cells (Figure 11C). 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 (Figure 12A and 13A), but was also observed when cells were in 10% serum (Figure 12B). Since this difference in S-phase may not completely account for the considerable increase in cell number with Mtbp and Myc co-overexpression, we also evaluated apoptosis by serum starving cells. Within 24 hours, 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 (Figure 13B). 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 (Figure 13C and D). 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 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

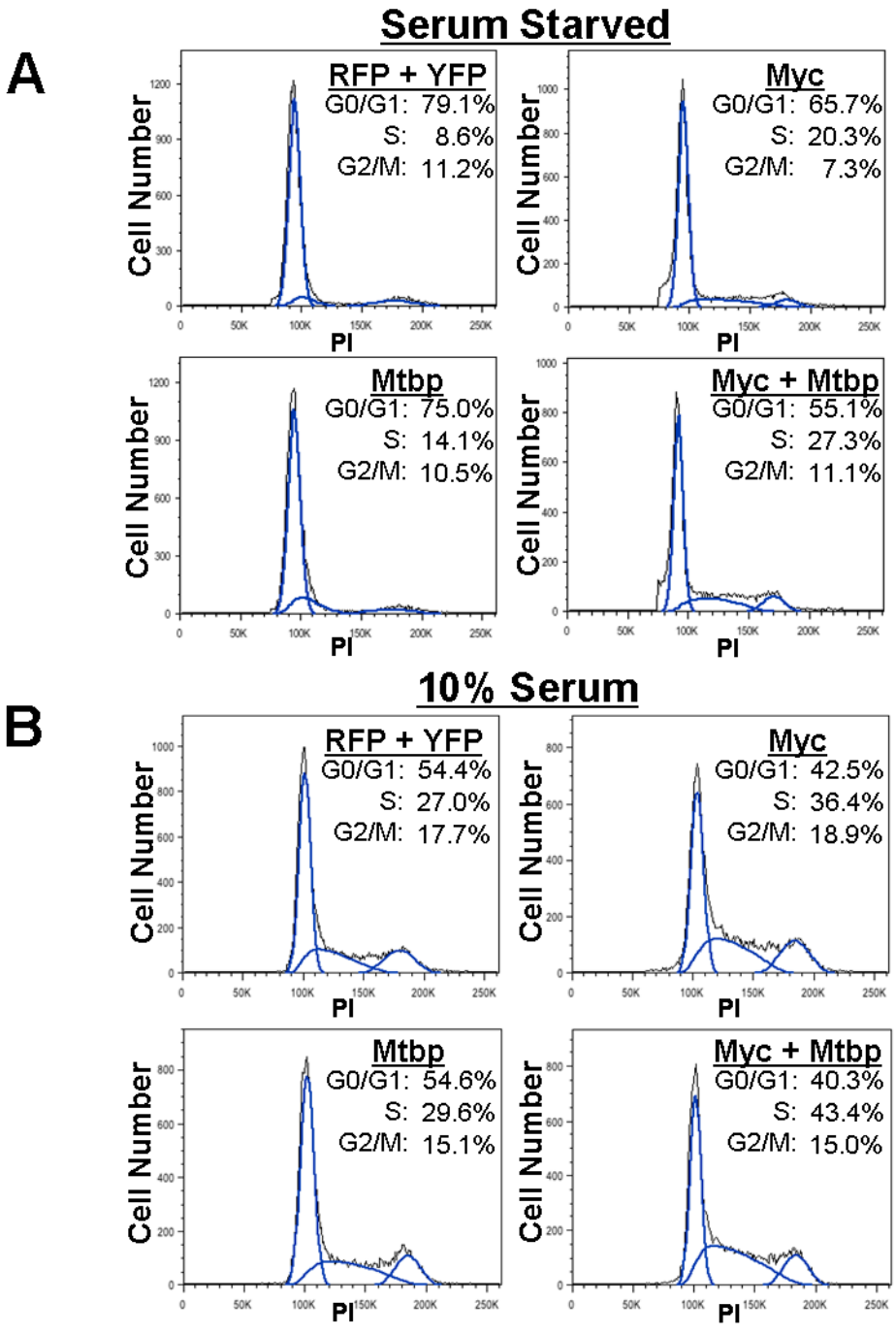

Figure 12. Mtbp promotes Myc-mediated cell cycle progression. (A&B) NIH3T3 cells infected with two bicistronic retroviruses encoding the indicated proteins were cultured under low serum conditions for 24 hours (A, see Figure 13A for quantification) or in 10% serum (B) and were analyzed by flow cytometry after propidium iodide staining of DNA. The percentage of cells in each phase of the cell cycle was determined by Dean-Jett-Fox analysis (blue line); representative histograms are shown.

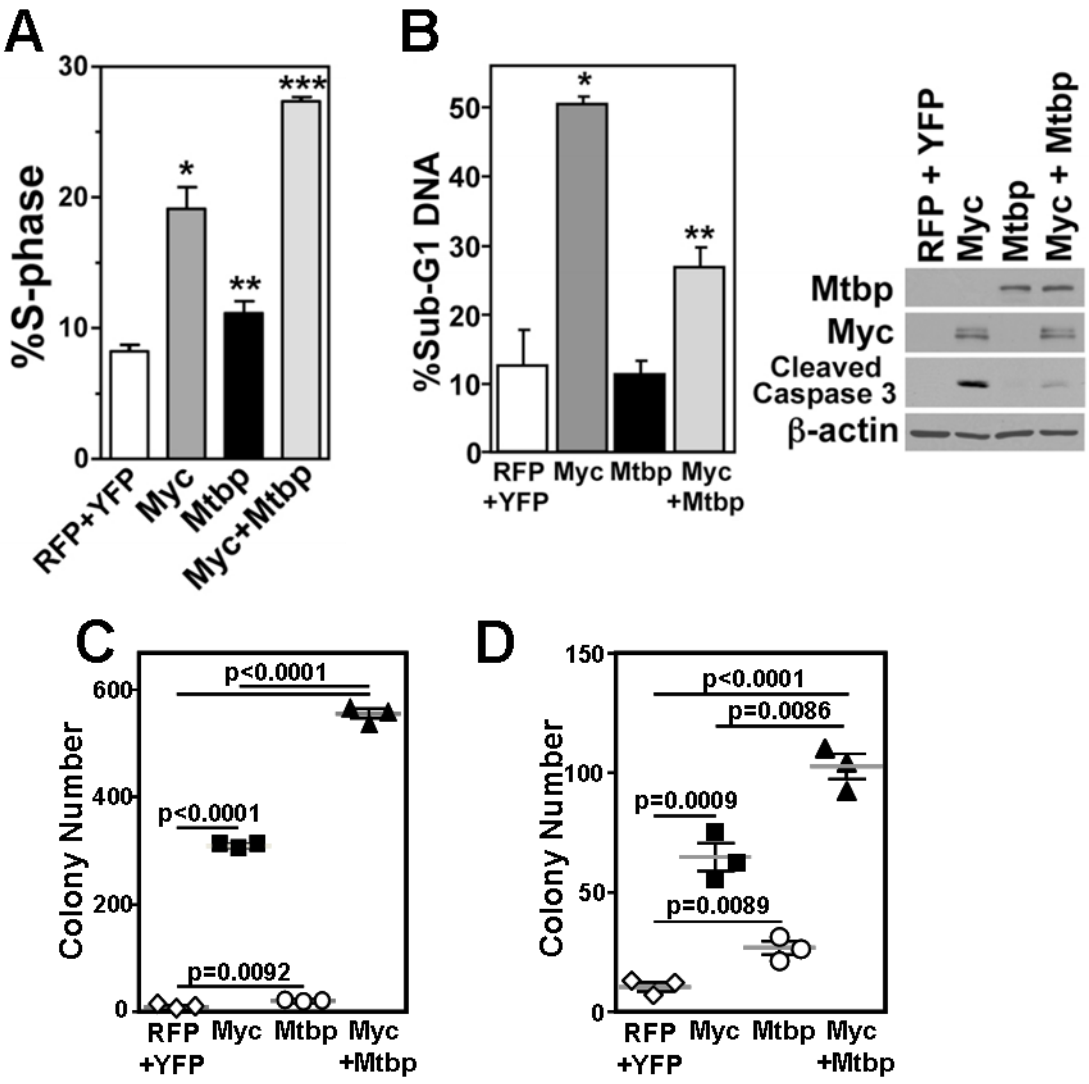

Figure 13. Mtbp promotes Myc-mediated cell cycle progression and transformation, inhibits apoptosis. (A&B) NIH3T3 cells infected with two bicistronic retroviruses encoding the indicated proteins were cultured under low serum conditions for 24 hours and were analyzed by flow cytometry after propidium iodide staining of DNA. (A) The proportion of live cells in S-phase (*p = 0.012 RFP+YFP vs. Myc, **p = 0.0296 RFP+YFP vs. Mtbp, ***p = 0.0191 Myc + Mtbp vs. Myc). (B) The proportion of total cells with sub-G1 DNA content (*p = 0.0025 RFP + YFP vs. Myc, **p = 0.0018 Myc vs. Myc + Mtbp). Western blot performed for the indicated proteins. (C) NIH3T3 cells from A&B (C) or immortalized non-tumorigenic human mammary epithelial (HMLE) cells (D, see Figure 11 C) subjected to soft agar colony assay with colony number quantified after 10 or 30 day, respectively. Error bars are standard deviation.

grew larger faster (Figure 14A) and weighed more upon extraction at day 34 (Figure 14B). 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 (Figure 11A), was recently linked to poor patient outcomes (Horiuchi *et al.*, 2012; Xu *et al.*, 2010). 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 ($p = 0.0314$; Figure 15), indicating MTBP levels influence the impact of MYC on patient prognosis. This trend was also observed in human lung and colon cancer (Figures 15). 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 2; Cerami *et al.*, 2012; Gao *et al.*, 2013). This occurred even though *MTBP* and *MYC* are 7.2 megabases apart at 8q24.12 and 8q24.21, respectively (Figure 16). 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.

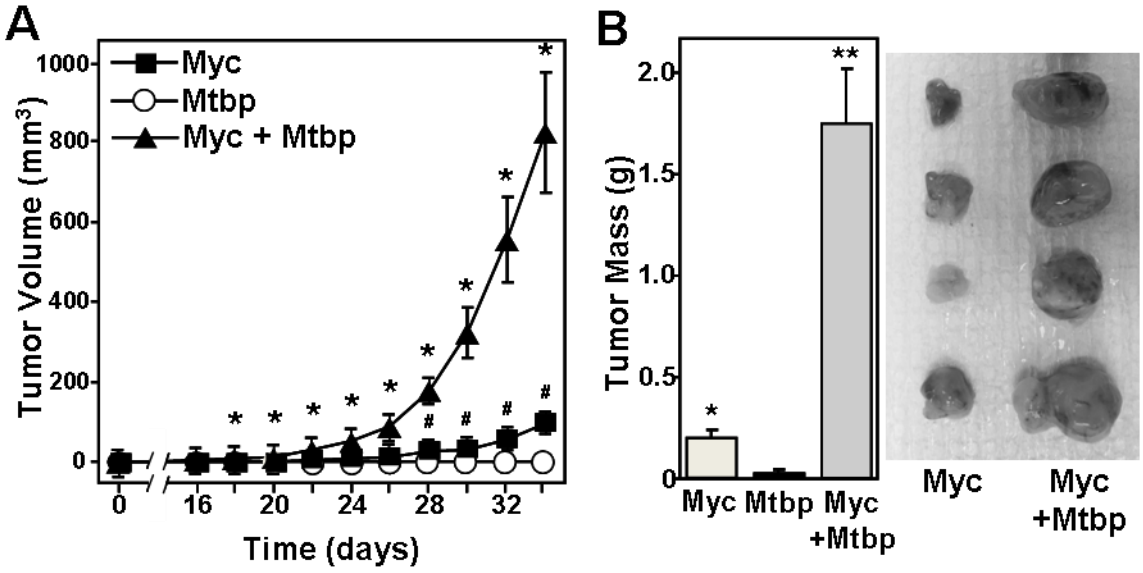

Figure 14. Increased Mtbp expression cooperates with Myc overexpression *in vivo*. (A) Equal numbers of NIH3T3 cells expressing Mtbp, Myc, or both were injected subcutaneously into the flanks of nude mice (n = 16/group), and tumor volume was measured at intervals (#$p < 0.001$ Myc vs. Mtbp, *$p < 0.0001$ 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. (A, B).

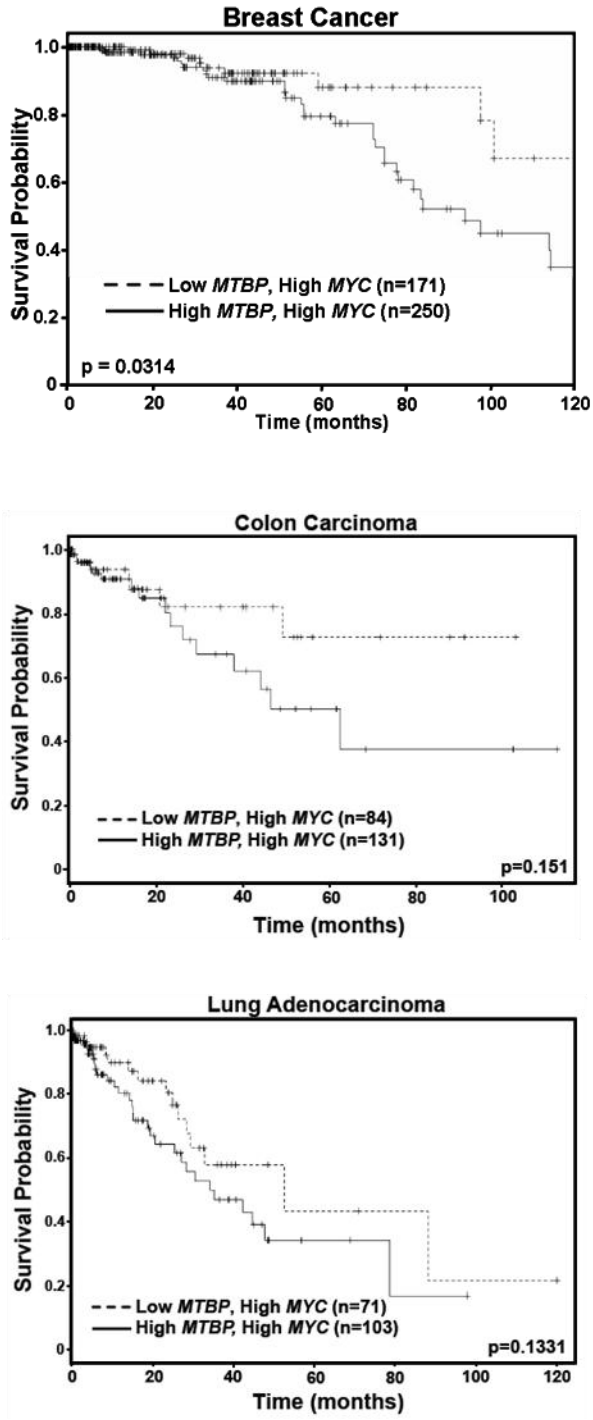

Figure 15. MTBP regulates MYC impact on cancer patient prognosis. Kaplan Meier survival curves from RNA-Seq mRNA expression data from TCGA database of indicated cancer patients with high *MYC* mRNA expression divided into low or high *MTBP* mRNA expression. Patient number indicated by n.

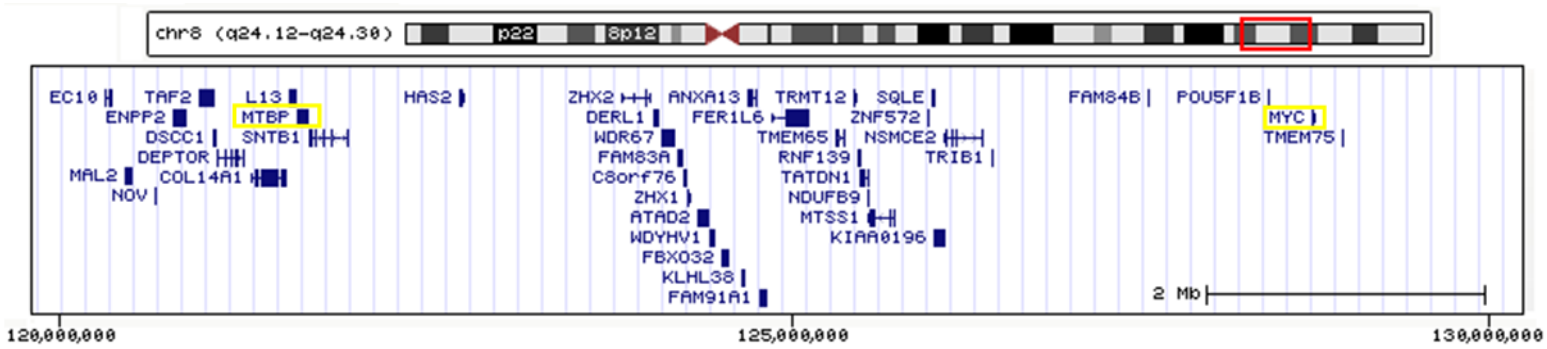

Figure 16. Location of *MTBP* and *MYC* on 8q24. Diagram of human chromosome 8 with the red box encompassing 8q24.12 to 8q24.30. The genes encoded and their location in this region are in the black box. *MTBP* and *MYC* are boxed in yellow and are 7.2 Mb apart. (Adapted from http://genome.ucsc.edu/index.html.)

C-terminus of Mtbp associates with and inhibits Myc

Since cancer patient data indicated MTBP and MYC co-overexpression reduces survival (Figure 15), 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 and no functional domains had been reported at this time, we divided Mtbp (aa 1-894) into thirds (Figure 17A). 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 (Figure 17 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 (Figure 17B). The commercially available Tip49 antibody was not of sufficient quality to conclusively determine Tip49 association. However, given the association between Mtbp and both Tip48 and Tip49 (Figure 7) and reports that Tip48 and Tip49 form heterocomplexes (Grigoletto *et al.*, 2011), 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 (Figure 17C). Further ChIP analyses showed the C-terminal Mtbp mutant, but not the central domain Mtbp mutant, enriched Myc-regulated promoter sequences (Figure 17D). Therefore, the C-terminus of Mtbp is sufficient to mediate the interaction with Tip48/Tip49, Myc, and Myc-bound promoters.

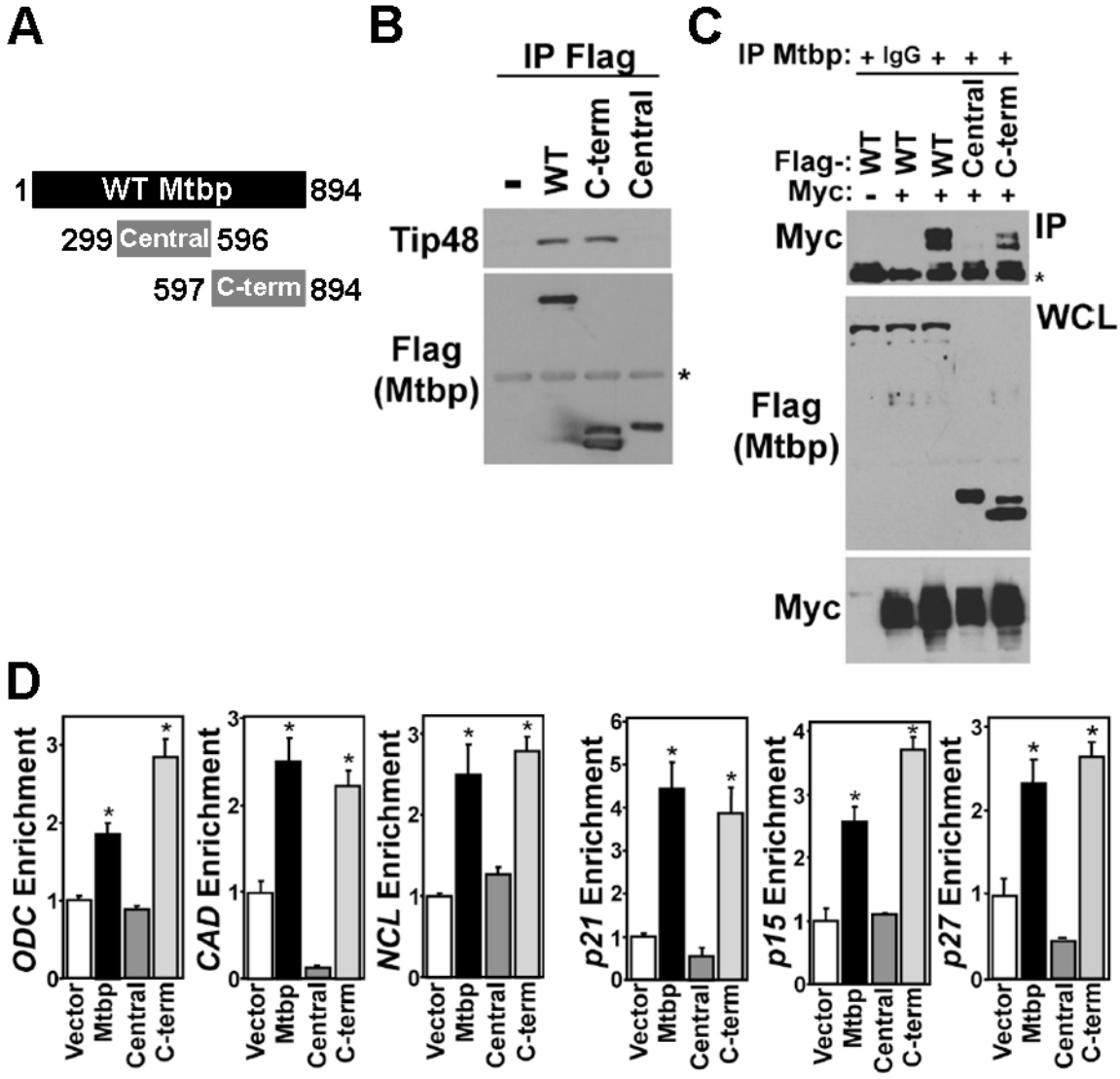

Figure 17. Mtbp C-terminus mediates association with Tip48, Myc and chromatin. (A) Diagram showing full-length wild-type (WT) Mtbp and the central (299-596) and C-terminal (597-894) Mtbp mutants. (B-D) 293T cells were transfected with vectors encoding Flag-tagged WT Mtbp and mutants, or empty vector control (-) and for (C) a vector that did (+) or did not (-) encode Myc. (B) Anti-Flag immunoprecipitation of whole cell lysates were Western blotted for Flag and endogenous Tip48. Asterisk denotes location of immunoglobulin heavy chain. (C) 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). (D) 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 (*$p \leq 0.01$ compared to vector).

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 (Figure 18A). In comparison, knockdown of *Mtbp* produced analogous results (Figure 19). The C-terminal Mtbp mutant also inhibited Myc-driven cellular proliferation (Figure 18B) and soft agar colony formation (Figure 18C), whereas the central domain Mtbp mutant had no significant effect. Similarly, *MTBP* knockdown in human or murine cells reduced proliferation (Figure 20). These results are consistent with our previous observations that decreased Mtbp expression due to a haploinsufficiency inhibited Myc-mediated pro-proliferative transcription and cellular proliferation (Odvody *et al.*, 2010). 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 (Barretina *et al.*, 2012; Kessler *et al.*, 2012). In both, the C-terminal Mtbp mutant significantly reduced proliferation compared to the central domain mutant or empty lentivirus control (Figure 21). 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.

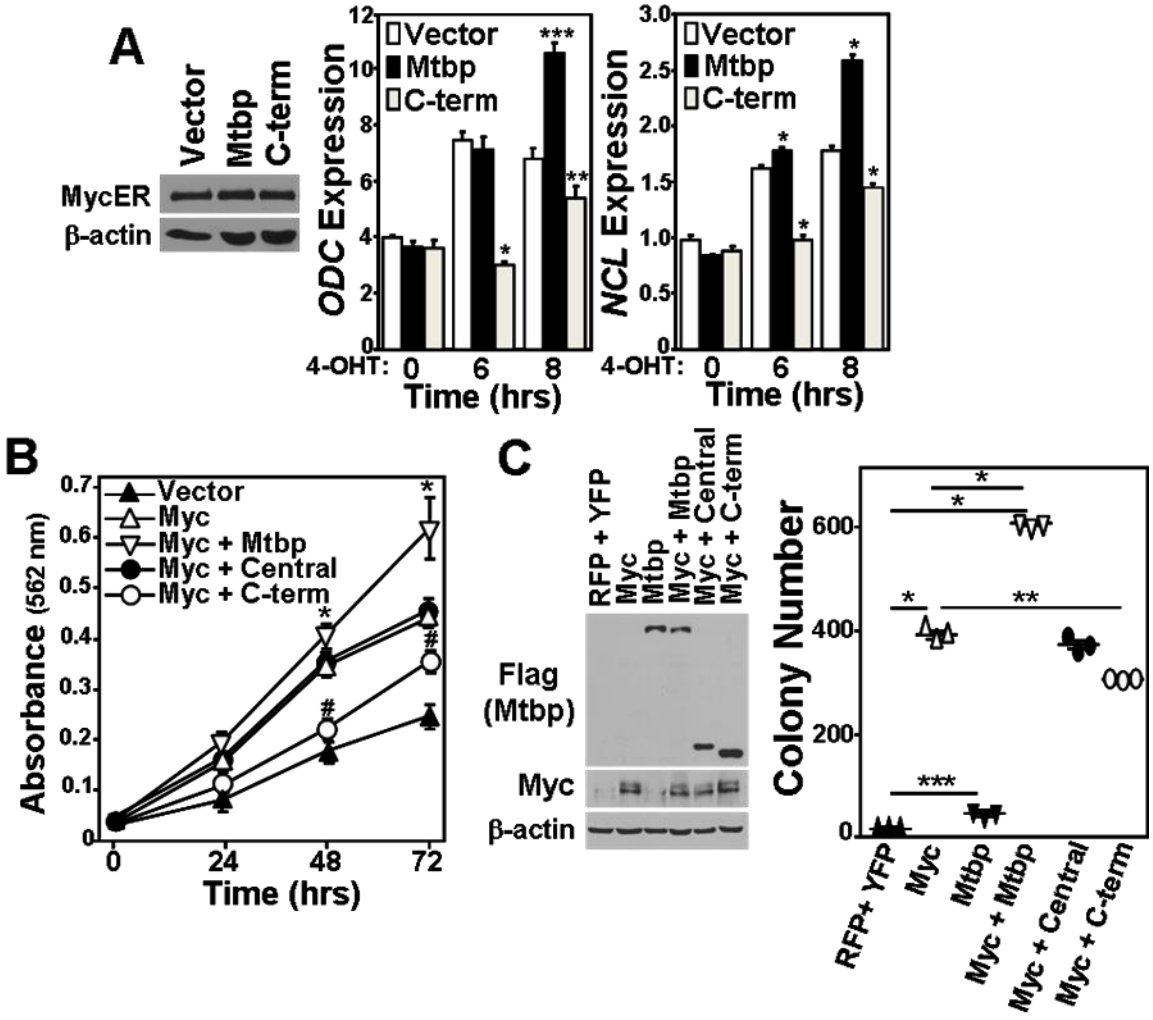

Figure 18. Mtbp C-terminal mutant inhibits Myc activity. (A) MycER expressing NIH3T3 cells were transfected with an empty vector or a vector encoding full-length Mtbp or the C-terminal Mtbp mutant. Western blots were performed. To activate MycER, 4-OHT was added to the cultures for the indicated time. 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). (B) 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). (C) 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).

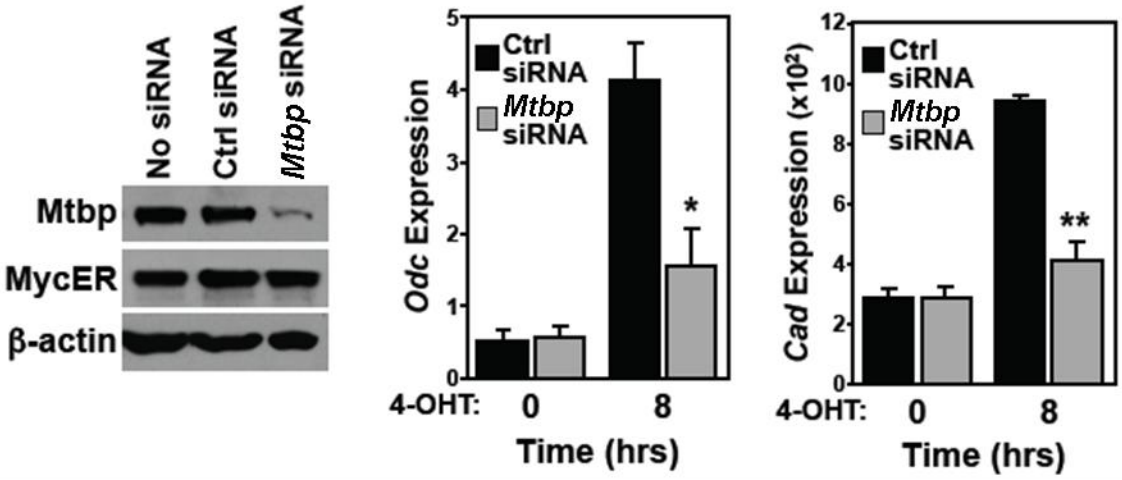

Figure 19. Myc-mediated transcription inhibited by Mtbp knockdown. MycER expressing NIH3T3 cells were transfected with non-targeting control siRNA or *Mtbp* siRNA. Western blots of whole cell lysates were performed. MycER was activated with 4-OHT for 0 or 8 hrs in cells in media containing 10% serum. qRT-PCR was performed for the indicated Myc target genes. All samples were normalized to β-*actin*. Error bars represent standard deviation; *p=0.0113 and **p=0.0005.

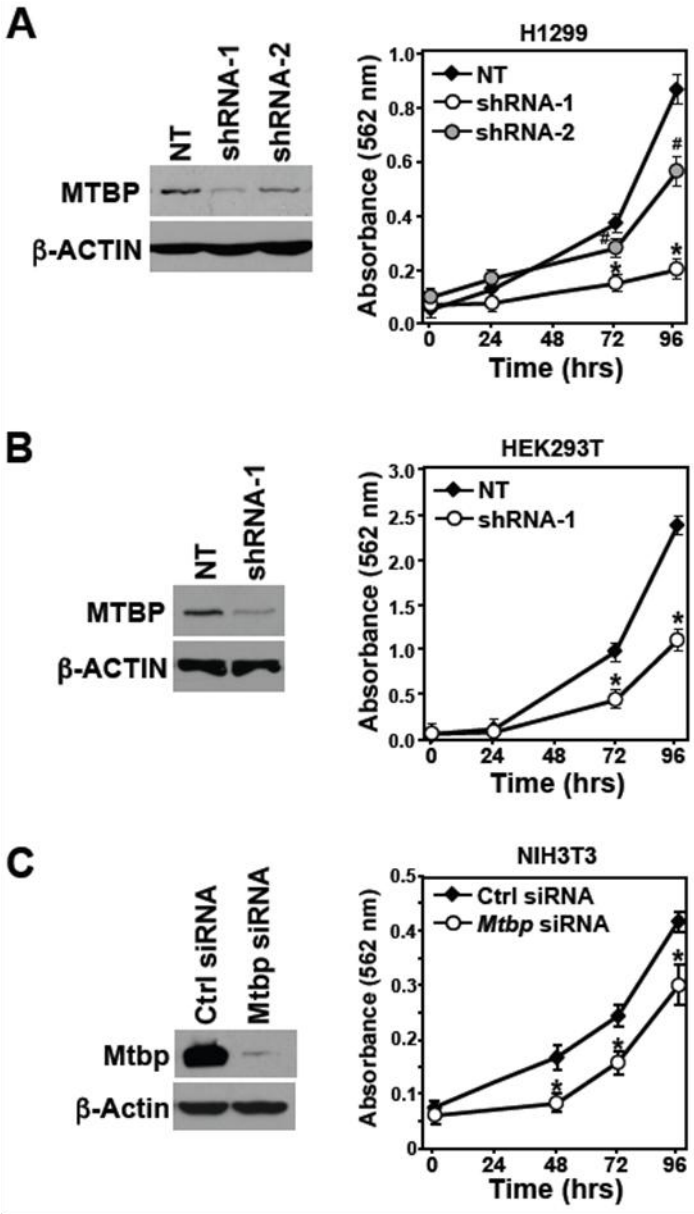

Figure 20. Mtbp/MTBP knockdown inhibits proliferation. (A&B) H1299 lung adenocarcinoma (A) and HEK293T (B) cells were transfected with vectors encoding non-targeting (NT) control shRNA or one of two different *MTBP*-specific shRNA (shRNA-1 or shRNA-2). Western blots of whole cell lysates were performed. Proliferation was monitored by MTT at intervals (*$p<0.0001$ NT vs. shRNA-1, #$p<0.0003$ NT vs. shRNA-2, $p<0.0001$ shRNA-1 vs. shRNA-2 24-96 hours for H1299 cells; *$p<0.0001$ for NT vs. shRNA-1 in HEK293T cells). (C) NIH3T3 cells were transfected with NT control siRNA or *Mtbp* siRNA. Western blots of whole cell lysates were performed. Proliferation was monitored by MTT at intervals (*$p<0.001$). Error bars represent standard deviation.

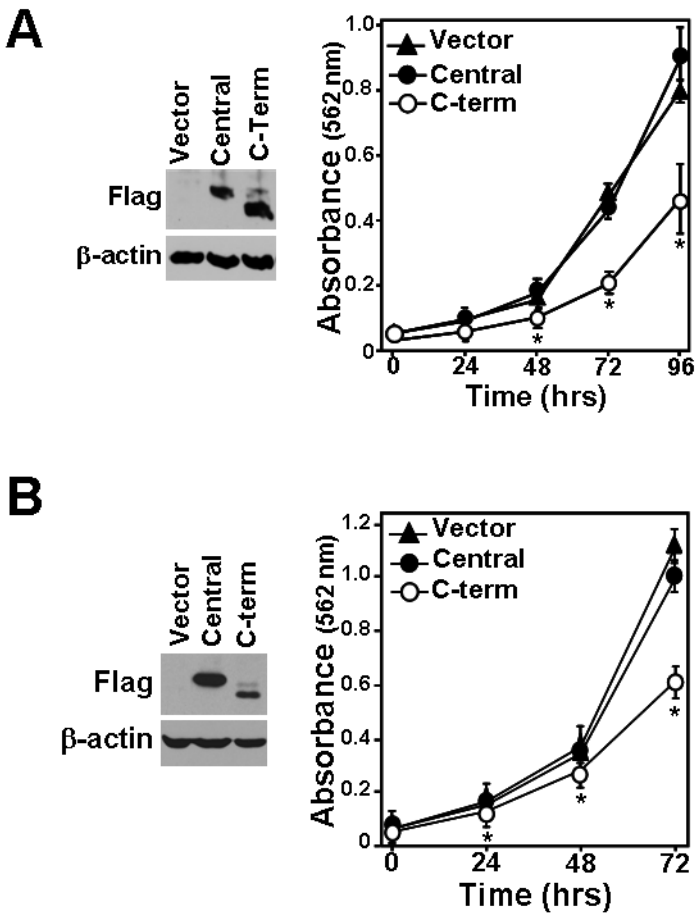

Figure 21. Mtbp C-terminal mutant inhibits breast cancer cell expansion. (A) MDA-MB-231 or (B) HCC1806 breast cancer cells expressing YFP alone (vector) or YFP with Flag-tagged central or C-terminal Mtbp mutants were subjected to MTT assays at 24 hour intervals (*$p < 0.001$ and *$p < 0.05$, respectively). Error bars are standard deviation. Western blot of whole cell lysates shown.

Discussion

Several studies suggested MTBP has a role in cancer development and possibly progression, but its function remained unresolved (Boyd *et al.*, 2000a; Iwakuma *et al.*, 2008; Odvody *et al.*, 2010). Initially, MTBP was thought to regulate the protein from which it received its name, Mdm2, a negative regulator of p53 (Boyd *et al.*, 2000a; Brady *et al.*, 2005); however, studies utilizing genetically engineered mice did not support this function of Mtbp *in vivo* (Brady *et al.*, 2005; Iwakuma *et al.*, 2008; Odvody *et al.*, 2010). 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 widely overexpressed in cancer and is part of a Tip48/Tip49 complex that binds Myc and regulates Myc-mediated transformation. 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 (Eilers & Eisenman, 2008; Meyer & Penn, 2008). 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 (Eilers & Eisenman, 2008; Frank *et al.*, 2003; Meyer & Penn, 2008; Wood *et al.*, 2000). For Tip48 and Tip49 specifically, they form a hexamer/dodecamer, bind Myc and facilitate Myc-mediated transformation (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Grigoletto *et al.*, 2011; Wood *et al.*, 2000). Although Tip48 and Tip49, through interactions with Myc, are critical for cell growth and proliferation during *Drosophila* and *Xenopus* development (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Grigoletto *et al.*, 2011), 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 directly bound by Tip48 and Tip49 (Wood *et al.*, 2000). Furthermore, we detected Mtbp in complex with Myc at promoters both transcriptionally activated and repressed by Myc, where others have also observed Tip48 and Tip49 in multiple cell types and species (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Frank *et al.*, 2003; Li *et al.*, 2010; Si *et al.*, 2010). 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, a complex critical for Myc-induced transformation (Wood *et al.*, 2000).

Mtbp alone was less oncogenic than Myc, an established powerful oncogene (Eilers & Eisenman, 2008; Meyer & Penn, 2008). 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 (Odvody *et al.*, 2010), indicating reduced levels of Mtbp inhibited the proliferative and transforming functions of Myc. Here, we provide further support for these observations and present mechanistic data that explains both the overexpression and the reduced expression effects of Mtbp on Myc activity and tumorigenesis. Specifically, our results show Mtbp forms a transcriptional complex with Myc through Tip48/Tip49, interacts with Myc at promoters, and significantly enhances Myc-mediated transcription of pro-proliferative genes, leading to increased proliferation and transformation. 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 also supports the conclusion that Mtbp directly facilitates Myc-induced transcription (Odvody *et al.*, 2010). Additionally, Myc controls the expression of many of its regulators, and Mtbp appears to be a direct transcriptional target of Myc (Fig 1A; (Odvody *et al.*, 2010). Therefore, the data strongly indicate Mtbp modulates Myc transcriptional function through their association, and together, this promotes 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 (Figure 4 and Tables 1-2). Moreover, we determined *MTBP*, which is 7.2

megabases apart from *MYC* on chromosome 8q24 (Figure 12; Boyd *et al.*, 2000b), 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*. 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 (Agarwal *et al.*, 2013; Iwakuma *et al.*, 2008), cancer cells were shown to downregulate proliferation and Myc in favor of movement (Liu *et al.*, 2012). One report does show decreased *MTBP* expression in a subset of head and neck cancer correlated with reduced survival (Vlatkovic *et al.*, 2011). 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 (Odvody *et al.*, 2010). MTBP was also recently linked to DNA replication origin firing and mitotic progression (Agarwal *et al.*, 2011; Boos *et al.*, 2013). 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 (Grigoletto *et al.*, 2011). 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 (Eilers & Eisenman, 2008; Meyer & Penn, 2008; Srinivasan *et al.*, 2013). Of note, MTBP was recently reported to interact with a DNA replication protein and be involved with DNA replication origins (Boos *et al.*, 2013). 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.

CHAPTER III

MTBP IS OVER-EXPRESSED IN TRIPLE NEGATIVE BRESAT CANCER AND CONTRIBUTES TO ITS GROWTH AND SURVIVAL

Work from this chapter was published in by Grieb *et al.* in *Molecular Cancer Research*.

Background and Significance

The use of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) as biomarkers is standard practice in the clinical management of breast cancer. Their expression directs the use of targeted therapeutics such as tamoxifen and trastuzumab that have dramatically improved patient survival. Unfortunately, such improvements in clinical outcomes have not been realized in the management of triple negative breast cancer (TNBC), a subset of breast cancers lacking *HER2* amplification and expression of ER and PR (Liedtke *et al.*, 2008). TNBC comprises 10-20% of breast cancer cases and is more commonly identified in younger women and those with African American or Hispanic heritage (Morris *et al.*, 2007). It is clinically aggressive, correlating with an increased risk of distant recurrence within three years following treatment and a significant decrease in overall patient survival, compared to receptor positive cases (Dent *et al.*, 2007; Haffty *et al.*, 2006). While there has been some success in exploiting novel molecular targets, such as PARP inhibitors in BRCA1 mutant tumors with errors in DNA break repair (Farmer *et al.*, 2005; Fong *et al.*, 2009), these cases are isolated and applicable to only select TNBCs. Other targets such as

mTOR, Src, and HER1 tested in phase II clinical trails have shown only minimal success (Carey *et al.*, 2012; Ellard *et al.*, 2009; Finn *et al.*, 2007). Thus, there is a need to identify and test the therapeutic efficacy of novel molecular targets in TNBC.

The Mdm2 (Two) Binding Protein (MTBP) was first identified as a potential tumor suppressor that binds Mdm2, a negative regulator of p53 (Boyd *et al.*, 2000a). However, subsequent genetic studies indicated it functions independent of Mdm2, and instead, contributes to tumor development induced by the Myc oncogene (Iwakuma *et al.*, 2008; Odvody *et al.*, 2010). Recently, MTBP has been implicated in regulating proliferation and cell cycle progression (Agarwal *et al.*, 2011; Boos *et al.*, 2013; Grieb *et al.*, 2014b; Odvody *et al.*, 2010). *MTBP* is a transcriptional target of MYC, and its protein expression increased in response to pro-proliferative signals and decreased upon growth factor withdrawal (Odvody *et al.*, 2010). In mouse models, *Mtbp* heterozygosity led to reduced levels of Mtbp protein and this inhibited Myc-induced B cell proliferation, resulting in a significant delay in lymphoma development (Odvody *et al.*, 2010). Furthermore, siRNA-mediated knockdown of *MTBP* was reported to delay cell cycle progression through the S and G2/M phases of the cell cycle (Agarwal *et al.*, 2011; Boos *et al.*, 2013). Therefore, MTBP appears to contribute to the development and possibly the maintenance of tumors through regulation of proliferation, but further investigation is needed.

Here, we report *MTBP* is overexpressed and amplified in breast cancer, correlating with decreased patient survival. Notably, *MTBP* mRNA expression was highest in TNBC. shRNA-mediated knockdown of *MTBP* in human TNBC cell lines inhibited their expansion and induced apoptosis, *in vitro*, as well as significantly reduced

tumor growth, *in vivo*. Our data reveal MTBP significantly contributes to breast cancer and is a potential novel therapeutic target in the treatment of TNBC.

Material and Methods

Patient Data

Patient survival and gene expression data for 844 breast cancers were accessed from The Cancer Genome Atlas public data portal (https://tcga-data.nci.nih.gov/tcga/) January-April 2013. 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. Log-rank tests were used to compare survival between groups. Box and whisker plots (box represents first and third quartiles, thick band is median value, and bars extend to +/- 1.58 the interquartile range divided by the square root of the number of samples), were applied to describe *MTBP* gene expression values. Groups were compared using a Wilcoxon rank sum test. Gene copy number alteration (CNA) and survival data for 913 breast cancers was obtained from the cBioPortal for Cancer Genomics (http://www.cbiopor2tal.org/public-portal/) May 2013.

Cell Culture, vectors, transfection, and infection

The human cell lines MDA-MD-231, HCC1806, and HCC1937 were cultured as described by the American Type Culture Collection (Manassas, VA) and were provided by Dr. Jennifer Pietenpol. Cells were transfected with Effectene (Qiagen, Germantown, MD) or were infected with retroviruses, as previously described (Zindy *et al.*, 1998).

MTBP shRNA 19mer sequences (shRNA1 GGAGAGTGTTCTAGCTATT or shRNA2 GAAACACAGTATTACCGAG) and non-targeting control (GACTTACGAGATCAGAAAG) were used in pSuper constitutive expression constructs (Oligoengine, Seattle, WA) and were adapted to the dox-inducible system (pInducer) generously provided by Dr. Thomas Westbrook (Meerbrey *et al.*, 2011) using the RNAi central shRNA retriever (http://cancan.cshl.edu/RNAi_central/RNAi.cgi?type=shRNA).

Proliferation, cell cycle, apoptosis and transformation assays

For measurement of proliferation, 1,000 to 5,000 cells were plated in triplicate and MTT assays were performed as per manufacture's protocol (Sigma, St. Louis, MO). Cell cycle and apoptosis (subG1 DNA content) were evaluated with FlowJo software (TreeStar Inc., Ashland, OR) following DNA staining with propidium iodide and flow cytometry. Apoptosis was also evaluated by assessing Annexin V binding (Life Technologies, Pittsburgh, PA) per manufacturer's protocol and Caspase 3 cleavage via Western blot (see below). Viability was assessed by Trypan Blue Dye exclusion. Soft agar assays were performed as previously described (Bouska *et al.*, 2008). For dox-inducible shRNA experiments, 0.5-1 μg/ml of dox was added to the cultures.

Mice

Female athymic nude mice (5-6 week old; Harlan, Indianapolis, IN) were injected subcutaneously in the flank with $3x10^6$ HCC1806 cells. Mice were housed with drinking water supplemented with 5% sucrose with or without 2 g/L of dox (Research Products

International Corp., Prospect, IL) that was changed every 48 hours beginning on the day of injection or 10 days later. Tumor volume was calculated from measurements with electronic calipers. At time of sacrifice, mice were photographed, and tumors were extracted, photographed and weighed. A piece of each tumor was frozen for Western blot analysis. All experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee and followed all federal and state rules and regulations.

Western blotting and quantitative real-time PCR analysis

For Western blotting, cells or tumors that were infected or transiently transfected (see above) were harvested after 48 hours or at indicated times and were lysed as previously reported (Bouska *et al.*, 2008; Zindy *et al.*, 1998). Equal amounts of protein were resolved by SDS-PAGE and Western blotted using antibodies specific for MTBP (B5, Santa Cruz Biotechnology, Santa Cruz, CA), cleaved Caspase 3 (D175, Cell Signaling Technology, Danvers, MA), MYC (C33, Santa Cruz Biotechnology) and β-actin (AC15, Sigma, St. Louis, MO). To evaluate mRNA expression, total RNA was isolated, cDNA was generated, and qRT-PCR for *MTBP* and *β-ACTIN* levels was performed as previously described (Odvody *et al.*, 2010). mRNA data are relative to *β-ACTIN* levels.

Statistical evaluation

Wilcoxon rank sum test (Figures 22A and 23A), log rank tests (Figure 22B-C), student's t-test (Figs 23B, 24--27), and Cox regression analysis were used to compare data. Error bars represent standard deviation (Figuress 23B, 24-26) or standard error of the mean (Figure 27).

Results

MTBP is overexpressed in human breast cancer and correlates with decreased patient survival and triple negative status

We previously detected MTBP/Mtbp overexpression in human lymphoma cell lines and primary murine lymphomas (Odvody *et al.*, 2010). *MTBP* was also reported amplified in colorectal carcinoma and multiple myeloma (Carrasco *et al.*, 2006; Martin *et al.*, 2007), as well as several human cancer cell lines (Barretina *et al.*, 2012), suggesting its overexpression contributes to human cancers. To specifically evaluate *MTBP* expression in human breast cancer, mRNA expression and patient survival data for 844 breast cancers from The Cancer Genome Atlas (TCGA) were assessed. *MTBP* was significantly elevated in breast cancer samples compared to normal breast tissue ($p=2.2X10^{-16}$; Figure 22A). When cancers were separated by their *MTBP* expression, those patients whose breast cancers had elevated *MTBP* expression exhibited reduced overall survival compared to patients whose breast cancers had lower levels of *MTBP* ($p=0.0337$; Figure 22B). A Cox regression analysis also showed that increased *MTBP* levels are significantly linked with worse patient survival ($p=0.033$). Moreover, *MTBP* was amplified in 19% of breast cancers, and this amplification decreased overall patient survival compared to tumors without amplified *MTBP* ($p=0.01955$; Figure 22C; (Cerami *et al.*, 2012; Gao *et al.*, 2013). These data indicate *MTBP* overexpression is common and thus, likely selected for during breast cancer formation and/or progression. The results also show that elevated levels of *MTBP* correlate with reduced breast cancer patient survival.

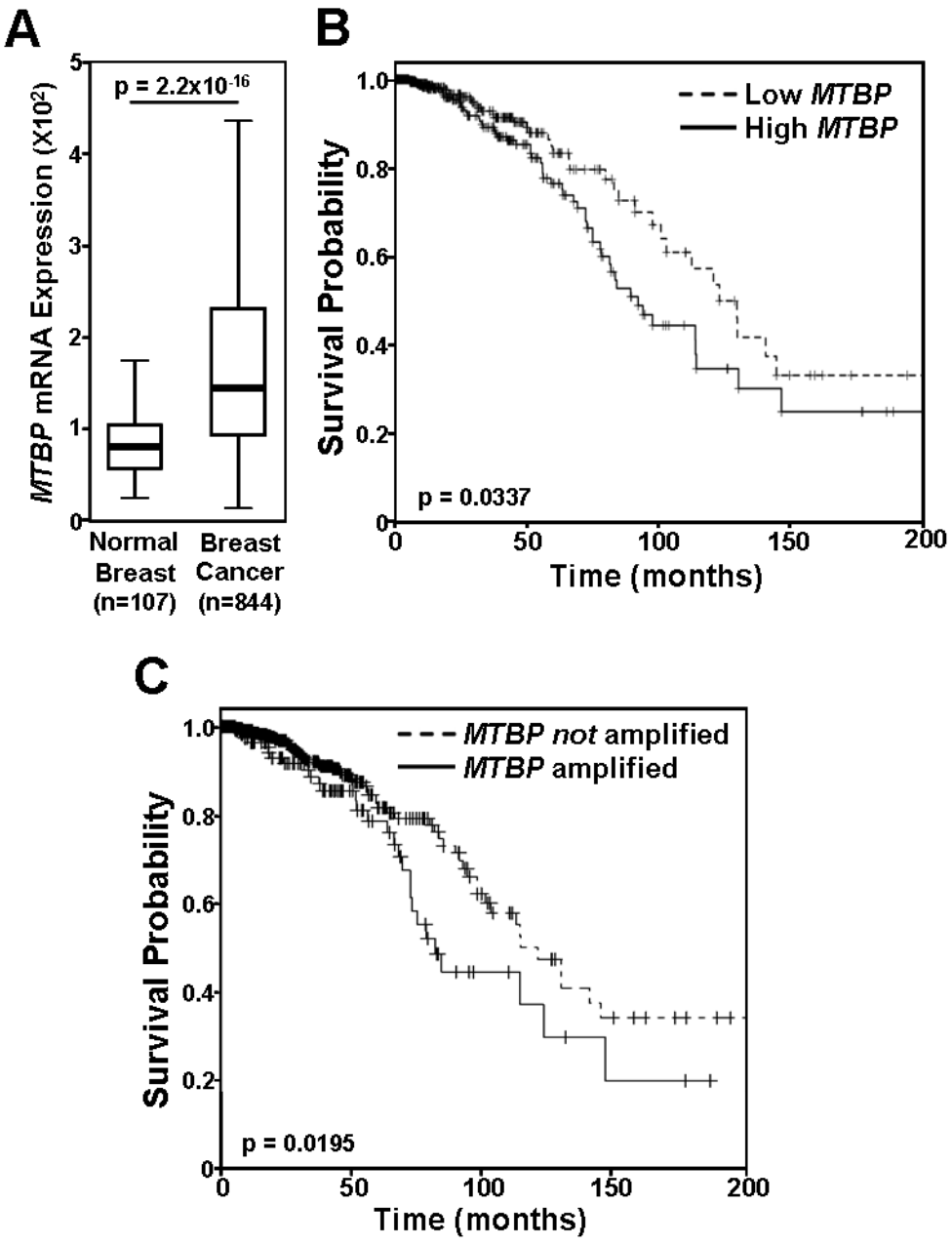

Figure 22. MTBP overexpression in breast cancer decreases survival. (A, B) RNA-Seq mRNA expression data for normal and breast cancer tissue from TCGA database. (A) Box and whisker plot of relative *MTBP* mRNA expression in normal and cancerous breast tissue with "n" indicating the number of samples. (B) Kaplan Meier survival curves for breast cancer patients divided by the median value into low and high *MTBP* mRNA expression (n=421/group). (C) Kaplan Meier survival curves of *MTBP* gene copy number in breast cancer samples with (n=171) and without (n=742) amplified *MTBP* from the cBio Portal for Cancer Genomics (22, 23). p-values calculated using a Wilcoxon rank sum test for A and a log-rank test for B-C.

To assess MTBP levels in different subtypes of breast cancers, we separated the TCGA breast cancer patient samples into clinically relevant subgroups: estrogen-receptor positive (ER+), HER2 positive (HER2+), and triple negative (TN: ER-, PR-, HER2-) tumors. While *MTBP* mRNA was elevated significantly in all three subgroups compared to normal breast tissue, the triple negative breast cancers (TNBC) expressed significantly more *MTBP* than the ER+ or HER2+ subgroups (Figure 23A). This finding was supported by the observation that *MTBP* mRNA was also significantly elevated in a panel of human TNBC cell lines compared to normal human mammary epithelial cells (HMECs; Figure 23B). To determine whether the increased mRNA levels translated into increased protein, the levels of MTBP protein were assessed. MTBP protein levels were elevated in all of the TNBC cells (Figure 23C). In comparison, the oncogenic transcription factor MYC, which has previously been shown to positively regulate *MTBP* expression and to be elevated in aggressive breast cancers, was also elevated in these same cells (Horiuchi *et al.*, 2012; Odvody *et al.*, 2010). Therefore, *MTBP* mRNA levels are the highest in patient samples of the clinically aggressive TNBC subtype, and TNBC cell lines have high levels of MTBP *mRNA* and protein.

Reducing MTBP levels inhibit TNBC cell proliferation

Considering Mtbp expression increases in response to pro-proliferative factors (Odvody *et al.*, 2010), and MTBP is highly overexpressed in TNBC, we questioned whether reducing levels of MTBP in human TNBC cells would alter their ability to proliferate. To begin to test this concept, we knocked down *MTBP* expression with two different *MTBP* shRNAs in the MDA-MB-231 and HCC1806 TNBC cell lines. While

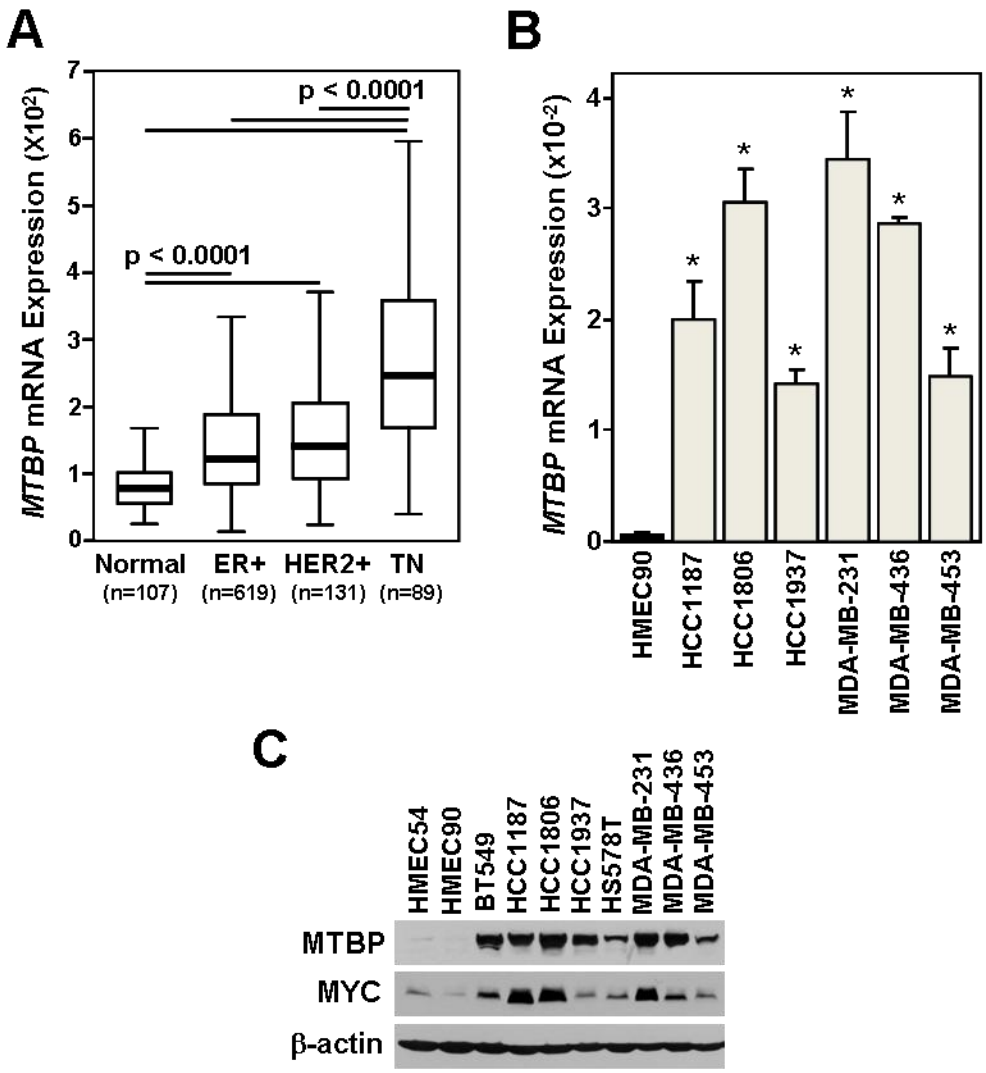

Figure 23. MTBP overexpressed in triple negative breast cancer. (A) Box and whisker plot of relative *MTBP* mRNA expression from TCGA RNA-Seq mRNA expression data, representing normal breast tissue and breast cancers divided into estrogen receptor positive (ER+), human epithelial growth factor receptor 2 positive (HER2+), or triple negative (TN: ER-, PR-, HER2-). The "n" indicates the number of samples. (B) Expression of *MTBP* mRNA measured by qRT-PCR in TNBC cell lines and normal human mammary epithelial cells (HMECs); *p≤0.0007. (C) Western blots of whole cell lysates of TNBC cell lines and HMECs for the proteins indicated.

both cell lines overexpress MTBP (Figure 23C), they represent distinct subtypes of TNBC (mesenchymal-like and basal-like, respectively), and HCC1806 cells have an *MTBP* amplification (Barretina *et al.*, 2012; Lehmann *et al.*, 2011). In both cell lines, reduced MTBP expression resulted in a decrease in proliferation that correlated with the amount of MTBP protein present, where *MTBP* shRNA1 was more effective at reducing MTBP protein levels than *MTBP* shRNA2 (Figure 24A). Similarly, anchorage-independent growth in soft agar was significantly reduced for both the MDA-MB-231 and the HCC1806 cells when *MTBP* was knocked down (Figure 24B). To ensure the observed effects were due to reduced MTBP expression, shRNA resistant murine Mtbp was co-expressed with *MTBP* shRNA1. The murine Mtbp rescued the ability of MDA-MB-231 cells to form colonies in soft agar in the presence of *MTBP* shRNA1, while cells co-transfected with vector control and *MTBP* shRNA1 still showed decreased colony formation (Figure 24C). These results indicate knockdown of *MTBP* inhibits TNBC expansion and anchorage-independent growth.

To further evaluate the anti-proliferative effects of *MTBP* knockdown in TNBC cells, *MTBP* shRNA1 was adapted to a lentiviral doxycycline (dox)-inducible system (Meerbrey *et al.*, 2011). Using the three TNBC cell lines MDA-MB-231, HCC1806, and the basal-like HCC1937 cells that also over-express MTBP (Figure 23C), we observed that dox-induced *MTBP* shRNA1 resulted in reduced MTBP protein expression within 24 hours (Figure 25A) and significantly decreased (37-40% reduced) proliferation within 72 hours (Figure 25B) in all three lines. The steady-state levels of MYC remained unchanged (Figure 25A). Expression of the shRNA resistant murine Mtbp allowed *MTBP* shRNA1 expressing MDA-MB-231 cells to continue growing at rates analogous to that of

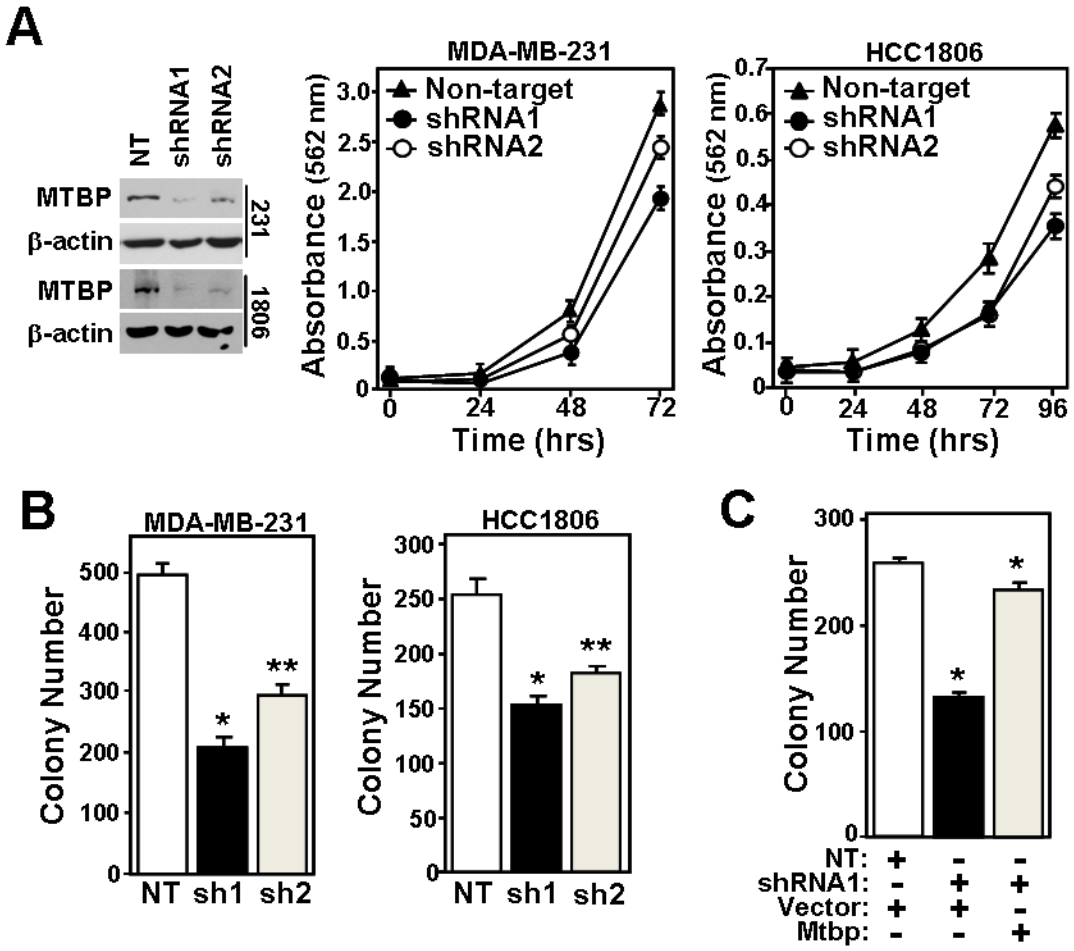

Figure 24. ***MTBP*** **knockdown inhibits proliferation and colony growth.** MDA-MB-231 or HCC1806 cells were transfected with constitutively expressing *MTBP* shRNA1, shRNA2, or non-targeting (NT) control shRNA vectors. (A) Whole cell lysates were Western blotted, and cells were subjected to MTT assays at 24 hour intervals (for MDA-MB-231 at 48-72 hrs $p<0.001$ for NT vs. shRNA1 or shRNA2 and $p<0.01$ for shRNA1 vs. shRNA 2; for HCC1806 $p<0.01$ NT vs. shRNA1 or shRNA2 at 24-96 hrs and $p=0.0013$ at 96 hrs). (B) Cells were subjected to soft agar colony formation assay (MDA-MB-231 *$p=0.0004$, **$p=0.0021$ and HCC1806 *$p=0.0036$, **$p=0.0136$ for NT vs. shRNA1 or shRNA2, respectively). (C) MDA-MB-231 cells transiently transfected with *MTBP* shRNA1 or non-targeting shRNA and shRNA1-resistant murine Mtbp or vector control were subjected to soft agar colony formation assay (*$p<0.0001$ for NT vs. shRNA1 and shRNA1 vs. shRNA1 + Mtbp).

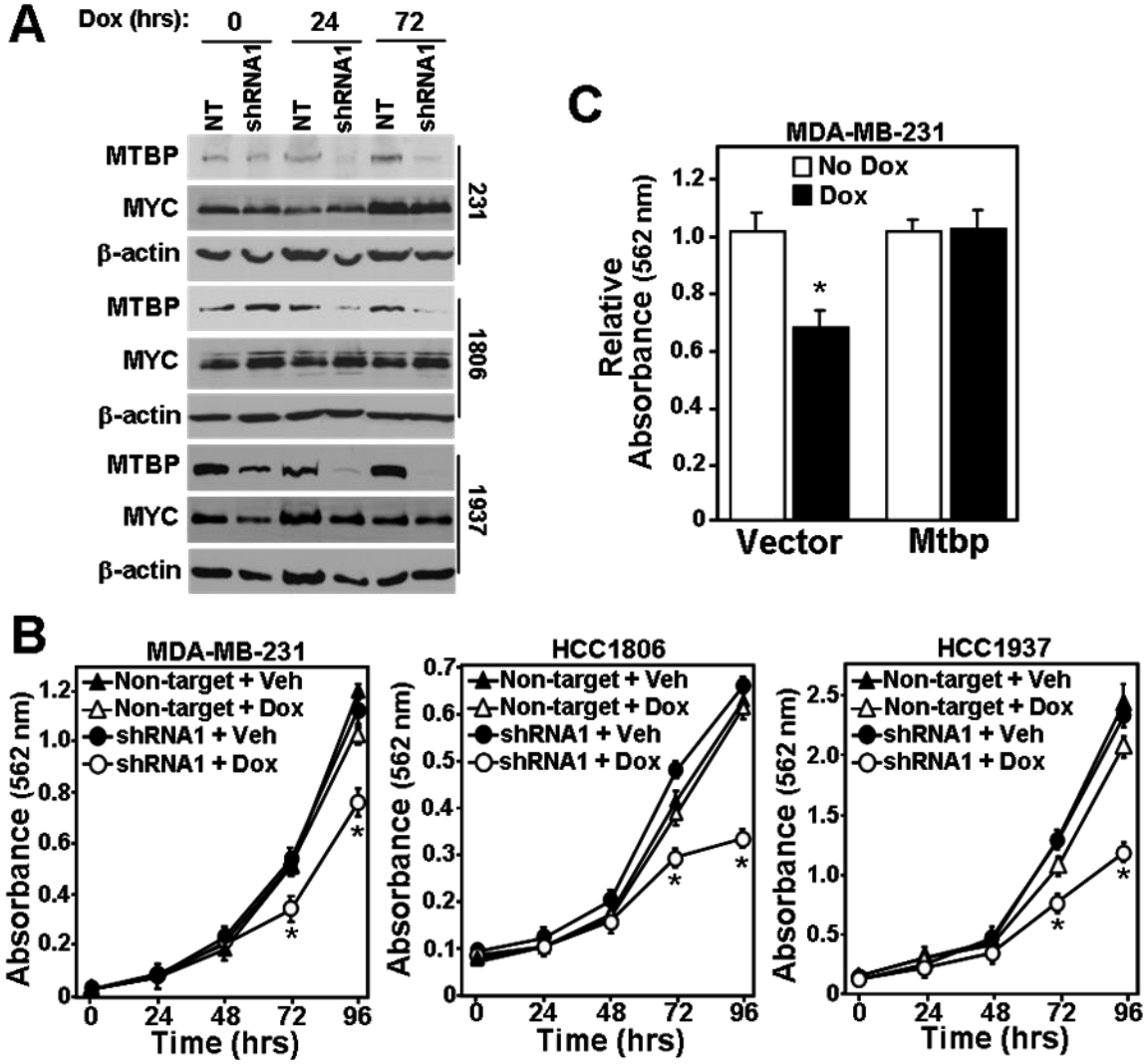

Figure 25. Inducible *MTBP* shRNA inhibits cell expansion. MDA-MB-231, HCC1806, and HCC1937 cells expressing doxycycline (dox) inducible *MTBP* shRNA1 or non-targeting (NT) shRNA control. (A) Western blots of whole cell lysates not exposed to dox or at intervals after the addition of dox. (B) Cells were cultured with dox or vehicle (veh) control and proliferation was monitored by MTT assay at 24 hr intervals (*$p<0.01$ shRNA1 + veh vs. shRNA1 + dox). (C) MTT assay of MDA-MB-231 cells expressing dox inducible *MTBP* shRNA1 transfected with shRNA1-resistant murine Mtbp or empty vector control after 72 hours in the presence of dox or vehicle control (no dox; *$p=0.0027$).

cells treated with vehicle control (Figure 25C). Therefore, disruption of MTBP expression with constitutive or inducible shRNA caused a reduction in the ability of TNBC cells to form colonies in soft agar and to proliferate.

MTBP knockdown induces apoptosis in TNBC cells

To investigate the biological reason *MTBP* knockdown inhibited the expansion of TNBC cells, HCC1806 cells expressing the dox-inducible *MTBP* shRNA1 or the non-targeting shRNA control were cultured with or without dox for 72 hours. At this time, there were visibly fewer adherent and more floating dox-treated *MTBP* shRNA1 expressing cells compared to vehicle control treated cells and non-targeting shRNA expressing cells cultured with or without dox (Figure 26A). Evaluation of the cell cycle revealed no significant difference in G1, S or G2/M distribution of the cells where *MTBP* shRNA1 had been induced with dox compared to vehicle control (Figure 26B). In contrast, dox-treated *MTBP* shRNA1 expressing HCC1806 cells had an increase in the percentage of cells with sub-G1 DNA content (Figure 26B). There was also a significant decrease in viability (Figure 26C) and an increase in Annexin V positive (Figure 26D) *MTBP* shRNA1 containing cells. Moreover, in HCC1806 cells with *MTBP* shRNA1, cleaved Caspase 3 was visible after the addition of dox compared to vehicle control or to those cells with the non-targeting shRNA control (Figure 26E). Thus, shRNA-mediated knockdown of *MTBP* in TNBC cells induced apoptosis, without detectable alterations in phases of the cell cycle.

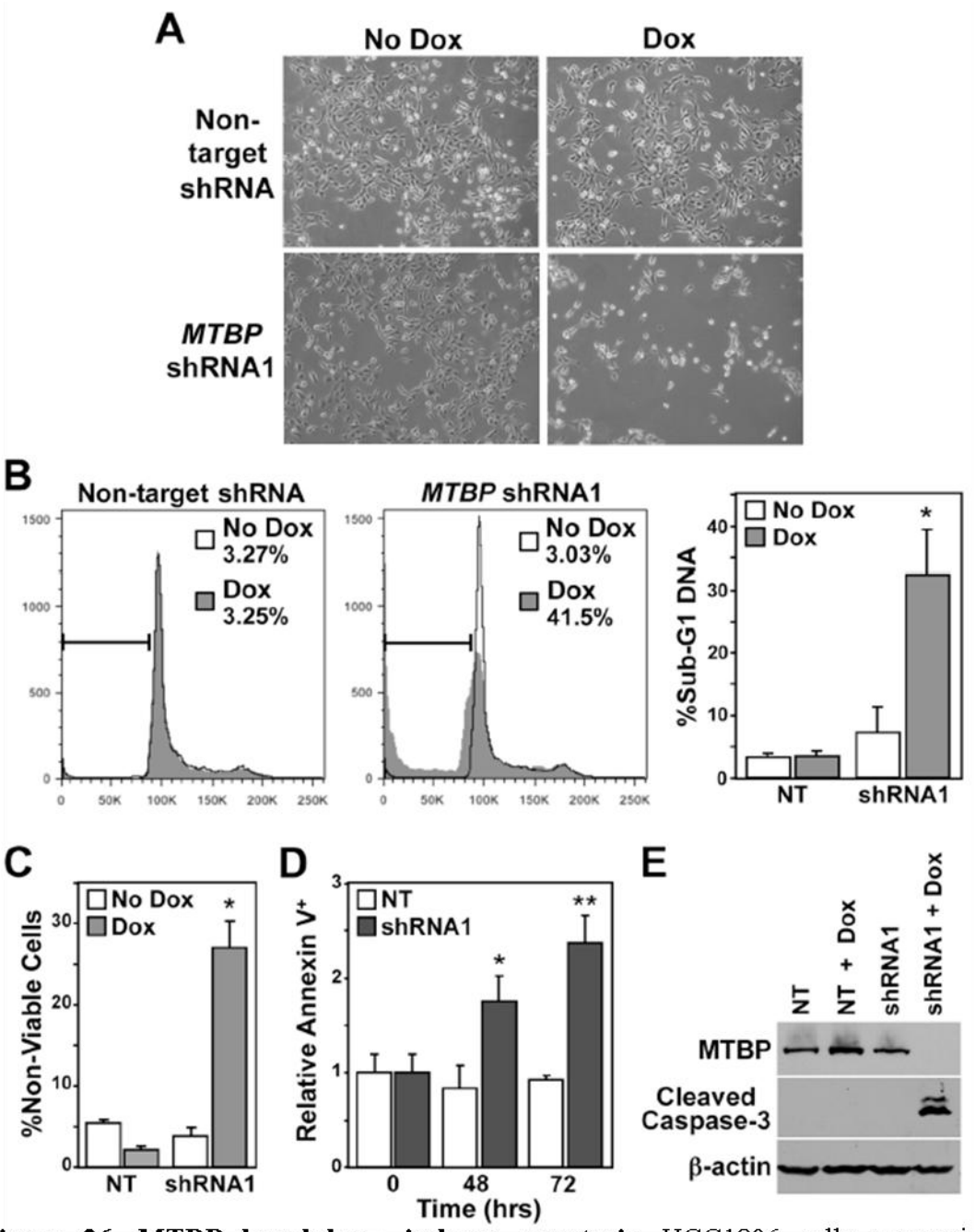

Figure 26. MTBP knockdown induces apoptosis. HCC1806 cells expressing doxycycline (dox) inducible *MTBP* shRNA1 or non-targeting (NT) shRNA incubated with dox or vehicle control (no dox) for 72 hours. (A) Representative light microscopy images. (B) The proportion of cells with sub-G1 DNA content was measured using flow cytometry and propidium iodide. Representative histograms with the percentage of sub-G1 DNA cells indicated (left); mean of data obtained shown on the right (*p=0.0008). (C) Trypan Blue Dye was used to determine cell viability (*p=0.0003). (D) The percentage of Annexin V positive cells relative to samples without dox was measured by flow cytometry (*p=0.034, **p=0.0069). (E) Western blots for the indicated proteins were performed on whole cell lysates.

MTBP loss inhibits TNBC growth, *in vivo*

To evaluate whether *MTBP* knockdown would alter TNBC growth *in vivo*, HCC1806 cells expressing dox-inducible *MTBP* shRNA1 were subcutaneously injected into the flanks of athymic nude mice. Their drinking water was supplemented with or without dox. By day 7, tumors in mice receiving dox to induce *MTBP* shRNA1 showed a statistically significant decrease in volume compared to tumors in control mice not receiving dox (24 mm^3 vs 43 mm^3; $p<0.0001$), and this difference continued to increase through the duration of the experiment (Figure 27A). At the time of sacrifice (day 21), tumors that expressed *MTBP* shRNA1 due to dox exposure were smaller in volume and weighed significantly less than the tumors from control mice (Figure 27A-C). There was a 70% reduction in tumor volume in the mice expressing *MTBP* shRNA1 compared to controls. These tumors showed reduced levels of MTBP protein, verifying *MTBP* shRNA1 expression persisted over the course of the experiment (Figure 27C). MYC protein levels were similar in all tumors (Figure 27C).

We also tested whether established TNBC tumors would be affected by knockdown of *MTBP*. Specifically, HCC1806 cells expressing the dox-inducible *MTBP* shRNA1 were injected into the flanks of nude mice at the same time as cells for the experiment described above and were allowed to grow. After 10 days when the tumors averaged 100-150 mm^3, these mice were given dox to induce *MTBP* shRNA1 expression. Within 72 hours these tumors were significantly smaller than tumors that were not exposed to dox (188 mm^3 vs 350 mm^3; $p=0.0057$); yet, tumors did not completely disappear (Figure 27A). Instead, after the initial decrease in tumor size, a significant reduction in the rate of tumor growth was observed that was analogous to the rate of

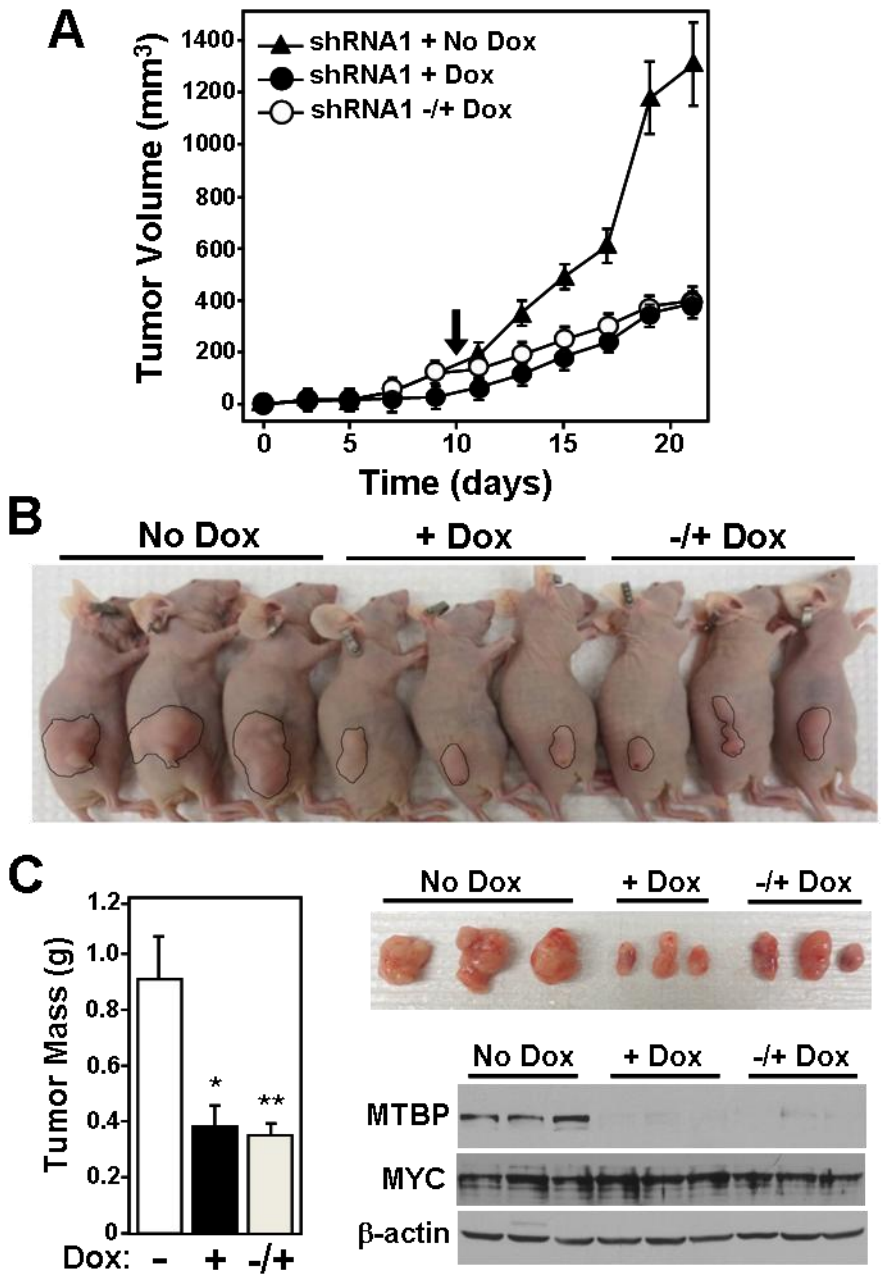

Figure 27. Knockdown of *MTBP* inhibits breast cancer growth *in vivo*. (A) HCC1806 cells were injected subcutaneously into the flanks of nude mice (n=10/group) on day 0. Mice received drinking water with (+Dox) or without dox (No Dox) at day 0, or water with dox starting at day 10 (-/+ Dox; indicated by arrow). Tumor volume was measured at intervals ($p<0.001$ for shRNA1 + No Dox vs. shRNA1 + Dox for days 7-21 and for shRNA1 + No Dox vs. shRNA1 -/+ Dox days 13-21). (B) On day 21, mice were sacrificed and photographed. A representative photo of all three treatment groups is shown with the tumors outlined in black. (C) Tumors were extracted, photographed and weighed (*$p=0.0058$ and **$p=0.0024$ compared to no dox). A representative photograph is shown. Protein lysates from representative tumors were western blotted for indicated proteins.

tumor growth for the mice that received dox on day one. At sacrifice (day 21), the tumors from the mice that received dox to induced *MTBP* shRNA1 beginning at day 10 were smaller and weighed significantly less than the tumors from mice that never received dox (Figure 27B-C). The tumors were similar in size and weight to the tumors from mice that been exposed to dox for the entire experiment. These data collectively indicate that targeting *MTBP*, reducing its expression, significantly limits the growth of TNBC cells *in vivo*, including established TNBC tumors.

Discussion

Studies have linked MTBP to cancer (Agarwal *et al.*, 2013; Carrasco *et al.*, 2006; Iwakuma *et al.*, 2008; Martin *et al.*, 2007; Odvody *et al.*, 2010), but little was known about MTBP in established cancer cells, particularly how it influenced proliferation, cellular survival, and patient outcomes. Here, we show *MTBP* is overexpressed in human breast cancer, and this correlates with significantly decreased patient survival. Notably, among the different breast cancer subtypes, we determined *MTBP* expression was highest in the TNBC subtype, which lacks targeted therapies and is known for being clinically aggressive (Dent *et al.*, 2007; Haffty *et al.*, 2006; Liedtke *et al.*, 2008). Experiments also revealed that reducing *MTBP* expression in human TNBC cell lines with shRNA significantly inhibited cell expansion by inducing apoptosis. The growth inhibitory effects of *MTBP* knockdown in TNBC cells were also observed *in vivo*, in xenografts and importantly, in established TNBC tumors. Therefore, this study identifies MTBP as an important indicator of poor breast cancer patient prognosis and triple negative status as

well as being critical for the growth and survival of TNBC cells. The results of this study support further investigation into MTBP as a novel therapeutic target in TNBC.

This study reveals that MTBP overexpression contributes in a significant way to human breast cancer and increases understanding of MTBP in cancer. Specifically, we previously, reported MTBP is overexpressed in human and murine B cell lymphomas (Odvody *et al.,* 2010). Others have shown the region of the genome encoding *MTBP* is amplified in colorectal cancer and multiple myeloma (Carrasco *et al.*, 2006; Martin *et al.*, 2007). Similarly, evaluation of copy number variation data from The Cancer Genome Atlas (TCGA) indicate *MTBP* is amplified in many types of human cancer (Cerami *et al.*, 2012; Gao *et al.,* 2013; Grieb *et al.*, 2014b). For breast cancer, we determined *MTBP* amplification occurred in 19% of the tumors, and this significantly correlated with decreased patient survival. Since most breast cancer deaths are associated with metastasis, the current analysis suggests *MTBP* overexpression is a potential novel indicator of aggressive breast cancers with increased metastatic potential that are more likely to result in patient death. However, this concept conflicts with experimental data that indicate decreased MTBP expression increases cell migration, invasion, and metastasis (Agarwal *et al.*, 2013; Iwakuma *et al.,* 2008). It is possible that both are correct if, as has been shown for MYC (Liu *et al.*, 2012), MTBP is temporarily downregulated when cancer cells move and is then upregulated after cancer cells seed metastatic sites and begin to proliferate again. However, there is also one report showing that decreased *MTBP* expression in a narrow subset of head and neck cancer correlated with reduced survival (Vlatkovic *et al.*, 2011). Thus, although MTBP could have a tissue-specific or cancer cell mutation-specific function, much of the data suggest that in

multiple hematopoietic and non-hematopoietic human cancers, including breast cancer, MTBP overexpression is selected for and contributes to cancer development and progression. The current study links *MTBP* overexpression in breast cancer to advanced disease and poor patient prognosis.

Data from multiple groups, including our own, suggest MTBP has a critical function in proliferation, and that this significantly contributes to tumor development (Agarwal *et al.*, 2011; Grieb *et al.*, 2014b; Odvody *et al.*, 2010). Specifically, we previously determined that an Mtbp haploinsufficiency suppressed proliferation mediated by the Myc oncogene, significantly inhibiting the ability of Myc to induce B cell lymphoma development (Odvody *et al.*, 2010). Moreover, *MTBP* mRNA and protein expression increased in response to oncogene expression (MYC and E2F1) or growth factor exposure, and *MTBP* was shown to be a transcriptional target of MYC (Odvody *et al.*, 2010). Others have indicated MTBP contributes to cell cycle progression by linking MTBP to DNA replication origins and mitotic progression (Agarwal *et al.*, 2011; Boos *et al.*, 2013). Additionally, we recently determined that elevated levels of MTBP resulted in enhanced cellular proliferation and transformation, *in vitro* and *in vivo* (Grieb *et al.*, 2014b). Here we show *MTBP* is overexpressed in breast cancers and its expression is the highest in the TNBC subtype. TNBCs are reported to have a higher proliferative index when compared to receptor positive high-grade invasive carcinomas (Han *et al.*, 2011). In addition, elevated MYC transcriptional activity, which is correlated with decreased breast cancer patient survival, is linked to increased proliferation in breast cancer, and the TNBC subtype has the highest MYC transcriptional activity (Horiuchi *et al.*, 2012). We recently determined patients with breast cancers that express high levels of both *MYC* and

MTBP have a worse prognosis than those with just high *MYC* expression (Grieb *et al.*, 2014b), suggesting cooperation between MYC and MTBP overexpression in breast cancer. Therefore, MTBP appears to be a pro-proliferative factor where its overexpression supports the increased proliferative capacity of cancer cells, which is associated with poor patient survival in many human cancers (Brown & Gatter, 2002; de Azambuja *et al.*, 2007; Han *et al.*, 2011; Hofmockel *et al.*, 1995; Isola *et al.*, 1990; King *et al.*, 1998; Martin *et al.*, 2004).

Deletion of *Mtbp* is embryonic lethal in mice, indicating it has an indispensable function in development (Iwakuma *et al.*, 2008). Here we show that MTBP also has an essential function in breast cancer cell survival. Knockdown of *MTBP* in human TNBC cell lines using constitutive or inducible *MTBP* shRNA severely limited TNBC growth, *in vitro* and *in vivo*, due to induction of apoptosis. This is similar to the oncogene addiction mediated cell death observed when an oncogene, such as MYC, is knocked down in cancer cells (Dang, 2012; Meyer & Penn, 2008; Soucek *et al.*, 2008), suggesting TNBC cancer cells can become reliant on MTBP for their continued growth and survival. Support of this concept was reported when knockdown of *MTBP* with siRNA in HeLa cells delayed DNA replication or mitosis and also led to cell death (Agarwal *et al.*, 2011; Boos *et al.*, 2013). Although we did not detect cell cycle changes with *MTBP* knockdown in TNBC cells, as was reported for HeLa cells (Agarwal *et al.*, 2011; Boos *et al.*, 2013), we did observe significant apoptosis resulting in reduced TNBC cell survival. The apoptosis that occurred upon *MTBP* knockdown resulted in a reduction in the ability of the TNBC cells to grow in soft agar and *in vivo* in mice. Notably, utilizing inducible *MTBP* shRNA revealed that, *in vivo*, established breast cancers rely on MTBP for their

continued growth. Therefore, our data identify MTBP as a protein TNBC cells need to survive and grow. Thus, MTBP is a potential novel therapeutic target in TNBC warranting further investigation. Moreover, additional studies are needed to examine whether MTBP has a similar essential function in other human malignancies, including receptor positive subtypes of breast cancers.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Dissertation Summary and Significance

The oncogenic transcription factor Myc represents one of the most important and far reaching oncogenes in cancer biology (Meyer & Penn, 2008; Tansey, 2014). It is overexpressed in the majority of human cancers and controls several critical cellular processes involved in tumorigenesis, tumor growth and cancer cell maintenance. Yet, even though Myc has been extensively studied for over three decades and reducing its activity has long been known to have therapeutic benefit in a wide variety of cancers (Felsher, 2010), we still lack a complete understanding of what factors regulate Myc activity and how this is orchestrated. Through this thesis project, my collaborators and I discovered Mtbp regulates Myc activity (Grieb *et al.*, 2014b). Prior to this work, little was known about the function of Mtbp as well as its relevance in established cancers. However, the current data indicate Mtbp is a pro-proliferative oncogenic protein commonly overexpressed in cancer. It associates with Myc at Myc-regulated gene promoters by binding the Myc transcriptional cofactors Tip48 and Tip49. Here, Mtbp functions as a cofactor that enhances Myc transcriptional activity, promoting downstream Myc-mediated cellular proliferation and transformation. Furthermore, using human triple negative breast cancer cells known to have high MYC activity, I observed MTBP expression is necessary for the maintenance of cancer cell growth and survival, providing evidence that MTBP represents a novel therapeutic target in cancer (Grieb *et al.*, 2014a).

As discussed below, these data significantly expand our understanding of Mtbp function, Myc biology and human cancer.

Proliferation and Cellular Transformation

Previous investigations on the function of Mtbp mainly examined the effects of decreased Mtbp expression. These reports by Dr. Eischen's lab and others revealed that a deficiency in Mtbp reduces proliferation and inhibits tumor development (Agarwal *et al.*, 2011; Boos *et al.*, 2013; Odvody *et al.*, 2010). Here, Mtbp knockdown similarly reduced the proliferation of murine fibroblasts. These observations though merely link Mtbp to proliferation, only providing sufficient evidence to suggest Mtbp is a limiting factor for cellular proliferation. In contrast, experiments described in chapter 2 utilize a novel approach and demonstrate increased Mtbp expression actually promotes proliferation. The data indicate this observation likely represents a highly conserved function of Mtbp, as it was observed in several cell types from multiple different species. Furthermore, Mtbp expression increased cellular transformation, *in vitro* and *in vivo*, albeit only moderately. These novel functions of Mtbp support and expand upon experiments with decreased Mtbp expression. They also offer new insight into previous observations. For example, Mtbp expression was shown to increase when cells were forced to proliferate by serum treatment or expression of oncogenes (Odvody *et al.*, 2010). *Mtbp* was also shown to be a Myc transcriptional activation target (Odvody *et al.*, 2010). In the context of the current analysis, these observations demonstrate Mtbp expression is likely increasing in response to growth signals in order to actively promote proliferation, not merely to support proliferation as previously suggested.

The novel pro-proliferative and oncogenic functions of Mtbp are relevant in cancer. Specifically, Mtbp more prominently increases proliferation when growth factors are limiting, suggesting that sustained or dysregulated Mtbp expression could serve as a mechanism for cells to survive and proliferate with limited external growth signals, hallmarks of cancer cells (Hanahan & Weinberg, 2000). This concept is supported by my discovery that MTBP mRNA and protein are overexpressed in diverse human cancers, as well as TCGA data that show *MTBP* is commonly amplified. These observations greatly expand upon previous reports that *MTBP* is amplified in multiple myeloma and colorectal cancer (Carrasco *et al.*, 2006; Martin *et al.*, 2007), but also suggest the oncogenic function of MTBP is conserved across tissue and cancer types. Moreover, in breast cancer patient data, *MTBP* mRNA was highest in triple negative breast cancers, which have increased proliferative capacity compared to other clinically aggressive breast cancers (Han *et al.*, 2011). Collectively, these data suggest elevated *MTBP* expression is selected for during cancer development to support the proliferation of cancer cells.

When the functional experiments and cancer patient data are examined together, it is apparent MTBP is oncogenic. However, Mtbp appears to be a relatively poor driver of cellular transformation, even though *MTBP* mRNA is widely overexpressed in human cancers. This suggests MTBP may cooperate with other oncogenes. Regardless, the data support the fundamental, yet significant, discovery that Mtbp expression promotes proliferation and cellular transformation *in vitro* and *in vivo*. These novel functions of MTBP appear to be highly conserved and are widely supported by available cancer patient data. They also provide unique insight into previous reports linking Mtbp expression to proliferation (Agarwal *et al.*, 2011; Boos *et al.*, 2013; Odvody *et al.*, 2010).

MTBP associates with Tip48, Tip49 and MYC

Taken together, previous reports (Agarwal *et al.*, 2011; Boos *et al.*, 2013; Odvody *et al.*, 2010) and the current analyses clearly indicate Mtbp regulates proliferation and transformation. However, at the beginning of this dissertation project, it was unknown how Mtbp regulated these cellular processes and few insights were available. Dr. Eischen's lab reported that decreased Mtbp expression inhibited pre-B cell proliferation and lymphoma development driven by a *Myc* transgene, likely due to decreased Myc transcriptional activity; however, *Mtbp* heterozygosity did not affect proliferation of wild-type pre-B cells (Odvody *et al.*, 2010). Thus, these results suggested Mtbp may be a limiting factor of Myc pro-proliferative transcriptional activity, but it was unclear why this occurred.

Significant insight was gained through experiments in chapter 2 that characterize a novel indirect association between Mtbp and Myc through Tip48 and Tip49. Mtbp has a similar nuclear distribution to Tip48 and, as suggested by *in vitro* binding assays, binds directly to both Tip48 and Tip49. While these observations are supported by a report that Mtbp possesses a nuclear localization sequence (NLS) in its C-terminus (Agarwal *et al.*, 2011), they opened the possibility that perhaps Mtbp could be in a complex with Myc. Tip48 and Tip49 are known to regulate Myc activity and were shown to bind Myc through the conserved MBII domain (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Li *et al.*, 2010; Si *et al.*, 2010; Wood *et al.*, 2000). Even so, Tip48 and Tip49 have been identified in other transcriptional complexes (Grigoletto *et al.*, 2011). Thus, it is significant that the association between Mtbp and Myc, which *in vitro* binding assays suggest is indirect, is

detectable with endogenous levels of protein in multiple cell types. Moreover, the C-terminal region of Mtbp and the MBII domain of Myc that mediate the association between Mtbp and Myc also mediate binding of Mtbp and Myc with Tip48/Tip49 (Wood *et al.*, 2000). The data also demonstrate the C-terminal mutant of Mtbp has a dominant negative effect on Myc activity (discussed below) offering functional support for the observed interactions. Collectively, these results indicate Mtbp forms a complex whereby it associates indirectly with Myc through its direct association with Tip48 and Tip49, all mediated by the C-terminus of Mtbp and MBII of Myc.

In addition to characterizing these protein-protein interactions, members of the Eischen lab and I detected Mtbp bound to chromatin. Specifically, Mtbp is in complex with Myc at gene promoters both transcriptionally activated and repressed by Myc. The C-terminal mutant of Mtbp also associates with these regions of DNA, further supporting the model described above. These results are consistent with reports demonstrating Tip48 and Tip49 associate with Myc regulated promoters in multiple cell types and species (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Frank *et al.*, 2003; Li *et al.*, 2010; Si *et al.*, 2010). Therefore, Mtbp, through its C-terminus, forms a novel transcriptional complex at DNA with the previously described Tip48-Tip49-Myc complex (Wood *et al.*, 2000).

The MBII domain of Myc is critical for its function as a transcription factor and oncogene (Meyer & Penn, 2008; Tansey, 2014). Some proteins that associate with Myc through MBII have been shown to regulate Myc stability, while others like Tip48 and Tip49 influence Myc activity (Wood *et al.*, 2000). These factors commonly affect known functions of Myc such as proliferation and transformation, which previous work and data

presented in chapter 2 indicate are regulated by Mtbp (Odvody *et al.*, 2010). However, since this dissertation project and pervious work in the Eischen lab indicate Mtbp levels do not alter Myc expression (Odvody *et al.*, 2010), and since Tip48 and Tip49 were previously shown to be critical for Myc function (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Li *et al.*, 2010; Si *et al.*, 2010; Wood *et al.*, 2000), the discovery that Mtbp associates with the Tip48-Tip49-Myc complex favors a model where Mtbp regulates Myc activity instead of stability. It also offers significant mechanistic insight into preliminary observations made by the Eischen lab using *Mtbp* heterozygous mice prior to the start of this dissertation project (Odvody *et al.*, 2010).

Even though this dissertation project focuses on the significant relationship between Mtbp and Myc, Tip48 and Tip49 have been shown to associate with and regulate the activity of other notable transcription factors, such as β-catenin/TCF and E2F1 (Dugan *et al.*, 2002; Feng *et al.*, 2003; Taubert *et al.*, 2004). Thus, independent of its association with Myc, the discovery that Mtbp directly binds Tip48 and Tip49 also opens the distinct possibility that Mtbp may have a broader function in regulating transcription (discussed in Future Directions).

MTBP cooperates with MYC

Myc transcriptionally regulates expression of an estimated 10 to 20% of genes within the genome, controlling many downstream cellular functions (Figure 2; Dang *et al.*, 2006; Meyer & Penn, 2008; O'Connell *et al.*, 2003; Patel *et al.*, 2004; Zeller *et al.*, 2006). Tip48 and Tip49, which the current analysis describes as novel binding partners of Mtbp, are known to regulate both Myc-mediated transcriptional activation and

repression (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Gallant, 2007; Li *et al.*, 2010; Si *et al.*, 2010). Moreover, a previous report by the Eischen lab as well as data from chapter 2 indicate reduced Mtbp expression inhibits the ability of Myc to transcriptionally activate its target genes needed to mediate downstream proliferation (Odvody *et al.*, 2010), suggesting Mtbp is a limiting factor for Myc-mediated transcription. In contrast, the majority of chapter 2 evaluates the effect of increased Mtbp expression on Myc transcriptional activity as well as subsequent proliferation and cellular transformation. An Eischen lab member and I made the significant discovery that Mtbp enhances the ability of Myc to transcriptionally activate its pro-proliferative target genes. Thus, these data suggest Mtbp is a novel transcriptional cofactor that positively regulates Myc activity.

Consistent with classifying Mtbp as a positive regulator of Myc, the data in chapter 2 further demonstrate Mtbp cooperates with Myc to promote proliferation and cellular transformation *in vitro* and *in vivo*, while inhibiting Myc-induced apoptosis. This suggests that in addition to enhancing Myc-mediated transcription, Mtbp may alter Myc activity to favor proliferation over apoptosis, a theory supported by several reports demonstrating that inhibition of Myc-mediated apoptosis is a critical function of factors that cooperate with Myc to promote cellular transformation (Meyer & Penn, 2008). Regardless, these functions of Mtbp appear to be conserved as Myc-induced proliferation and transformation are enhanced by Mtbp in multiple species and tissue types. Moreover, the data are consistent with previous observations in the Eischen lab that a deficiency in Mtbp inhibited Myc-driven pre-B cell proliferation and B cell lymphoma development in a mouse model (Odvody *et al.*, 2010).

The conclusion that Mtbp positively regulates Myc activity is further supported by available cancer patient data. *MTBP* and *MYC* are frequently selected for co-amplification in human cancers, even though they are 7.2 megabases apart on chromosome 8q24 (Boyd *et al.*, 2000b), suggesting this confers a previously unrecognized benefit to cancer cells. In support of this concept, MTBP expression among breast cancers was highest in the triple negative subtype, which has increased MYC transcriptional activity (Horiuchi *et al.*, 2012) and a higher proliferative capacity than clinically aggressive ER+ and HER2+ breast cancers (Han *et al.*, 2011). Furthermore, breast cancer patients whose tumors have elevated expression of both *MYC* and *MTBP* exhibit worse survival than patients with tumors expressing high levels of *MYC* and low levels of *MTBP*, a trend also observed in colon and lung cancers. These observations suggest MTBP is making MYC a more effective oncogenic transcription factor, increasing cancer cell proliferation and contributing to an aggressive disease phenotype.

While increased MYC expression is often linked to poor patient prognosis (Iwakawa *et al.*, 2011; Kluk *et al.*, 2012; Pagnano *et al.*, 2001; Spencer & Groudine, 1991; Varley *et al.*, 1987), this is not always the case (Green *et al.*, 2012; Xu *et al.*, 2010). In these instances, MYC mRNA and even protein expression may not reflect the ability of MYC to enact its transcriptional program, due to the complex multilayered regulation of MYC expression and activity. Instead, evaluating the expression of over 350 MYC target genes to determine MYC transcriptional activity has been shown to accurately predict cancer patient outcomes (Horiuchi *et al.*, 2012). However, the current analyses suggest simply examining MYC and MTBP expression may serve as a surrogate

measure of MYC transcriptional activity to predict patient outcomes. These observations combined with work in chapter 3 examining the effects of shRNA-mediated knockdown of MTBP in TNBC cells suggest MTBP may represent a novel biomarker and therapeutic target in cancer, particularly those with dysregulated MYC (discussed in next section).

Collectively, the data demonstrate Mtbp enhances Myc activity. Thus, I propose a model whereby at Myc-regulated gene promoters the C-terminus of Mtbp mediates its binding to Tip48/Tip49 that are bound to the MBII domain of Myc, allowing Mtbp to promote Myc-mediated transcriptional activity, proliferation, transformation and tumor growth (Figure 28). This model is further supported by experiments conducted with mutants of Mtbp. Specifically, the C-terminal mutant of Mtbp that associated with Myc, Tip48 and Myc-bound gene promoters, appeared to function as a dominant negative inhibitor of Myc, reducing Myc-mediated transcriptional activation of pro-proliferative target genes, proliferation and transformation, as well as the proliferation of human breast cancer cells possessing high MYC transcriptional activity (Horiuchi *et al.*, 2012). This provides functional evidence that the C-terminus of Mtbp mediates its association with the Tip48-Tip49-Myc complex and highlights the critical importance of Mtbp in Myc function. Moreover, several reports demonstrate Tip48 and Tip49 are important for Myc transcriptional activation and repression, as well as downstream proliferation and transformation (Bellosta *et al.*, 2005; Etard *et al.*, 2005; Grigoletto *et al.*, 2011; Li *et al.*, 2010; Si *et al.*, 2010; Wood *et al.*, 2000).

While the proposed model provides significant mechanistic insight into previous observations regarding the impact of Mtbp expression on Myc-mediated processes

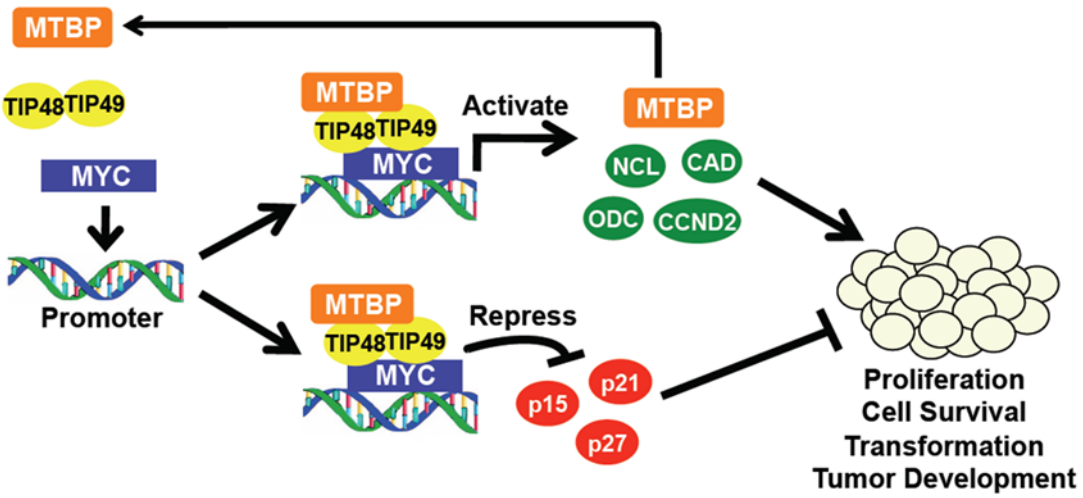

Figure 28. Working model of MTBP function. MTBP binds TIP48 and TIP49, which bind MYC at promoters of genes MYC transcriptionally activates that are pro-proliferative (CAD, CCDN2, NCL, and ODC). MTBP also associates with MYC at promoters of genes that MYC transcriptionally represses that are anti-proliferative (p15, p21, p27). The consequences of both of these actions are that MYC induces proliferation, transformation, and tumor development. MYC also transcriptionally upregulates MTBP (Odvody *et al.*, 2010), causing more MTBP to be available to regulate MYC-mediated transcription (feed-forward regulatory loop). When there are elevated levels of MTBP, as is the case in many human cancers (Tables 1 and 2), MYC has increased proliferative and transforming abilities and cells are less susceptible to MYC-induced apoptosis.

(Odvody *et al.*, 2010), it also provides an alternate explanation for other functions attributed to Mtbp that were reported prior to this thesis work becoming public. Recently, Mtbp was detected in other DNA binding complexes and implicated in the regulation of DNA replication origin firing (Boos *et al.*, 2013) as well as proper segregation of chromosomes during mitosis (Agarwal *et al.*, 2011). In addition to transcription, Myc directly regulates DNA replication by associating with the pre-replication complex and facilitating DNA replication initiation (Cole & Cowling, 2008; Dominguez-Sola *et al.*, 2007; Srinivasan *et al.*, 2013). Moreover, Myc was recently shown to maintain its association with chromatin during mitosis (Yang *et al.*, 2013), offering an alternate explanation as to why Mtbp is detectable at chromatin in prometaphase (Agarwal *et al.*, 2011). Dysregulation of Myc results in an abnormal G2/M checkpoint and subsequent errors in chromosome segregation (Felsher *et al.*, 2000; Li & Dang, 1999; Menssen *et al.*, 2007; Sheen & Dickson, 2002), similar to those observed by Agarwal *et al.* following modulation of Mtbp expression (Agarwal *et al.*, 2011). More specifically, downmodulation of Mtbp was shown to mimic a decrease in mitotic arrest-deficient 2 (Mad2) expression (Agarwal *et al.*, 2011), which has been shown to be a transcriptional activation target of Myc (Menssen *et al.*, 2007). Thus, while Mtbp may regulate cellular processes described by others (Agarwal *et al.*, 2011; Boos *et al.*, 2013), the data available for Myc combined with the proposed model highlight the distinct possibility that functions previously attributed to Mtbp may be through its regulation of Myc activity.

In addition to providing alternative mechanistic explanations, the discovery that Mtbp regulates Myc activity is also significant, because Myc regulates a wide variety of

cellular processes (Figure 2). Even though this dissertation largely focuses on the ability of Mtbp to regulate proliferation and transformation, it is possible that as a novel regulator of Myc transcriptional activity, Mtbp may influence other cellular processes downstream of Myc.

In short, my work describes a novel complex formed by Mtbp and Myc, allowing Mtbp to regulate Myc transcriptional activity and downstream cellular processes. This is a fundamental discovery and clearly has potentially far reaching significance. In this same vein, I had the opportunity to collaborate with Dr. Tansey who has recently discovered a novel interaction between Myc and WDR5, a protein involved in recruiting DNA methyltransferases to gene promoters (Smith *et al.*, 2011). They have shown that this complex, which lacks methyltransferase activity, helps recruit Myc to its target genes. A mutation in Myc that disrupts its interaction with WDR5 (MYC^{WBM}; data not shown) significantly inhibits the ability of Myc to promote cellular transformation *in vivo* (Figure 29). Thus, even though Myc has been extensively studied over the last three decades, there is still a great deal to learn about the regulation of Myc and how this impacts both normal and cancer cell biology.

MTBP in cancer

While *Mtbp* heterozygosity was shown to significantly delay Myc-driven B cell lymphoma development in a mouse model (Odvody *et al.*, 2010), little was known about the expression or function of MTBP in cancer at the start of this dissertation project. Available *in silico* data suggested *MTBP* is amplified in multiple myeloma and colorectal

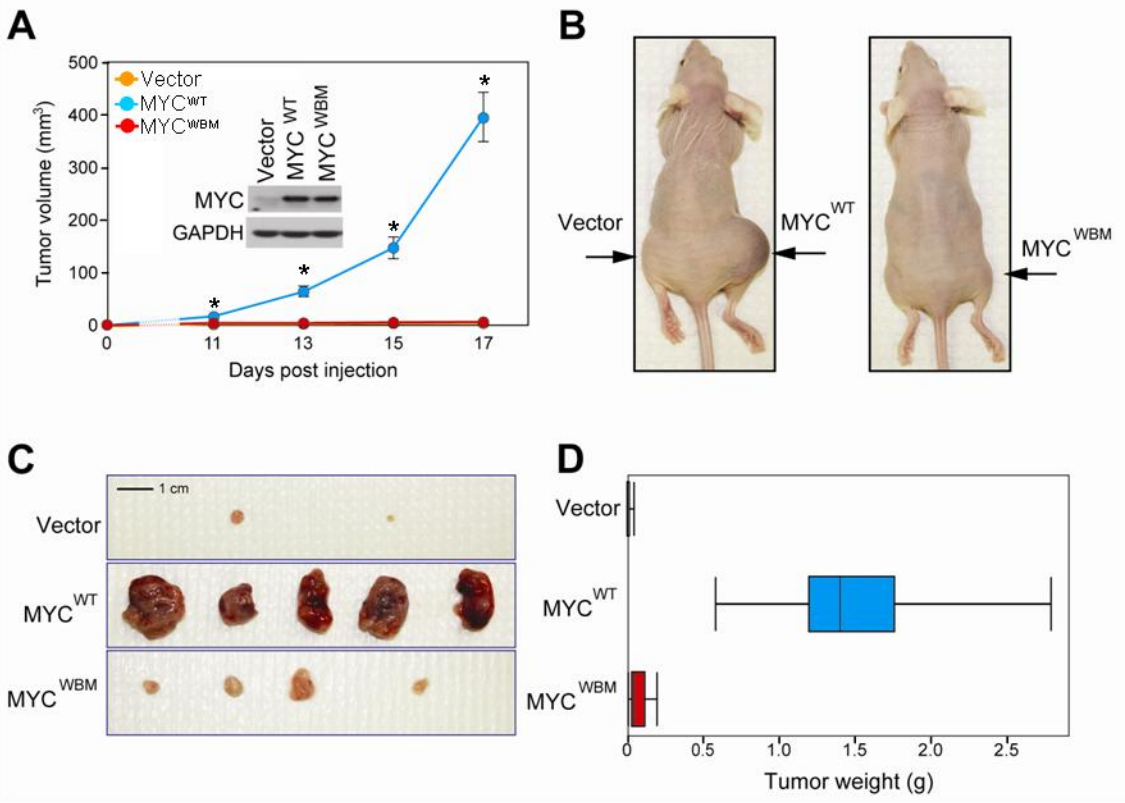

Figure 29. WDR5 is necessary for MYC-mediated transformation, *in vivo*. (A) NIH3T3 cells expressing wild-type MYC (MYC^{WT}), a MYC with a WDR5 binding mutation (WBM) unable to associate with WDR5 (MYC^{WBM}), or a vector control were western blotted (inset) and injected into the flanks of athymic nude mice at day zero. Tumor volume was calculated at intervals using measurements taken with electronic calipers (n = 10 flanks per sample, *$p < 0.001$ by Student's t-test for MYC^{WT} vs. vector or MYC^{WBM}). (B-D) At 18 days following injection, mice were sacrificed and representative photographs were taken (B). Tumors were excised, photographed (C) and weighed (D). (I would like to acknowledge Dr. Lance Thompson who I assisted in these experiments and Dr. William Tansey for sharing these data.)

cancer (Carrasco *et al.*, 2006; Martin *et al.*, 2007). My collaborators and I greatly expanded these initial observations and determined *MTBP* is commonly overexpressed and amplified in a diverse array of human cancers. Notably, elevated *MTBP* expression is associated with decreased breast cancer patient survival and is highest in the clinically aggressive TNBC subtype. In contrast, loss of MTBP expression in a narrow subset of SCCHN correlated with decreased patient survival (Vlatkovic *et al.*, 2011). This could be explained by reports that Mtbp functions as a metastasis suppressor (Agarwal *et al.*, 2013; Iwakuma *et al.*, 2008), corresponding with a recent report that cancer cells downregulate proliferation and Myc in favor of movement (Liu *et al.*, 2012). Yet, metastasis is a major cause of cancer-related death, particularly in breast cancer where data indicate MTBP negatively impacts patient survival (Mehlen & Puisieux, 2006). It remains unclear if these data represent tissue- or cell-specific functions of MTBP, but the data from chapter 2 indicate the function of MTBP is highly conserved and the majority of cancers appear to have increased *MTBP* expression. Thus, MTBP is a protein with oncogenic function that is overexpressed to varying degrees in several forms of human cancer, negatively impacting cancer patient survival.

In addition to being overexpressed in human cancers, MTBP appears to be important for cancer cell growth and survival. While previous investigations examined the effects of decreased Mtbp expression on cancer development (Odvody *et al.*, 2010), I examined the effects of reducing MTBP expression in cancer cells as described in chapter 3. In TNBC cells, shRNA-mediated knockdown of MTBP reduced cellular expansion by inducing apoptosis without affecting MYC expression, *in vitro* and *in vivo*. Notably, MTBP knockdown did not increase the proportion of cells in late S or G2/M phases of

the cell cycle, as was reported in HeLa cells (Agarwal *et al.*, 2011; Boos *et al.*, 2013). However, a decrease in proliferation was observed when the C-terminal Mtbp mutant that functioned as a dominant negative inhibitor of Myc was expressed in TNBC cells, which have increased MYC transcriptional activity (Horiuchi *et al.*, 2012). This suggests MTBP knockdown may have reduced MYC activity and triggered apoptosis associated with oncogene addiction, commonly observed when MYC activity or expression is reduced in cancer cells (Felsher, 2010).

The discovery that MTBP is necessary for cancer cell maintenance and survival highlights the possibility of using MTBP as a therapeutic target. This approach is supported by experiments from chapter 3 that demonstrate reducing MTBP expression significantly slows the growth of established tumors, *in vivo*. Even though the majority of data is specific to TNBC (MTBP knockdown also reduces proliferation in H1299 human lung carcinoma cells), a therapy against MTBP could have broad reaching effectiveness, as MYC activity is regulated by MTBP. MYC is overexpressed and/or dysregulated in the majority of human caners (Vita & Henriksson, 2006). Reducing MYC activity or expression has been shown to have a therapeutic benefit in several different forms of cancer (Felsher, 2010; Giuriato *et al.*, 2006; Jain *et al.*, 2002; Pelengaris *et al.*, 2002; Shachaf *et al.*, 2008; Shachaf *et al.*, 2004; Wu *et al.*, 2007), even in cancers not driven by MYC itself (Soucek *et al.*, 2008; Soucek *et al.*, 2013). Moreover, the observation that the C-terminal MTBP mutant acts as a dominant negative inhibitor of MYC and inhibits TNBC cell proliferation, suggests that disrupting the function and/or formation of the MTBP-TIP48-TIP49-MYC complex may be feasible and effective.

Future Directions

My dissertation work has significantly advanced our understanding of Mtbp function and its role in cancer. The model that Mtbp is pro-proliferative and a novel regulator of Myc activity provides a possible mechanistic explanation for many functions attributed to Mtbp by the Eischen lab and others (Agarwal *et al.*, 2011; Boos *et al.*, 2013; Odvody *et al.*, 2010). However, the data within this dissertation also raise several important questions that are discussed below and will serve as a foundation for many future investigations.

Given the discovery Mtbp is a novel regulator of Myc, it is now increasingly important to understand how Mtbp itself is regulated. Previous work from the Eischen lab demonstrates *Mtbp* is a Myc transcriptional activation target that is responsive to growth signals (Odvody *et al.*, 2010), but it is unknown how or even if Mtbp is post-translationally regulated. Available data suggest this is probable. Western blots of Mtbp with a polyclonal antibody (Figure 4A for example) show multiple bands that likely represent Mtbp, suggesting post-translational modification. As discussed in the introduction, post-translational modifications to Myc alter its activity and stability (Hann, 2006; Tansey, 2014). Like Myc, Mtbp appears to have a relatively short half-life on the order of a few hours (Odvody *et al.*, 2010). If Mtbp is post-translationally modified, identifying these modifications and examining their impact on Mtbp stability may offer insight into how Mtbp expression is regulated and potentially reveal novel mechanisms to explain its dysregulation in cancer. Modification could also affect the ability of Mtbp to

associate with the Tip48-Tip49-Myc complex, impacting its ability to regulate Myc. Moreover, the current analysis suggests Mtbp is chromatin bound and exclusively nuclear, but others report observing low levels of Mtbp in the cytoplasm (Agarwal *et al.*, 2013; Vlatkovic *et al.*, 2011). If this is true, do modifications of Mtbp affect its localization, offering an additional layer of regulation? If this line of research is pursued, *Mtbp* expression constructs with point mutations of affected residues would be interesting experimental tools to probe the significance of individual sites, particularly when used to reconstitute Mtbp expression in cells from *Mtbp* conditional knockout mice (discussed below).

In addition to how Mtbp itself is regulated, it is unclear at this time precisely how mechanistically Mtbp regulates Myc activity, although available data provide some insight. Tip48 and Tip49 have been associated with processes and protein complexes that epigenetically modify DNA, allowing for transcriptional activation and repression (Grigoletto *et al.*, 2011). For example, Tip48 and Tip49 are in complex with the histone acetyl transferase Tip60 and are necessary for its function, enabling modulating of Myc-transcriptional activity (Frank *et al.*, 2003; Jha *et al.*, 2008). Moreover, Tip48 and Tip49 were implicated in histone variant switching, opening DNA at promoters and transcriptional start sites (Choi *et al.*, 2009). It is possible Mtbp facilitates these activities through functioning as a scaffold protein for Tip48 and Tip49, which the data suggest both bind Mtbp directly. Alternatively, Mtbp could harbor an unknown enzymatic function (even though extensive evaluation of the Mtbp amino acid sequence throughout this dissertation project did not reveal any functional domains) or the ability to recruit additional cofactors with its N-terminal domain, considering the C-terminal domain of

Mtbp retained the ability to associate with Tip48, Myc and chromatin but inhibited Myc activity. At this time, each theory represents a plausible model that requires additional experimental investigation to validate or refute.

As regulatory mechanisms surrounding Mtbp and Myc become illuminated, it may be interesting to determine how Mtbp and its novel regulatory factors control other cellular functions that require tight regulation of Myc activity, such as stem cell biology and cellular metabolism (Dang, 2013; Eilers & Eisenman, 2008). Identification of these factors will also make it possible to evaluate the effects of minutely modulating Myc activity. Given the prominence of Myc as an oncogene, this may only modify the risk of cancer development; however, it has the potential to impact the aging process itself. While Myc has not been directly linked to aging, it controls many cellular processes that are classically associated with aging, such as replicative stress induced senescence, apoptosis, stem cell maintenance, genomic instability and metabolism (Dang, 2013; Kuningas *et al.*, 2008; Meyer & Penn, 2008; Tansey, 2014; Vijg & Campisi, 2008). For example, Myc increases reactive oxygen species (ROS) formation (Dang *et al.*, 2005; Vafa *et al.*, 2002), and decreases tissue repair over time (Factor *et al.*, 1997; Waikel *et al.*, 2001). Myc has also been shown to regulate or be regulated by prominent factors in the aging field, such as mTOR and SIRT1 (Laplante & Sabatini, 2012; Yuan *et al.*, 2009). Data we have recently generated demonstrate $Mtbp^{+/-}$ mice exhibit increased longevity compared to $Mtbp^{+/+}$ littermate matched control mice (Figure 30). Based on the model supported by this thesis project, the *Mtbp* heterozygous mice may have systemically reduced Myc transcriptional activity, supporting a novel role for Myc in organismal

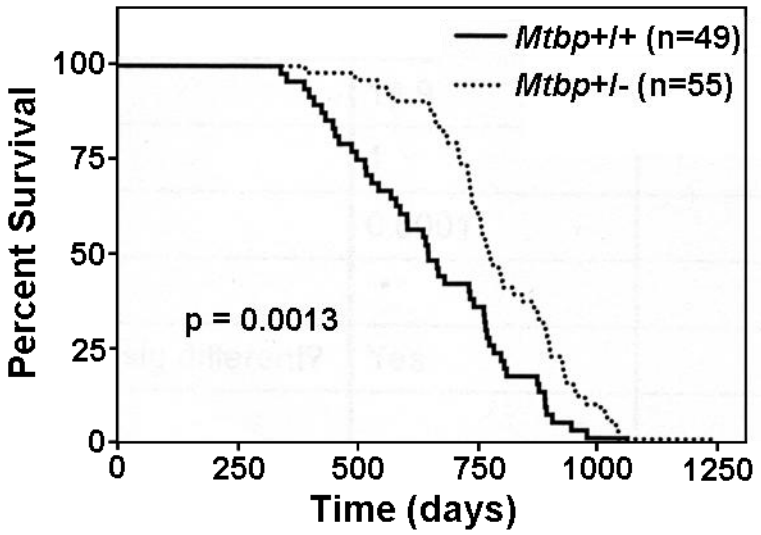

Figure 30. ***Mtbp*** **heterozygous mice exhibit increased longevity.** Kaplan Meier survival curve of littermate-matched $Mtbp^{+/+}$ and $Mtbp^{+/-}$ mice. Number of mice indicated by n. Log rank test used to calculate statistical significance.

aging. Thus, in addition to cancer, proteins that regulate Myc activity, like Mtbp, may become important focuses of research in the field of aging and lifespan extension.

As knowledge of how Mtbp regulates Myc expands, it will be important to address whether functions previously attributed to Mtbp, such as DNA replication origin firing and mitosis (Agarwal *et al.*, 2011; Boos *et al.*, 2013) occur through Myc (see discussion in previous section). Yet, even though this dissertation project focused on Mtbp regulation of Myc, I cannot rule out Myc-independent functions of Mtbp or even that Mtbp could have indirect effects on Myc function. The novel Mtbp binding proteins Tip48 and Tip49 have been described in several transcriptional complexes (Grigoletto *et al.*, 2011). For example, they regulate β-catenin/TCF transcriptional activity, which is often dysregulated in colon cancer and is known to positively regulate Myc function (Feng *et al.*, 2003). Tables 1 and 2 indicate *MTBP* is overexpressed and amplified in human colorectal cancer. Here, in addition to directly enhancing MYC activity, which is important in colon cancer (He *et al.*, 1998), MTBP could also be potentiating β-cantenin/TCF transcriptional output, increasing downstream oncogenic signaling and possibly indirectly enhancing Myc function. A similar case for indirect effects of Mtbp on Myc could also be made with the transcription factor E2F1, which is also regulated by Tip48/Tip49 and serves as a positive regulator of Myc (Dugan *et al.*, 2002).

In addition to binding transcription factors, Tip48/Tip49 have been linked to multiple forms of epigenetic modification (Grigoletto *et al.*, 2011), raising the possibility that Mtbp may have a more global effect on transcriptional output, indirectly affecting the transcriptional activity of several growth promoting transcription factors under a variety of conditions. Large scale RNA or ChIP-Seq experiments may help reveal these and

other potential Myc-independent functions of Mtbp. For example, Mtbp association with the genome may overlap with near complete complementarity to Myc, or be localized at additional sequences, perhaps corresponding to binding of other transcription factors or epigenetic modifications.

Regardless of how MTBP regulates MYC or whether it has MYC-independent functions, the data presented within this dissertation provide evidence that MTBP may represent a viable therapeutic target in cancer. This was only directly tested extensively in TNBC cells, which have elevated MYC transcriptional activity (Horiuchi *et al.*, 2012). To help determine if MTBP represents a therapeutic target in other cancers driven by MYC, I propose evaluating the role of Mtbp in cancer cell maintenance and survival using a genetically defined Myc-driven cancer model, such as Eμ-*myc* mice that overexpress Myc in B cells and develop B cell lymphoma (Adams *et al.*, 1985). I have already laid the groundwork for these important studies with newly generated *Mtbp* conditional knockout mice received from a collaborator. Specifically, I isolated, cultured and cryopreserved Eμ-*myc* lymphomas from $Mtbp^{+/+}$, $Mtbp^{+/-}$, $Mtbp^{+/flox}$, $Mtbp^{flox/flox}$, and $Mtbp^{flox/-}$ mice. In future experiments, a regulatable Cre can be used to evaluate the effects of Mtbp loss both *in vitro* and *in vivo*. Such a study combined with this thesis work would offer strong evidence that MTBP represents a drug target in cancers reliant on MYC.

In addition to lymphoma, future studies should continue to investigate MTBP in breast cancer. While chapter 3 focuses on TNBC, the data demonstrate *MTBP* expression is elevated in ER+ and HER2+ breast cancers. Further analysis indicates ER+ breast cancer patients whose cancers have high *MTBP* expression exhibit decreased survival

compared to those with low *MTBP* expression (Figure 31). Beyond breast cancer, data presented in chapter 2 demonstrate MTBP expression is dysregulated in a wide variety of cancers. Given these observations and studies demonstrating the therapeutic effect of decreasing MYC expression or activity in cancer (Felsher, 2010), MTBP warrants investigation as a broad therapeutic target in human cancer. Thus, it remains possible that MTBP is an as yet untapped broadly applicable therapeutic target, as has been proposed for MYC (Sodir & Evan, 2011; Tansey, 2014).

Should future efforts reveal MTBP is a viable therapeutic target in cancer, it will warrant design of small molecules or peptides that mimic the effect of MTBP knockdown observed here. Data presented in chapter 2 using the C-terminal mutant of Mtbp suggest this might be possible. Although not directly tested, the C-terminal Mtbp mutant could be inhibiting Myc activity by displacing endogenous Mtbp from the Mtbp-Tip48-Tip49-Myc complex, inhibiting proper formation/function of the complex at chromatin, or reducing the pool of unidentified cofactors also bound by the C-terminal mutant of Mtbp. Hypothetically, if the domain of Mtbp that mediates its association with Tip48/Tip49 can be narrowed to a small peptide sequence, generating a crystal structure of this interaction might reveal a binding pocket receptive to small molecule disruption. Future investigation into the transcriptional and post-translational regulation of MTBP might also reveal novel mechanisms to disrupt MTBP. Moreover, other reports demonstrate that decreasing the expression or interfering with the ATPase function of Tip48/Tip49 in hepatocellular carcinoma decreases tumor growth and viability (Haurie *et al.*, 2009; Menard *et al.*, 2010). Thus, alternative investigation into compounds that disrupt the ATPase function of Tip48/Tip49 as well as the association between Myc and

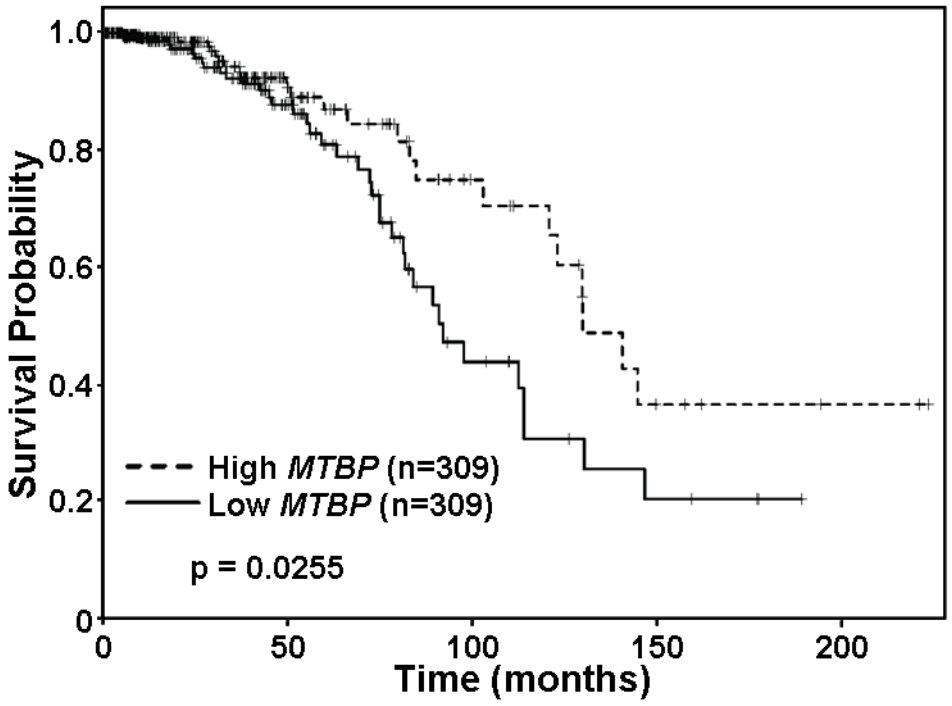

Figure 31. MTBP regulates survival of ER+ breast cancer patients. Kaplan Meier survival curve from TCGA RNA-Seq mRNA expression data for ER+ breast cancers. Patient number indicated by n. Log rank test used to calculate statistical significance.

Tip48/Tip49, or the assembly of Tip48/Tip49 hexamers or dodecomers might also have therapeutic value. While ultimately the efficacy of these hypothetical compounds in treating cancer cannot be predicted, a significant concern would be toxicity. Myc, Tip48, Tip49 and Mtbp are all embryonic lethal (Bereshchenko *et al.*, 2012; Davis *et al.*, 1993; Etard *et al.*, 2005; Iwakuma *et al.*, 2008). Myc also regulates the growth and maintenance of many normal tissues throughout the body (Korinek *et al.*, 1998; Murphy *et al.*, 2005). Fortunately, mouse models examining the effects of whole body Myc inhibition suggest these treatments are tolerable, although care should be taken in extending these conclusions to human patients (Soucek *et al.*, 2008) and to Tip48/Tip49, which have functions beyond Myc (Grigoletto *et al.*, 2011).

In conclusion, this dissertation research has significantly advanced our understanding of MTBP. The observations described within this dissertation demonstrate MTBP is a novel pro-proliferative and oncogenic transcriptional cofactor of MYC, one of the most important oncogenes in cancer. MTBP is also overexpressed in many human cancers and likely represents a novel therapeutic target with potentially far reaching effects. These highly significant discoveries should spur future research that perhaps one day will improve cancer patient care.

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