Regulation of p53, p21, ARF, BIM, and BAX by the Transcription Factor Trip-Br1

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K. Virginia Lehmkuhl-Dakhwe

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by

K. VIRGINIA LEHMKUHL-DAKHWE

has been approved for
the Department of Biological Sciences
and the College of Arts and Sciences by

__________________________
Donald L. Holzschu
Associate Professor of Biological Sciences

__________________________
Benjamin M. Ogles
Dean, College of Arts and Sciences
ABSTRACT

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Regulation of p53, p21, ARF, BIM, and BAX by the Transcription Factor Trip-Br1

(150 pp.)

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Cyclins and cyclin-binding proteins interact to contribute to cell cycle regulation and aberrations in their functions/associations can contribute to inappropriate cell proliferation and cancer. Retroviral and cellular cyclins physically interact with the transcription factor Trip-Br1 as demonstrated in yeast-2-hybrid analyses. In-vitro, Trip-Br1 associates with cellular cyclins A and B and the walleye dermal sarcoma virus retroviral cyclin (WDSV rv-cyclin). These interactions are potentially important since cyclins and Trip-Br1 have been implicated in cell-cycle regulation. The WDSV rv-cyclin, rescues cyclin-deficient yeast from growth arrest and is a potent stimulator of eukaryotic cell proliferation. Trip-Br1 is implicated in the G1-S transition of the cell cycle where Trip-Br1 antagonizes the inhibitory function of p16INK4a bound to cyclin D/cdk4 allowing phosphorylation of the retinoblastoma protein (RB, p105). Trip-Br1 also functions in association with the transcription factors E2F-1 and DP-1 to transactivate the b-myb promoter in reporter assays. Given the functional characteristics of Trip-Br1 in the context of the b-myb promoter, I anticipate that Trip-Br1 may also regulate other E2F-responsive genes, implicating it in regulation of a diverse assemblage of genes involved in both pro-cell-cycle and pro-apoptotic functions. I hypothesize that
the results of overexpression of Trip-Br1 on cell proliferation is dependent on the cellular context, namely the complement of intact E2F-responsive genes.

Here, I investigate the differential regulation of pro-apoptotic genes in normal and in cells overexpressing Trip-Br1 and Trip-Br1 mutants. I show that overexpression of Trip-Br1 transactivates an artificial promoter containing 6 E2F-responsive elements and Trip-Br1 is potentially implicated in the regulation of p21 and p14 ARF promoter constructs and this regulation is dependent on an intact Trip-Br1 heptad repeat protein domain. I show that ectopic expression of Trip-Br1 increases BAX protein and mRNA levels from the BAX native locus, possibly contributing to mitochondrial-dependent cell death. Additionally, I investigate the effects of overexpression of cyclins A and B on the Trip-Br1-mediated transactivation of p53, 6xE2F, p21, ARF, and BIM promoters and investigate the significance of a putative Trip-Br1 cyclin B/cdk1 phosphorylation site on the transactivation of these promoters. Finally, I report results of comparative microarray analyses performed in cells expressing normal and ectopic Trip-Br1.

Approved: _____________________________________________________________

Donald L. Holzschu

Associate Professor of Biological Sciences
For My Parents
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<tr>
<td>ARF</td>
<td>Alternative Reading Frame</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia Telangiectasia Mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia Telangiectasia and Rad3 related</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast Cancer</td>
</tr>
<tr>
<td>BRG</td>
<td>Brahma-related gene</td>
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<tr>
<td>BRM</td>
<td>Brahma</td>
</tr>
<tr>
<td>CBP</td>
<td>Cyclic adenosine monophosphate (cAMP) response element-Cinding Protein (CREB)-binding protein</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell division cycle</td>
</tr>
<tr>
<td>cdk</td>
<td>Cyclin Dependent Kinase</td>
</tr>
<tr>
<td>Chk</td>
<td>Checkpoint kinase</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin-dependent Kinase Inhibitor</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-Transferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HDM2/MDM2</td>
<td>Human Double Minute 2/Mouse Double Minute 2</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatin Protein 1</td>
</tr>
<tr>
<td>KRAB</td>
<td>Krueppel-associated box</td>
</tr>
<tr>
<td>KRIP</td>
<td>KRAB-A interacting protein 1</td>
</tr>
<tr>
<td>Mcm</td>
<td>Minichromosomal maintenance protein</td>
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<tr>
<td>MPF</td>
<td>Maturation Promoting Factor</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PHD</td>
<td>Plant Homology Domain</td>
</tr>
<tr>
<td>RB</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RBL</td>
<td>Retinoblastoma-Like</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<td>SEI</td>
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<tr>
<td>Sp</td>
<td>Specificity Protein</td>
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<tr>
<td>Tc</td>
<td>Tetracycline</td>
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<tr>
<td>THD</td>
<td>Trip Homology Domain</td>
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<tr>
<td>Thr</td>
<td>Threonine</td>
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<td>Trip-Br1</td>
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<td>Tyr</td>
<td>Tyrosine</td>
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<td>WDSV</td>
<td>Walleye Dermal Sarcoma Virus</td>
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1. GENERAL INTRODUCTION

1.1 Overview of Dissertation

Novel cyclin-protein interactions are potentially relevant to normal cell division and uncontrolled cell proliferation/cancer. Using the yeast 2-hybrid system, it has been demonstrated that retroviral and cellular cyclins interact with the transcription factor Trip-Br1. Trip-Br1 can promote cell division by overriding the inhibitory function of the cyclin dependent kinase inhibitor (CDKI), p16INK4a, bound to the cell cycle promoting complex cyclin D/cyclin-dependent kinase 4 (cdk4), allowing hyperphosphorylation of the retinoblastoma protein (RB) (Li et al., 2004b; Sugimoto et al., 1999).

Hyperphosphorylation of RB contributes to E2F-1- and DP-1-mediated transactivation of a series of genes required for cell cycle progression. The function of Trip-Br1 in this context was demonstrated using the E2F-responsive b-myb promoter in luciferase reporter assays (Hsu et al., 2001). Given the functional characteristics of Trip-Br1 in the context of the b-myb and other E2F-responsive promoters, I hypothesize that Trip-Br1 may regulate a diverse assemblage of genes involved in both pro-cell-cycle and pro-apoptotic functions and that the outcome of ectopic expression of Trip-Br1 on cell proliferation is dependent on the complement of intact E2F-responsive genes. For example, in NIH3T3 fibroblasts that are null for the tumor suppressor p19ARF, it has been shown that overexpression of Trip-Br1 gives rise to inappropriate cell proliferation. I hypothesize that in NIH3T3 fibroblasts, the general stimulation of E2F-responsive promoters by Trip-Br1 promotes cell proliferation (Tang et al. 2005). However, in cells where the ARF locus is intact, for example in HeLa cells, if transcription of the ARF
locus is stimulated by Trip-Br1, then ARF-induced signaling cascades would result in cell growth arrest (Watanabe-Fukunaga et al., 2005).

To contribute to the understanding of the functional characteristics of Trip-Br1, I have conducted a mutational analysis of Trip-Br1 and associated proteins. I have generated a series of Trip-Br1 mutant proteins and tested their function in different promoter contexts. I begin reporting my findings with a brief review of the eukaryotic cell cycle with emphases on characterization of cyclins and RB- and E2F-family members. I present an overview of the literature pertaining to Trip-Br family proteins with emphasis on Trip-Br1 and its putative functional domains. I then investigate the differential regulation of pro-apoptotic genes in normal and in cells overexpressing Trip-Br1 and Trip-Br1 mutants. These results will be addressed in the next five sections of this dissertation. In sections 2 and 3, I show that overexpression of Trip-Br1 affects cell proliferation and transactivates an artificial promoter containing 6 E2F-responsive elements. This transactivation is dependent on the heptad hydrophobic domains in Trip-Br1 and DP-1. I also present data that support published results demonstrating that Trip-Br1 is potentially implicated in the transcriptional regulation of the cell cycle regulator p21 and the translational modulation of the tumor suppressor p53. In section 4, I show that Trip-Br1 transactivates a p14ARF promoter construct and this regulation is also dependent on intact heptad repeat protein domains in Trip-Br1 and DP-1. Additionally, I show that ectopic expression of Trip-Br1 increases BAX protein and mRNA from the BAX native locus, possibly contributing to mitochondrial-dependent cell death. In section 5, I investigate the effects of overexpression of cyclins A and B on the Trip-Br1-
mediated transactivation of 6xE2F, p21, ARF, and BIM promoters and characterize the significance of a putative Trip-Br1 cyclin B/cdk1 phosphorylation site on the transactivation of these promoters. Finally, in section 6, I report a summary of results of comparative microarray analyses performed in cells expressing endogenous and ectopic Trip-Br1.

1.2. Eukaryotic cell cycle

The eukaryotic cell cycle is a series of coordinated events where check-point passage results in cell content duplication and mitotic division (Figure 1).

![Figure 1: Regulation of the eukaryotic cell cycle.](image)

Cyclin D (CycD)/cdk4/cdk6 regulate the G1-S transition by phosphorylating RB (retinoblastoma protein, p105) allowing transcription of genes required for DNA replication and cell division. Cyclin E/cdk2 hyperphosphorylates RB. The G2-M transition is regulated by cycB/cdk1. (Adapted from Collins et al. 1997 and Hsu et al. 2001).
The cell cycle is divided into four stages: G1 (gap1), S (DNA synthesis), G2 (gap 2), and M (mitosis). During G1, the cell prepares for DNA synthesis and, during S-phase, has aneuploid DNA contents between 2N and 4N. During G2, the cell increases in volume and prepares for mitosis (nuclear division). Cells in G0 are not actively dividing but have the potential to re-enter the cell cycle. Morphologically, the cell cycle can be divided into interphase (including G1, S, and G2) and mitosis (including prophase, metaphase, anaphase, and telophase) (Schafer 1998).

In association with their cyclin-dependent kinase (cdk) binding partners, cyclins are the primary regulatory molecules of the cell cycle phosphorylation cascades. Cellular cyclins are transiently expressed and degraded at specific times in the cell cycle. Combinations of cyclins/cdks have unique substrates that when phosphorylated, efficiently and specifically regulate cell cycle progression (Collins et al., 1997). Cyclin D, with cyclin E, is the critical regulator of the G1 to S phase transition of the cell cycle (Figure 1, G1). Cyclin A regulates DNA synthesis (Figure 1, S) and cyclin B is critical for entry into mitosis (Figure 1, G2-M) (Collins et al. 1997). Detailed descriptions of the functions of cyclins D, E, A and B in cell cycle regulation will be presented in sections 1.3, 1.4, 1.5 and 1.6.

In addition to cyclins and cdks, there are a number of other proteins that regulate cell cycle progression. Proto-oncogenes, including some cyclins, function to promote cell proliferation whereas tumor suppressors limit cell division by arresting cells in either G1 or G2 or by inducing programmed cell death. Inappropriate bypass of cell cycle check-points, often due to incorrect expression or regulation of cell cycle modulators,
results in abnormal cell proliferation and cancer (Sherr, 1993). Loss of function mutations in tumor suppressors lead to the cancer phenotype (Osborne et al. 2004). For example, the earliest identified tumor suppressor, p53, is mutated in over 50% of lung (Toyooka et al. 2003), colon (Iacopetta 2003), ovarian (Schuijer and Berns 2003), and skin (Giglia-Mari and Sarasin 2003) cancers. A critical function of the p53 tumor suppressor is to arrest cells in G1 in response to DNA damage, allowing time for DNA repair before DNA replication in S-phase (Little et al. 1995). The tumor suppressor p53 has also been implicated in G2 arrest, although its mode of action in this context is less well understood. Detailed discussion of p53 and its downstream targets will be presented in section 2.1.

Proto-oncogenes promote cell division and can contribute to cancer when they are mutated to become aberrantly or constitutively active in the cell (Osborne et al. 2004). For example, the cell cycle regulator cyclin D is a proto-oncogene whose normal function is to promote progression from the G1 to S phase of the cell cycle (see section 1.3 for detailed information). Overexpression of cyclin D is, however, associated with breast and prostate cancers (Aatomaa et al 1999; Bartkova et al. 1994; Drobnjak et al. 2000).

1.3. Cyclins D and E and the eukaryotic cell cycle

Growth factors trigger cells in G1 and G0 to enter the cell cycle. In response to mitogenic stimulation D- (D1, D2, D3) type cyclin expression is up-regulated, initiating the G1-S transition. Various combinations of D-type cyclins are expressed in different cell types (Sherr and Roberts, 2004). The cyclin D-dependent kinases (cdk4 and cdk6) specifically phosphorylate retinoblastoma (RB) family members. Phosphorylation of RB
by cyclin D/cdk4 initiates transcription of E2F-responsive genes and expression of a battery of genes required for initiation and passage of the G1-S transition of the cell cycle (see section 1.4 below) (Trimarchi and Lees, 2002). In late G1 phase, following phosphorylation of RB, cyclin D is rapidly degraded (Sherr and Roberts, 2004).

Cyclin E/cdk2 activity is maximal at the G1- S-phase transition and regulates entry into S-phase (Clurman et al., 1996; Dulic et al., 1992). Once the E2F transcriptional program is initiated, active cyclin E- and A2-dependent cdk2 complexes accumulate and hyperphosphorylate and maintain RB in its hyperphosphorylated form, independent of cyclin D-dependent kinases, and cells progress through mitosis in a mitogen-independent manner (Sherr and Roberts, 2004).

**1.4. E2F- and RB (pocket) family proteins and the eukaryotic cell cycle.**

E2F family members, in association with their RB-family binding partners, regulate a diverse assemblage of genes that are involved in DNA replication and cell cycle progression. There are at least eight members of the E2F-family (see Figure 2), with various functions that, in association with RB-family proteins, regulate the timely expression of E2F-responsive genes (Sherr and Roberts, 2004).
During G1 of the cell cycle, RB family proteins [RB (p105), RBL1 (p107), and RBL2 (p130)] repress transcription from E2F-responsive promoters by binding to E2F-family transcription factors (Trimarchi and Lees, 2002). E2F family members bind to RB via an 18 amino acid sequence within the transactivation domain of E2F family members. E2F-interaction with RB blocks the ability of E2Fs to recruit transcriptional machinery. The E2F-RB complex can interact with chromatin remodeling complexes like histone deacetylases (HDACs), BRG- and BRM-based nucleosome remodeling factors, lysine histone methyltransferase like SUV39H1 and transcription factors such as heterochromatin protein 1 (HP1) (Bannister et al., 2001; Brehm et al., 1998; Flemington et al., 1993; Harbour and Dean, 2000; Helin et al., 1993a; Lachner et al., 2001; Nielsen et al., 2001; Rayman et al., 2002; Strobeck et al., 2000; Vandel et al., 2001). Via these associations, E2F-RB complexes repress gene expression in a two-step process. Firstly,
RB-E2F complexes repress actively transcribed genes by recruiting HDACs to deacetylate lysine 9 of histone H3, then, the SUV39H1-HP1 complex replaces the HDAC, where SUV39H1 methylates H3K9 forming a binding site for the repressor HP1. These associations facilitate modification of adjacent histone tails and may propagate the repressive signals to adjacent loci (Trimarchi and Lees, 2002). When RB is phosphorylated by cyclin D/cdk4 complexes, hyperphosphorylated RB dissociates from E2F-complexes, initiating transcription of genes required for cell cycle progression.

E2F-family transcription factors were originally characterized as activators of genes required for cell cycle progression (DeGregori et al. 1995). Consensus E2F-binding sequences (TTTCCCGCC(A/T)(A/T)(A/T)) are found in the promoters of genes involved in DNA synthesis (dihydrofolate reductase, thymidine kinase, and DNA polymerase-α), in cell cycle-regulation (cyclins A, D, and E), and in cellular proto-oncogenes (c-myc, N-myc, epidermal growth factor receptor, c-myb, and b-myb) (DeGregori et al., 1995; Hara et al., 1993; Henglein et al., 1994; Lam and Watson, 1993; Nevins, 1992; Parisi et al., 2002; Robertson and Jones, 1998; Sala et al., 1994). E2F-1, E2F-2, and E2F-3 are the “Activator E2Fs” and E2F1-3 triple knockout cells are defective for S-phase entry (Du and Pogoriler, 0000; Wu et al., 2001). The activator E2Fs bind almost exclusively to RB (not the related proteins RBL1 or RBL2).

Since the initial characterization of the activator E2Fs, other E2F-family members with diverse functions have been identified. E2F-4 and E2F-5 are “Repressor E2Fs”, associating with all three pocket proteins and binding to E2F-responsive promoters in the G0/G1 phase of the cell cycle. The repressor E2Fs function by recruiting chromatin-
remodelling complexes, histone-and DNA modifying enzymes such HDAC and DNA methyltransferases (Attwooll et al., 2004a). E2F-4 and E2F-5 lack nuclear localization signals and are excluded from the cell nucleus during S-phase. They localize to the nucleus after complex formation with the pocket proteins (Attwooll et al., 2004a). It appears that the repressor complexes RBL2/E2F-4 predominate in cells where E2F targets are repressed, and these are replaced with activator complexes containing E2F-1, E2F-2 and E2F-3 at mid-to-late G1 phase causing induction of E2F-dependent transcription (Takahashi et al., 2000; Wells et al., 2000).

E2F-6 is unique in that it lacks transactivation and RB family member protein binding domains typical of E2Fs 1-5; and E2F6 interacts with Polycomb proteins to repress transcription from some promoters (Attwooll et al., 2004b; Oberley et al., 2003). E2F-7 and E2F-8 also appear to repress specific promoters and harbor a duplication of the E2F DNA-binding domain (Christensen et al., 2005; Maiti et al., 2005). While E2Fs6-8 appear to repress transcription from E2F-responsive promoters, their specific functions in the regulation, if any, of the cell cycle are still to be elucidated.

Recent investigations show that members of the E2F-family are also essential for regulating apoptotic pathways. Ectopic expression of E2F-1, E2F-2 or E2F-3 can activate E2F-regulated tumor-suppressor genes (p14/p19ARF, p73, Apaf-1, and caspases) leading to apoptosis (Bates, 1998; Hiebert et al., 1995; Lin et al., 2001; Powers et al., 2004; Stevaux and Dyson, 2002).
1.5. Cyclin A and DNA synthesis

Cyclin A interacts with both cdks 1 and 2 and is required for initiation of DNA synthesis and entry into mitosis (see Figure 1, S-, G2-, and M-phases) (Bell, 2002). Eukaryotic DNA replication begins with binding of a six-subunit origin recognition complex (ORC) onto the chromatin (Bell, 2002; Joseph A. Bogan, 2000). Proteins Cdc6 and Cdt1 load helicases Mcm 2 to 7 onto the ORC-chromatin complexes to form a pre-replication complex (pre-RC). Pre-RCs are activated when the Mcm10 protein binds (Wohlschlegel et al., 2002). Cyclin A/cdk2 phosphorylation releases Cdc6 and it is replaced by Cdc45 through functions of Cdc7/Dbf4 and cyclin E/cdk2. Cdc45 escorts DNA polymerase-α-DNA primase to the origin and RNA-primed DNA synthesis is initiated (Nguyen et al. 2001). Not only is the timing of DNA replication critical, it is also essential that the genome be replicated once and only once per replication cycle. Cyclin A/cdk1 hyperphosphorylates the ORC and reduces its affinity for chromatin in metaphase, thereby ensuring that genome re-replication does not occur (Li et al., 2004a). Cyclin A is degraded early in mitosis (Sherr and Roberts, 2004).

1.6. Cyclin B and Mitosis

Cyclin B synthesis increases during late S-G2 phase and it interacts with cdk1. Newly formed cyclin B/cdk1 complexes (also called the MPF, maturation-promoting factor) (Doree and Hunt, 2002) are maintained in an inactive state by inhibitory phosphorylation of cdk1 at Thr14 and Tyr 15 by WEE1 and MYT1. In addition, the complex is subject to constant nuclear exclusion through Crm1-mediated nuclear export. At prophase, cyclin B/cdk1 is activated following rapid nuclear accumulation and cdc25
phosphatase mediated dephosphorylation of Thr14 and Tyr 15 on cdk1 (Jin et al., 1998). Cyclin B/cdk1 plays a key role in chromosome separation during anaphase. 

Chromosome separation is initiated by cleavage of cohesin (SCC1) by the protease separase. Cyclin B/cdk1 phosphorylates cohesin to maintain it in an inactive state (Stemmann et al., 2001). Separase is also regulated by the inhibitor, securin, and both securin levels and cdk1/cyclin B kinase activity drop at the beginning of anaphase due to ubiquitin-dependent proteolytic degradation, mediated by the anaphase-promoting complex (Holloway et al., 1993; Murray, 1995; Wheatley et al., 1997).

1.7. Identifying novel cyclin binding partners

In sections 1.2 to 1.6, I have addressed the critical role that cyclins play in regulation of the cell cycle. Novel cyclin binding partners continue to be identified and characterized, contributing to our understanding of both normal cell division and uncontrolled cell proliferation/cancer. Recently, a novel piscine retroviral cyclin was identified (LaPierre et al., 1998a; LaPierre et al., 1999; LaPierre et al., 1998b). WDSV (walleye dermal sarcoma virus) rv-cyclin rescues cyclin-deficient yeast from G1 cell cycle arrest (LaPierre et al., 1998a; LaPierre et al., 1998b) and transgenic mice expressing the WDSV rv-cyclin display severe dermal hyperplastic and dyplastic skin lesions (Lairmore et al., 2000). Additionally, the WDSV rv-cyclin was found to interact with cdk8, the partner of cyclin C (Rovnak et al., 1998). Since there is voluminous data demonstrating that retroviral oncogenes induce cell proliferation by interacting with proteins that interact with cellular proto-oncogenes it seemed reasonable that the WDSV-cyclin, while very divergent from cellular cyclins, would be a good probe for the
identification of novel cyclin binding partners. It was hypothesized that since WDSV rv-cyclin has low sequence homology to cellular cyclins but its tertiary structure is predicted to be conserved with cellular cyclins, interactions based only on conserved 3-dimensional structure would be identified and these interactions would be highly conserved with cellular cyclins (Holzschu, personal communication). Therefore, the WDSV-rv cyclin was used as bait in a mouse genome yeast-2-hybrid screen to search for cellular cyclin-binding partners (Yuan unpublished). Two clones that were obtained in the WDSV yeast-2-hybrid screen have been shown to interact with cellular cyclins, the transcription factors TBX-2 and Trip-Br1 (Yuan unpublished). TBX-2, in association with the WDSV-rv cyclin, and cellular cyclins A and B, transcriptionally repress the tumor suppressor ARF (Kim, personal communication). Trip-Br1 (transcriptional regulator interacting with the PHD-bromodomain) is intriguing in that it contains a potential cyclin A-binding site (Hsu et al., 2001), interacts in-vitro with cellular cyclin A, B, D, and E (our unpublished data) and is expressed during the G1 and S phases of the cell-cycle (Hsu et al., 2001). Moreover, there is evidence that Trip-Br1 is involved in the G1-S transition in the eukaryotic cell cycle (Hsu et al., 2001; Li et al., 2004b; Sugimoto et al., 1999).

1.8. Trip-Br family proteins

Trip-Br1 is the founding member of a family of four proteins that are related by protein sequence. The Trip-Br family members are: Trip-Br1 (SEI-1), Trip-Br2 (SEI-2, Y127), Trip-Br3 (CDC4A, SEI-3, Hepp), and RBT1 (Figure 3) (Calgaro et al., 2002; Cho et al., 2000; Hayashi et al., 2006; Hsu et al., 2001; Watanabe-Fukunaga et al., 2005).
1.9. Trip-Br family members

Trip-Br1 was identified in two independent yeast two-hybrid screens using two different proteins as bait. Hsu et al. 2001 identified mouse Trip-Br1 in a yeast-2 hybrid screen using the PHD bromodomain of the transcription factor KRIP-1 as the bait protein. Human Trip-Br1 (SEI-1-Selected with INK4a-1) was identified in a yeast-two-hybrid screen using the cdk inhibitor p16INK4a as bait (Sugimoto et al., 1999). Trip-Br1 expression parallels that of cyclin A during the eukaryotic cell cycle (Hsu et al., 2001). In mice, Trip-Br1 is expressed at low levels in the brain, thymus, lung, spleen, kidney, colon, and bone marrow (Watanabe-Fukunaga et al., 2005). See subsequent sections for detailed description of Trip-Br1.

Trip-Br2 (Y127) was first identified by Nagase et al. (1995), and putative functional domains have been proposed based on homology to Trip-Br1 (see subsequent sections) (Hsu et al., 2001). Expression profiles of Trip-Br2 in mouse tissues parallel
those of Trip-Br1 (Watanabe-Fukunaga et al., 2005). Trip-Br2 function remains to be
determined, although by analogy to Trip-Br1, it may be involved in cell cycle regulation.

Considerable work has been done to characterize the most recently identified
member of the Trip-Br family, Trip-Br3 (SEI-3, CDC4, Hepp). In mice, Trip-Br3 mRNA
is abundant in the thymus, spleen, and bone marrow and is moderately abundant in the
brain, lung, kidney, and colon (Watanabe-Fukunaga et al., 2005). During embryonic
hematopoiesis, Trip-Br3 expression is repressed in mouse fetal liver hematopoietic stem
cells but is induced in hematopoietic progenitor cells. During adult hematopoiesis, Trip-
Br3 is repressed in bone marrow hematopoietic stem cells but is induced as bone marrow
hematopoietic stem cells differentiate into progenitors. Trip-Br3 is also expressed in
mature lymphoid and myeloid cell types. These studies suggest that Trip-Br3 is involved
in the regulation of hematopoietic stem cell progenitor differentiation (Abdullah et al.,
2001). In humans, Trip-Br3 is transcribed at very low levels in heart, lung, spleen, and
thymus and at a higher level in muscle (Abdullah et al., 2001). Mechanistically, Trip-Br3
is a repressor of E2F-dependent transcriptional activation as demonstrated by Trip-Br3-
mediated suppression of E2F1–3-induced reporter activity. Intriguingly, Trip-Br3 is
transcriptionally regulated by E2F-family genes, suggesting that Trip-Br3 may auto-
regulate its own transcription, and may be regulated by Trip-Br1 (see subsequent
sections). The Trip-Br3 promoter contains an E2F-responsive element near the
transcriptional initiation site that is necessary for the E2F-induced activation of Trip-Br3
transcription. Chromatin immunoprecipitation analyses support these data. Additionally,
expression of small interfering RNA (siRNA) targeted at Trip-Br3 results in increased
rates of cell growth and DNA synthesis, suggesting that Trip-Br3 is a negative regulator of the cell cycle (Hayashi et al., 2006).

A fourth member of the Trip-Br family of proteins is RBT1. RBT1 is most closely related to Trip-Br2 and has been shown to bind to subunit (RPA32) of replication protein A. RBT1 is implicated in DNA replication, repair, and recombination (Cho et al., 2000).

1.10. Trip-Br-family member functional domains

Hsu et al. 2001 described three putative functional domains based on Trip-Br1 and Trip-Br2 sequences and referred to these domains as trip homology domain-1 (THD-1), trip homology domain-2 (THD-2), and trip homology domain-3 (THD-3). THD-1 encompasses a cyclin A binding domain and a heptad repeat domain typical of E2F-family members (see Figure 3). Residues 67-108 of E2F-1 are sufficient for interaction with cyclin A/cdk2 (Krek et al., 1994) and while the putative cyclin binding domain of Trip-Br family proteins do not have the conserved Histidine-X-Leucine (HXL) motif reported to be required to bind a hydrophobic patch in cyclin A (as described in Schulman et al. 1998), Trip-Br proteins are hypothesized to interact with cyclin A. The functional significance of this association is unknown, although by analogy to the regulatory function of E2F-1/cyclinA/cdk2, it may be crucial to the timely expression of E2F-responsive promoters during the cell cycle (see subsequent sections).

The THD-1 hydrophobic heptad repeat was also identified based on homology to the heptad repeat of E2F- and DP-family members (Hsu et al., 2001). While the heptad periodicity of Trip-Br proteins is unique, it has been suggested that Trip-Br1 and Trip-
Br2 can form homodimers and heterodimers. Trip-Br1 is thought to interact with DP-1 through the heptad hydrophobic repeat region, allowing DP-1 to recruit Trip-Br1 to promoter-bound E2F-1 (Hsu et al., 2001). These interactions have not been characterized in detail and warrant thorough investigation. The hydrophobic heptad repeat of Trip-Br family proteins has also been described as a SERTA motif (for SEI-1, RBT1, and TARA). SERTA refers to a 40-amino acid motif that is conserved in Trip-Br-family proteins and in a novel member of the trithorax group (TrxG), TARA, recently identified in *Drosophila*. TARA has been implicated in maintenance/remodeling of chromatin structure to regulate the transcription of the TrxG target genes (Calgaro et al., 2002).

THD-2 encompasses amino acids 76-167 and has no homology to other known proteins and the functional significance of this region remains to be determined (Hsu et al., 2001). THD-3 encompasses amino acids 167-236 of Trip-Br1 (the carboxy terminus) and has homology to regions in other proteins that contain a PHD-bromo interacting domain (identified based on amino acid identity with MDM2). The PHD-bromo interacting domain is thought to facilitate Trip-Br1 interaction with PHD-bromodomain-containing transcription factors like KRIP-1 and p300/CBP (Hsu et al., 2001). Additionally, Trip-Br3 appears to be dependent on the THD-3 domain for suppression of E2F1-regulated promoter activity (Hayashi et al., 2006).

1.11. Characterization of Trip-Br1 and associated proteins

During G1, hypophosphorylated RB is bound to E2F-/DP- complexes, inhibiting transcription of E2F-responsive genes (see Figure 4, G1). Phosphorylation of RB by cyclin D-cdk4/cdk6 results in the release of RB from E2F, allowing transcription of genes
required for DNA replication and cell division and driving cell proliferation (Figure 4, Late G1) (Dyson, 1998). Since the activity of cyclin D-cdk4/cdk6 is fundamental to regulating the G1 check-point, cdk4/6 activity is also regulated, in part by the cyclin-dependent kinase inhibitor (CKI), p16INK4a. P16INK4a binds cdk4 and cdk6, rendering the cyclin D/cdk complex inactive and unable to phosphorylate RB (Figure 4, see initial tertiary complex) (Serrano et al. 1993). Trip-Br1 antagonizes the inhibitory function of p16INK4a bound to cyclin D/cdk4, thereby allowing phosphorylation of RB and release of cells from the G1 checkpoint (Figure 4, step 1). Trip-Br1 does not appear to modulate cyclin D/cdk6 activity (Sugimoto et al., 1999).

Trip-Br1 is also a co-activator of the E2F-1/DP-1 complex. When hyperphosphorylated RB dissociates from E2F-1/DP-1, promoter-bound E2F-1/DP-1 complexes recruit Trip-Br1 through physical association with DP-1 (Figure 4, step 6). DP-1-bound Trip-Br1 may recruit a number of other protein binding partners, e.g. members of the Kruppel-associated box (KRAB)-mediated repressors, KRIP-1 (TIF1B and TIF1a), and the co-activator/adaptor p300/ CBP (Hsu et al., 2001). KRIP-1 transcription factors enhance the activation of the b-myb promoter by the E2F-1/DP-1/Trip-Br1 complex (Hsu et al., 2001). P300/CBP are known to interact with Trip-Br1 in-vitro although their ability to transactivate the b-myb promoter was not investigated. Interestingly, it has been shown that in some cells, the timing of replacement of the repressor complex RBL2/E2F-4 with the “Activator E2Fs” correlates with the dissociation of histone deacetylases (HDACs) from promoters, the subsequent appearance of histone acetyltransferases (HATs) (p300, CBP, P/CAF or Tip60), the
hyperacetylation of histone H3 and histone H4, and the induction of E2F-dependent transcription (Caretti et al., 2003; Ferreira et al., 2001; Rayman et al., 2002; Takahashi et al., 2000; Taubert et al., 2004; Wells et al., 2000). It is tempting to speculate that Trip-Br1 may be involved in the integration of these signals.

Recently, the oncogenic potential of Trip-Br1 was demonstrated in NIH3T3 mouse fibroblasts where ectopic expression of Trip-Br1 gave rise to anchorage-independent growth and tumor formation in nude mice (Tang et al. 2005). Also, Trip-Br1 knock-down cells and knock-out cell lines are impaired for serum-induced cyclin E expression and do not enter S-phase and cellular proliferation, implicating Trip-Br1 in pro-cell proliferation pathways (Sim et al., 2006; Sim et al., 2004). The general function of Trip-Br1 at E2F-responsive promoters was demonstrated in reporter assays using an

**Figure 4:** Trip-Br1 acts at two positions in the G1-S transition. Trip-Br1 antagonizes the inhibitory effects of p16INK4a to promote phosphorylation of RB (1). Trip-Br1 is also recruited to E2F-responsive promoters through interaction with DP-1 (5). Trip-Br1 may then recruit other proteins to the promoter to further regulate the promoter (6). Re-drawn from Hsu et al. 2001.

Recently, the oncogenic potential of Trip-Br1 was demonstrated in NIH3T3 mouse fibroblasts where ectopic expression of Trip-Br1 gave rise to anchorage-independent growth and tumor formation in nude mice (Tang et al. 2005). Also, Trip-Br1 knock-down cells and knock-out cell lines are impaired for serum-induced cyclin E expression and do not enter S-phase and cellular proliferation, implicating Trip-Br1 in pro-cell proliferation pathways (Sim et al., 2006; Sim et al., 2004). The general function of Trip-Br1 at E2F-responsive promoters was demonstrated in reporter assays using an
artificial promoter consisting of 6 E2F-binding elements and the b-myb promoter (Gupta et al., 2003; Hsu et al., 2001). Since E2F-transcription factors are implicated in regulating a diverse assemblage of genes involved in both pro-cell cycle and pro-apoptotic functions, it is appealing to consider that Trip-Br1 may also have wide regulatory role. Intriguingly, Trip-Br1 has also been implicated in cell growth arrest pathways where overexpression of Trip-Br1 in HeLa cells leads to cell growth arrest, evidently through accumulation of the tumor-suppressor p53 (see blow) (Watanabe-Fukunaga et al., 2005). Although Trip-Br family proteins stimulate p53 directed reporter expression in a p53-protein and p53-responsive element-dependent fashion, the precise mechanism by which Trip-Br1 contributes to p53 protein stabilization is unclear. Trip-Br1 does not co-immunoprecipitate with p53, or with components of the p53-regulating proteins, such as MDM2, p19ARF, and E6AP and overexpression of Trip-Br1 leads to p53-independent growth inhibition, suggesting that Trip-Br1 is also involved in p53-independent pro-apoptotic mechanisms. It is hypothesized that p21 is responsible for the reduction in cell viability observed in this context although the specific mechanism of regulation is unknown (Watanabe-Fukunaga et al., 2005).

There is evidence that Trip-Br1 plays a role in the G1-S transition of the cell cycle (Sugimoto et al. 1999; Hsu et al. 2001). Here, I investigate novel functions for Trip-Br1 in cell cycle regulation. I investigate the possibility that Trip-Br1 may be a multi-functional protein that acts to co-ordinate the timely expression of E2F-responsive genes.
1.12. General Objectives

To investigate the function of Trip-Br1 in cell proliferation and transcriptional regulation, I have taken the following approaches:

1. Tested the effects overexpression of Trip-Br1 on cell growth and on p53 and p21 protein and mRNA expression (Section 2).

2. Characterized the functional domains of Trip-Br1 using mutational analyses, protein binding and transcriptional reporter assays (Section 3).

3. Determined whether Trip-Br1 contributes to the transcriptional regulation of the tumor suppressor p14ARF (Section 4).

4. Investigated the significance of Trip-Br1-association with cyclins in transcriptional regulation (Section 5).

5. Performed comparative microarray analyses between cell expressing ectopic and endogenous Trip-Br1 (Section 6).
2. EFFECTS OF OVEREXPRESSION OF TRIP-BR1 ON CELL GROWTH

2.1. Introduction

Trip-Br1 has been implicated in cell cycle regulation, functioning primarily at the G1-S transition. It has recently been demonstrated that overexpression of Trip-Br1 in HeLa derivatives (designated HAM4-8 and PG8 cells) results in reduction in cell viability (Watanabe-Fukunaga et al. 2005). It was initially hypothesized that, in p53 +/+ cells, cell growth arrest occurs through accumulation of the tumor-suppressor p53. This conclusion was based on the following observations: 1. Trip-Br1, in a p53-dependent manner, transactivates a minimal promoter containing 14 tandem repeats of the p53 responsive element. Trip-Br1 does not affect this promoter if a p53 dominant negative is co-overexpressed in these assays, suggesting that the Trip-Br1 effect on the promoter is p53-dependent. 2. Based on immunoblot and realtime-PCR analyses, Trip-Br1 appears to stabilize p53 protein, but does not induce expression of p53 mRNA. That is, overexpression of Trip-Br1 increases p53 protein via a post-transcriptional mechanism leading to up-regulation of the downstream p53 target, p21. Attributing Trip-Br1-induced cell growth arrest to p53-dependent pathways alone is however, an incomplete model since it was also reported that Trip-Br1 induces reduction in cell viability in cells constitutively expressing siRNA against p53. These data suggest that Trip-Br1 may be involved in both p53-dependent and p53-independent cell regulatory pathways and this warrants further investigation (Watanabe-Fukunaga et al. 2005).

The tumor suppressor p53 is a homotetrameric transcription factor that is activated by DNA damage or by inappropriate mitogenic signaling. Its activation and
accumulation can trigger cell-cycle arrest or apoptosis (Quelle et al., 1995; Tao and Levine, 1999). The transcription factor p53 coordinates cellular signals to mediate cell cycle arrest (Pellegata et al., 1996), apoptosis (Shen and White, 2001) and differentiation (Chylicki et al., 2000; Eizenberg et al., 1996). There are several well characterized mechanisms that regulate the activity of p53 involve protein modification and/or localization. The ATM/ATR-Chk1/Chk2 DNA damage pathway activates p53 by phosphorylation leading to disruption of the p53-HDM2/MDM2 association, allowing accumulation of p53 (Chehab et al., 2000). In addition, the tumor suppressor p14ARF indirectly regulates p53 by sequestering HDM2 to the nucleolus, limiting HDM2-mediated degradation of p53, allowing increased p53 activity (see subsequent sections). Additionally, several other proteins (e.g. SUMO-1 (Rodriguez et al., 1999) and Parc (Nikolaev et al., 2003)) modulate p53 activity through post-translational modifications (Luo et al., 2004). While the transcriptional regulation of p53 is less well understood, p53 is known to transcriptionally up-regulate itself via p53-responsive elements in the p53 promoter (Sun et al., 1995; Tuck and Crawford, 1989; Wang and El-Deiry, 2006).

Once activated, p53 induces transcription of many genes involved in cell cycle arrest and apoptosis. Generally, p21 expression is transcriptionally up-regulated in response to p53. p21 is a member of the CIP/KIP family of cyclin-dependent kinase inhibitors (CDKI) that inhibits cyclinE/cdk2 and to a lesser extent cyclinD/cdk4 and is involved in pathways that sustain the G2 checkpoint after DNA damage to arrest cell division (Brugarolas et al., 1999). Paradoxically, p21 also appears to stabilize the interaction between cdk4/cdk6 and cyclin D to promote phosphorylation of RB-family
proteins (LaBaer et al., 1997). It has also been shown that overexpression of p21 can result in G1- (Niculescu et al., 1998) and S-phase arrest (Bunz et al., 1998; El-Deiry et al., 1994; Niculescu et al., 1998). The importance of p21 as a tumor suppressor has been demonstrated by the observation that p21-deficient cells fail to undergo cell cycle arrest in response to p53 activation after DNA damage (Waldman et al., 1995) and p21-deficient mice develop spontaneous tumors at an average age of 16 months (Martin-Caballero et al., 2001).

In addition to p53, there are numerous other factors that transcriptionally regulate p21, including E2F-1/E2F-3, Sp1/Sp3, Smads, Ap2, and BRCA1 and some of these pathways are p53-independent (reviewed in (Gartel et al., 2000; Gartel and Tyner, 1999)). p21 is also regulated post-transcriptionally by modulation of mRNA stability (Gorospe et al., 1998; Shu et al., 2006; Wang et al., 2000), by ubiquitin-dependent and -independent protein degradation (Bao et al., 2002; Coulombe et al., 2004; Lee et al., 2006) and by epigenetic silencing (Chen et al., 2000; Roman-Gomez et al., 2002; Zhu et al., 2003). Along with its role in the DNA damage response, p21 is also implicated in terminal differentiation, replicative senescence, and protection from p53-dependent and -independent apoptosis (Di Cunto et al., 1998; Paramio et al., 2001).

The complexity of the regulation of p21 illustrates that it is a multi-faceted protein that functions to integrate cellular signals and the role of Trip-Br1 in the regulation of p21 warrants further investigation. As alluded to before, Watanabe-Fukunaga et al. 2005 conducted a series of experiments to test whether the apparent Trip-Br1-induced reduction in cell viability is dependent on the p53-p21 pathway (Watanabe-Fukunaga et
al., 2005). Based on reporter assays, it was shown that Trip-Br1 regulates a minimal p53 promoter in a p53-dependent manner. These results have limitations because the reporter constructs that were used in these assays do not contain the p53 promoter, but an artificial construct that harbors tandem p53 binding sites (p53-Luc from Stratagene®). To investigate whether Trip-Br1 transactivates the wild-type p53 promoter in a p53-dependent fashion, I cloned a segment of the p53 promoter and conducted a series of luciferase assays to determine the effects of Trip-Br1 overexpression on p53 transcription. In addition, I determined whether overexpression of Trip-Br1 and its putative binding partners E2F-1 and DP-1 affect the p53 promoter. Using a series of HeLa derivative cell lines, some with inducible expression of Trip-Br1 (designated HAM derivative cell lines, reviewed below), it was also reported that overexpression of Trip-Br1 inhibits cell growth in a p53-independent manner, illustrating the complexity of cell cycle regulation by Trip-Br1 (Watanabe-Fukunaga et al., 2005). I also obtained HAM derivative cell lines and conducted cell growth assays and real-time PCR to confirm results regarding the effects of Trip-Br1 on cell growth reported by Wantanabe-Fuku

2.2 Results

2.2.1 Reporter assays show that Trip-Br1 does not contribute to transcriptional regulation of p53.

Watanabe-Fukunaga et al. (2005) reported that Trip-Br1 transactivates a minimal promoter containing p53 binding elements in tandem (p53-Luc from Stratagene) in a p53-protein-dependent fashion. They did not, however, address the effects of overexpression
of Trip-Br1 with its binding partners E2F-1 and DP-1 in this context. To investigate these observations further, I cloned a segment of the p53 promoter and tested the function of Trip-Br1 and its putative binding partners in this context (Figure 5). This approach has advantages over published accounts since our construct includes 503 bases of the wild-type p53 promoter (designated p53(503)-Luc) (Figure 5A), rather than tandem repeats of p53 responsive elements in an unrelated minimal promoter context. To test the effects of overexpression of Trip-Br1 and its binding partners, E2F-1 and DP-1, on p53(503)-Luc, HeLa cells were transfected with the reporter and combinations of E2F-1, DP-1, Trip-Br1, and p53. My data agree with published results suggesting that Trip-Br1 alone does not affect the p53 promoter (Figure 5B, compare lanes 1 and 2). Additionally, overexpression of Trip-Br1 binding partners, E2F-1 and DP-1, or E2F-1, DP-1, and Trip-Br1 do not affect expression from the p53 promoter (see Figure 5B, compare lanes 1, 2, 3, and 4). Unlike published results, however, Trip-Br1 did not stimulate p53 directed reporter expression in a p53-protein dependent fashion. Overexpression of p53 transactivates the promoter by 15-fold and addition of Trip-Br1, E2F-1/DP1, or E2F-1/DP-1/Trip-Br1 does not affect the transactivation of the p53 promoter by p53 (Figure 5B, compare lanes 1, 5, 6, 7, and 8).
Figure 5: Effects of overexpression of Trip-Br1 and binding partners E2F-1 and DP-1 on transactivation of the p53 promoter.  

A. Sequence of p53(503)-Luc used in these assays. Residues required for p53-transactivation of the promoter are indicated by * (Tuck and Crawford 1989; Wang and El-Deiry 2006). B. p53 overexpression transactivates the p53 promoter construct whereas Trip-Br1 and binding partners do not affect p53(503)-Luc. Results are average of two experiments +standard deviation.
The discrepancy between my results and published reports could be due to either the differences in promoter contexts and/or the amount of Trip-Br1 plasmid transfected in each study. Published studies reported p53-dependent transactivation of 14 tandem repeats of the p53 responsive elements by overexpression of Trip-Br1 from 800 ng of plasmid (Watanabe-Fukunaga et al. 2005). My experiments were performed using 503 bases of the wild-type p53 promoter with transfection of 200 ng of Trip-Br1.

2.2.2. Ectopic expression of Trip-Br1 contributes to reduced cell viability in HAM3 derivatives.

To further address the effects of Trip-Br1 on cell growth and on the p53-p21 pathway, Watanabe-Fukunaga et al. 2005 constructed a series of HeLa and U2OS derivatives that express ectopic Trip-Br1 in a doxycycline (DOX, tetracycline derivative)-inducible manner. I was able to collaborate with Watanabe-Fukunaga et al. and obtained these cell lines to pursue further investigations. Watanabe-Fukunaga et al. constructed the HAM derivatives as follows: HeLa and U2OS derivatives expressing inducible Trip-Br1 were constructed using the tetracycline (Tc)-controlled transactivation (rtTA) system for generating cells that express a gene of interest in a DOX-inducible manner. The parental HeLa derivatives (HAM3 cells) were constructed by introducing the pArtTAuro plasmid into HeLa cells (Table 1 lists HAM derivatives described here). To establish cell lines that express Flag-Trip-Br1 in a DOX-inducible manner, HAM3 cells were co-transfected with hygromycin-resistant (pMiw-Hph) and Flag-Trip-Br1 driven by a promoter with a tetracycline responsive element. Hygromycin-resistant clones expressing DOX-inducible Trip-Br1 were selected and a clone expressing
particularly high Trip-Br1 when induced was designated HAM4-8. To determine whether the apparent reduction in cell viability is attributable to a p53-dependent apoptotic pathway, a p53 knockdown cell line was established by introducing p53-directed siRNA into HAM4-8 to generate the PG8 cell line. A scrambled siRNA was introduced into HAM5-1 cells to generate a cell line designated “vector” cells. U2OS derivatives U5-1 and U4-5 were constructed using similar techniques to those used to construct HAM5-1 and HAM4-8 cell lines (Table 1).
Table 1: List of HeLa and U2OS derivative cell lines used to characterize the effects of overexpression of Trip-Br1 on cell growth and the p53-p21 pathway (Watanabe-Fukunaga et al., 2005). HAM3 cells exposed to DOX express endogenous Trip-Br1 and p53. HAM4-8 cells exposed to DOX express ectopic Trip-Br1 and endogenous p53. HAM5-1 cells exposed to DOX express endogenous Trip-Br1 and p53. PG8 cells are HAM4-8 derivatives constitutively expressing p53-specific siRNA. PG8 cells exposed to DOX express ectopic Trip-Br1 and reduced p53 expression. Vector cells harbor a scrambled siRNA and express endogenous Trip-Br1 and p53. U2OS derivatives are also listed. * indicates wild-type expression normal for HeLa cells. # indicates wild-type expression normal for U2OS cells.

<table>
<thead>
<tr>
<th>HeLa derivative</th>
<th>Trip-Br1 expression with DOX induction.</th>
<th>p53 status</th>
<th>ARF status</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAM3</td>
<td>-wild-type*</td>
<td>-wild-type*</td>
<td>-wild-type*</td>
</tr>
<tr>
<td>HAM4-8</td>
<td>-Ectopic -Harboring integrated TRE-Trip-Br1</td>
<td>-wild-type*</td>
<td>-wild-type*</td>
</tr>
<tr>
<td>HAM5-1</td>
<td>-wild-type* -Harboring integrated empty TRE</td>
<td>-wild-type*</td>
<td>-wild-type*</td>
</tr>
<tr>
<td>PG8</td>
<td>-Ectopic -HAM4-8 is parental cell line.</td>
<td>-Reduced -Constitutive expression of siRNA against p53.</td>
<td>-wild-type*</td>
</tr>
<tr>
<td>Vector</td>
<td>-wild-type* -HAM5-1 is parental cell line.</td>
<td>-wild-type*</td>
<td>-wild-type*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>U2OS derivative</th>
<th>Trip-Br1 expression with DOX induction.</th>
<th>p53 status</th>
<th>ARF status</th>
</tr>
</thead>
<tbody>
<tr>
<td>U4-5</td>
<td>-Ectopic -Harboring integrated TRE-Trip-Br1</td>
<td>-wild-type#</td>
<td>-loss of function</td>
</tr>
<tr>
<td>U5-1</td>
<td>-wild-type# -Harboring integrated empty TRE</td>
<td>-wild-type#</td>
<td>-loss of function</td>
</tr>
</tbody>
</table>
To further investigate the effects of Trip-Br1 on cell growth, I performed a series of assays comparing the number of viable cells over time in cells ectopically expressing Trip-Br1 (HAM4-8 and PG8 cells) to cells expressing endogenous levels (HAM5-1 and vector cells). Approximately 1x10^5 HAM4-8, PG8, HAM5-1, and “vector” cells were seeded in 12-well plates, and either mock-induced or induced with 0.5 ug/ml DOX. After 96 hours in culture, the number of cell colonies formed following treatment, in each cell line, was recorded. The number of colonies formed in cell lines that overexpress Trip-Br1 in response to DOX (HAM4-8 (p53 normal) and PG8 (p53 knock-down)) is lower than in cell lines where Trip-Br1 is not induced in response to DOX (HAM5-1 and “vector”). Figure 6A shows a representative example of crystal violet staining of colonies formed in each treatment, in each cell line. Figure 6B is a summary of the average number of colonies (rounded to the nearest colony) formed in three independent growth assays. There are clearly fewer colonies formed in HAM4-8 and PG8 cells induced with 0.5 ug DOX than in mock-induced HAM4-8 or PG8 cells or in induced or mock-induced HAM5-1 or “vector” cells. These data confirm results reported by Wantanabe-Fukunaga et al 2005 showing that the observed Trip-Br1-induced reduction in cell viability is p53-independent since both HAM4-8 and PG8 cells show similar response (reduced cell viability) to ectopic expression of Trip-Br1.
Figure 6: Effects of overexpression of Trip-Br1 on cell growth. A. Representative crystal violet staining of cell colonies formed in HAM derivatives induced and mock-induced with DOX. B. Summary of average number of colonies formed in HAM derivatives induced and mock-induced with DOX (Average of 3 experiments, results rounded to nearest colony). C-F. Results of MTT assays in HAM derivatives induced with DOX for 12 (C), 48 (D), 96 (E), and 120 (F) hours. Data analyzed using one-way ANOVA (SPSS). *p<0.05, **p<0.001, ***p<0.0001. G. Representative DOX induction of Trip-Br1 in HAM4-8 cells. Upper band is presumably phosphorylated Trip-Br1 (see section 5.4).
To further confirm growth assay results, I performed MTT cell viability assays to confirm the number of viable cells in each treatment in each cell line over time (Figure 6C-F). MTT cell viability assays are based on the reduction of tetrazolium salts by viable cells. A yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells (generating NADH and NADPH) and the resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. HAM4-8, PG8, HAM5-1, and “vector” cells were seeded in 12-well culture plates and treated with DOX (0.5 ug/ml) for 12, 48, 96, and 120 hours. At specified time points, cells were treated with MTT reagent and absorbance of cell extracts measured in each case. Absorbance at 570 nm is proportional to the number of viable cells present in culture at the time of cell harvest.

Figure 6C shows comparison of the number of viable HAM4-8, HAM5-1, PG8, and vector cells present 12-hours post DOX-induction. At 12 hours, there is no significant difference in number of viable cells among the cell types. Figure 6D shows comparison of the number of viable HAM4-8, HAM5-1, PG8 and vector cells present 48-hours post DOX-induction. At 48 hours post-induction, there are significantly fewer viable PG8 cells compared to HAM5-1 and vector cells. Figure 6E shows comparison of the number of viable HAM4-8, HAM5-1, PG8 and vector cells present 96-hours post DOX-induction. At 96 hours, there are significantly fewer viable HAM4-8 and PG8 cells compared to HAM5-1 and vector cells. Figure 6F shows comparison of the number of viable HAM4-8, HAM5-1, PG8 and vector cells present 120-hours post DOX-induction. At 120 hours there are significantly fewer viable HAM4-8 and PG8 cells compared to
HAM5-1 and vector cells. To ensure that DOX treatment efficiently induced Trip-Br1 expression in HAM4-8 and PG8 cells and not in HAM5-1 and vector cells, I probed for Trip-Br1 in HAM derivatives induced with DOX at 12, 48, 96, and 120 hours. Figure 6G shows efficient induction of Trip-Br1 by DOX in HAM4-8 cell used in growth assays. Over time, (12-120 hrs) Trip-Br1 expression increases in HAM4-8 cells induced with DOX. Similar results were obtained for induction profiles in PG8 cells and Trip-Br1 was not induced in HAM5-1 or vector cells as expected (data not shown). These data support reports documented by Watanabe-Fukunaga et al. (2005), suggesting that Trip-Br1 may be involved in p53-independent reduction in cell viability.

2.2.3. Ectopic expression of Trip-Br1 contributes to increased p21 mRNA and protein expression.

To further characterize the effects of overexpression of Trip-Br1 on p53 and p21 expression, I performed real-time PCR analyses to measure p53 and p21 mRNA induction in cells expressing normal and ectopic Trip-Br1 (Figure 7).
Figure 7: Effects of overexpression of Trip-Br1 on p53 and p21 mRNA and protein.
A. Realtime PCR data showing that Trip-Br1 mRNA is efficiently induced in DOX-treated HAM4-8 and PG8 cells (average of 2 experiments ± standard deviation. B. Realtime PCR data showing that PG8 cells express lower levels of p53 mRNA relative to other HAM derivative cell lines (single experiment). C. Realtime PCR results showing that p21 mRNA is induced in response to ectopic Trip-Br1 expression (average of 3 experiments ± standard deviation). D. Western blot showing that Trip-Br1 induces p21 protein in both p53-null and p53-positive cells.

Figure 7A shows that addition of 0, 0.5, and 1.0 ug/ml DOX induces no change, and 3-, and 5-fold expression of Trip-Br1 mRNA in HAM4-8 cells (Figure 7A, light, medium, and dark grey bars labeled HAM4-8) and no change and 3.5-, and 4.2-fold induction of Trip-Br1 expression in PG8 cells (Figure 7A, bars labeled PG8 cells).
expected, DOX had no effect on Trip-Br1 expression in HAM5-1 and “vector” cells (Figure 7A, bars labeled HAM5-1 and “vector”). Figure 7B shows expression of p53 in cells induced with DOX. In agreement with reports by Watanabe-Fukunaga et al. 2005, ectopic expression of Trip-Br1 had no effect on p53 mRNA expression (Figure 7B, compare light, medium, and dark grey bars in HAM4-8 cells). Control cell lines did not show variability in p53 mRNA levels with DOX treatment either (Figure 7B, compare light, medium, and dark grey bars in HAM5-1 and in “vector” cells). In PG8 cells (harboring constitutively expressing p53-directed siRNA), p53 mRNA levels are reduced 10-fold relative to cell lines not harboring the p53-directed siRNA (Figure 7B, compare p53 expression in all treatments among all cell lines). These data demonstrate the effectiveness of the p53-directed siRNA in reducing p53 mRNA levels in PG8, relative to other HAM3 derivative cell lines. Figure 7C shows expression of p21 in HAM3 derivative cell lines. With addition of 0, 0.5, and 1.0 ug/ml DOX, p21 mRNA levels are induced 1-, 2.5-, and 5-fold in HAM4-8 cells (Figure 7C, compare light, medium, and dark grey bars labeled HAM4-8) and 1-, 15-, and 20-fold in p53 knock-down PG8 cells (Figure 7C, compare light, medium, and dark grey bars labeled PG8). Since Trip-Br1 overexpression induces up-regulation of p21 in both p53 “normal” HAM4-8 and in p53 knock-down PG8 cells, these data indicate that Trip-Br1 is implicated in p53-independent transcriptional regulation of p21. These data are supported by immunoblot data probing for p21 expression (Figure 7D). Clearly, p21 protein is up-regulated in both p53 “normal” HeLa cell and p53-null H1299 cells. These data suggest that Trip-Br1 is
implicated in p53-independent regulation of p21 (Figure 7D, compare p53 and p21 protein levels in HeLa cells and p21 protein levels in H1299 cells).

2.2.4. Conclusions

Reporter, cell growth, and real-time PCR analyses are consistent with data presented by Watanabe-Fukunaga et al. 2005 showing that Trip-Br1 is potentially implicated in cell growth arrest processes and these processes may be independent of p53 and may involve modulation of the cell cycle regulator p21.

2.3 Discussion

The ability of Trip-Br1 to reduce cell viability has been demonstrated by Watanabe-Fukunaga et al., 2005 and my data agree with their results. We have demonstrated that Trip-Br1 does not affect a promoter construct harboring 503 bases of the p53 promoter or p53 expression from the native locus. While p53 protein accumulates in p53 +/+ cells in response to Trip-Br1, the reduction in cell viability is not dependent on p53. This is apparent in p53 knock-down cells where overexpression of Trip-Br1 results in fewer viable cells over time. These data suggest that Trip-Br1 is implicated in p53-independent cell cycle regulatory pathways (Watanabe-Fukunaga et al., 2005), and that p21 may be involved. This hypothesis has not been pursued in detail and warrants further investigation.

I have demonstrated that overexpression of Trip-Br1 induces increased p21 mRNA and protein and this effect is not dependent on p53. There are a number of mechanisms by which p21 has been shown to be regulated transcriptionally, independently of p53 (reviewed in Gartel et al., (2000); Gartel and Tyner, (1999)). Based
on work identifying putative functional domains of Trip-Br1, there are at least three potential mechanisms by which Trip-Br1 could feasibly contribute to the transcriptional regulation of p21. Firstly, Trip-Br1 is hypothesized to be recruited to E2F-responsive promoters via physical interaction with DP-1, and the p21 promoter is known to be E2F-responsive (see sections 1.10 and 1.11). Secondly, it is also possible that Trip-Br1 interacts with p300, and may be recruited to Sp-binding elements through these associations and it is well-documented that p21 is regulated by Sp-family transcription factors (see section 2.1). Thirdly, Trip-Br1 harbors a putative cyclin-binding domain and interaction between cyclins, Trip-Br1, and associated factors may regulate p21 transcription. Finally, there is a region of Trip-Br1 that shows no homology to other proteins, but may have functions in transcriptional regulation that are yet to be identified.

The putative functional domains of Trip-Br1 have not been confirmed through mutational analyses and these experiments are critical to thorough understanding of Trip-Br1 function. I have therefore initiated a genetic analysis of the functional domains of Trip-Br1. Results of these investigations and their significance in the regulation of the p21 promoter are presented in the next section (section 3).
3. MUTATIONAL ANALYSES OF TRIP-BR1 FUNCTIONAL DOMAINS

3.1. Introduction

Overexpression of Trip-Br1 limits cell proliferation, and p21 has been implicated in these cellular processes. Thorough understanding of Trip-Br1 activity in this and other contexts requires the putative Trip-Br1 functional domains to be confirmed experimentally. Only then, is it possible to identify the regions of the Trip-Br1 polypeptide that are required for the regulation of the p21 and other promoters. The putative functional domains of Trip-Br1 have been described in previous sections and are represented in Figure 8.

![Diagram of the functional regions of Trip-Br1.](image)

Studies have suggested that promoter regulation by Trip-Br1 is via the heptad repeat (in association with DP-1), by interaction with PHD-bromodomain-containing proteins, and/or via other regions that are in the uncharacterized THD2 or putative cyclin binding regions. To systematically test the contribution of each protein domain in the transactivation of specific promoter elements, I have constructed a series of mutant Trip-Br1 proteins with deletion in the heptad repeat, the THD-2 domain, the acidic
transactivation domain, or the cyclin-binding domain. I have tested each of these proteins in a battery of reporter assays to ascertain the contribution of each protein domain to promoter activity. In this and in section 4, I characterize the heptad repeat, the PHD-bromo-interacting, and the THD-2 domains. Analyses of the putative cyclin-binding domain will be deferred until section 5.

3.2. Trip-Br1 functions at E2F-responsive promoters

Since E2F-transcription factors are implicated in regulating a diverse assemblage of genes involved in both pro-cell-cycle and pro-apoptotic functions (Hershko and Ginsberg, 2004), it is appealing to consider that Trip-Br1 may function in an analogous fashion, having both pro-apoptotic and pro-cell cycle functions, depending on the promoter context. Supporting this notion, the general activity of Trip-Br1 at E2F-responsive promoters was demonstrated in reporter assays using an artificial promoter consisting of 6E2F-binding elements (Gupta et al. 2003).

The heptad hydrophobic repeat domain of Trip-Br1 (amino acids 43-76) is hypothesized to be necessary for Trip-Br1/DP-1 interaction and required for Trip-Br1-mediated transactivation of E2F-responsive promoters, although this has not been confirmed experimentally. The heptad hydrophobic repeat (leucine zipper) motif often mediates protein dimerization and was first described in c-Myc, c-Jun, and the yeast gene regulatory protein GCN4 (Landschulz et al., 1988). The hydrophobic heptad repeat harbors leucine at every seventh amino acid residue (designated the A position) and there are generally nonpolar amino acids (leucine, isoleucine, and valine) within the heptad repeat at the fourth position (D) (Figure 9). X-ray crystallography and X-ray scattering
support that the secondary structure of a heptad repeat is a coiled coil (O'Shea et al., 1991; Rasmussen et al., 1991).

Figure 9: Schematic of the hydrophobic heptad repeat protein domain. A and D are positions of hydrophobic residues. Re-drawn from: http://cis.poly.edu/~jps/coilcoil.html

DP-family transcription factors harbor a hydrophobic heptad repeat required for dimerization with E2F-family members. The DP family of transcription factors contains three members, DP1, DP2, and DP3 (see Figure 10). DP-family are homologous in the DNA binding/heterodimerization domain but their C-termini are divergent (Helin et al., 1993b; Ormondroyd et al., 1995; Qiao et al., 2007; Wu et al., 1995).

Figure 10: Schematic of DP-1, DP-2 and DP-3 protein domains showing heptad repeat and DNA binding domains. Re-drawn from Qiao et al. (2007).
All three DP-family members contribute to gene expression by interacting with E2F proteins where heterodimerization of E2F-family and DP-family proteins is essential for high affinity DNA binding and efficient transcriptional regulation by E2Fs (Helin et al., 1993b; Huber et al., 1993; Krek et al., 1993; Qiao et al., 2007).

As heterodimers, the E2F-DP-1 and E2F-DP-2 complexes bind to the consensus E2F DNA recognition site. Heterodimer binding leads to either activation or repression of the target gene, depending on whether the bound E2F family member is an activator or repressor E2F (see general introduction). DP-3 is unique in that it binds to E2F proteins, but does not bind to E2F consensus sequence and DP3-E2F complexes inhibit E2F-mediated transcriptional activation. It has been found that ectopic expression of DP-3 impairs the G1 to S phase transition rather than facilitating the transition as DP-1 does (Qiao et al., 2007). As cofactors for E2Fs, DP proteins have been reported to cause transformation of cells in conjunction with activated ras, indicating a proto-oncogenic potential.

Glutathione-S-transferase (GST)-pull-down assays have demonstrated that Trip-Br1 interacts strongly with DP-1 but not with E2F-1 although mutational analyses confirming the requirement of the heptad repeat for this interaction have not been performed (Hsu et al., 2001). We have constructed mutant Trip-Br1 and DP-1 proteins that lack the hydrophobic heptad repeat and have tested their ability to interact (using GST-pull-down) and to transactivate E2F-responsive promoter (6xE2F-Luc and others).
3.3 Results

3.3.1. Trip-Br1-DP-1 interaction is dependent on intact hydrophobic heptad repeats.

Figure 11 shows an alignment of the heptad repeats of E2F- and DP- family members and the putative heptad repeat of Trip-Br1. Residues in orange indicate heptad periodicity characteristic of the dimerization domains E2F-, DP-, and Trip-Br1 proteins (Hsu et al., 2001). Using the Kunkle method of site-directed mutagenesis, I constructed Trip-Br1 and DP-1 mutants that lack the heptad repeat domains indicated in Figure 11.

![Figure 11: Alignment showing heptad repeat of E2F1, DP1, and Trip-Br1.](image)

References are indicated in the figure. Shaded regions show hydrophobic residues at positions 1 and 7.

To determine whether the interaction between DP-1 and Trip-Br1 is dependent on the heptad repeat regions, I conducted a series of GST-pull-down assays to test for protein-protein interaction between wild-type and mutant proteins (Figure 12). GST-pull-down assays show that wild-type Trip-Br1 and DP-1 interact (Figure 12A, lane 3).
whereas Trip-Br1 does not interact with DP1ΔHEP, a mutant DP-1 protein that lacks the heptad repeat domain, or the GST tag alone (Figure 12A, lane 2 and lane 1).

![Figure 12B](image_url)

**Figure 12:** GST-pull-down assays showing the dependence of Trip-Br1/DP-1 interaction on intact hydrophobic heptad repeat regions of both A) DP-1 and B) Trip-Br1. A. GST, GST-DP-1ΔHEP, and GST-DP-1 were combined with myc-Trip-Br1 (rows 1 and 2 show expression of input proteins). Myc-Trip-Br1 was pulled down by GST-DP1 but not by GST or GST-DP-1ΔHEP (row 3). B. Myc-Trip-Br1, myc-TRIP1ΔHEP, myc-TRIP1ΔTHD2, and myc-TRIP1Δ17 were combined with GST-DP-1 (rows 1 and 2 show expression of input proteins). Myc-Trip-Br1, myc-TRIP1ΔTHD2, and myc-TRIP1Δ17 were pulled down by GST-DP1, but myc-TRIP1ΔHEP was not (row 3).

Figure 12B shows results of binding assays between wild-type DP-1 and Trip-Br1 and TRIP mutants. Wild-type DP-1 does not interact with TRIPΔHEP, a Trip-Br1 mutant that lacks the heptad repeat (Figure 12B, lane 2), whereas wild-type DP-1 interacts with wild-type Trip-Br1, TRIPΔTHD2, and TRIPΔ17 (Figure 12B, lanes 1, 3, and 4). These data show that the interaction between Trip-Br1 and DP-1 is dependent on both the heptad repeat of Trip-Br1 and DP-1.
3.3.2. Trip-Br1-mediated promoter transactivation is dependent on the Trip-Br1 and DP-1 hydrophobic heptad repeats.

In order to determine whether the Trip-Br1 and DP-1 hydrophobic heptad repeats are required for Trip-Br1-mediated transactivation of an E2F-responsive promoter, we carried out reporter assays using the 6xE2F-Luc promoter construct (Gupta et al., 2003). Transient transfection assays show that overexpression of E2F-1/DP-1 and E2F-1/DP-1/Trip-Br1 transactivate the 6xE2-Luc by 14- and 40-fold respectively (Figure 13A, compare lanes 1, 2, and 3). Figure 13B shows that maximal promoter activity is dependent on overexpression of E2F-1, DP-1 and Trip-Br1 where overexpression of E2F-1 or Trip-Br1 alone result in 9- and 7-fold activation of 6xE2F-Luc (Figure 13B, compare lanes 1, 2, and 4). As expected, overexpression of DP-1 alone does not transactivate the promoter (Figure 13B, compare lanes 1 and 3).

To address the dependence of maximal Trip-Br1-mediated expression of 6xE2F on DP-1 functional domains, a series of assays were performed using wild-type DP-1, DP-1ΔDBD (DNA-binding-deficient DP-1 mutant, kindly provided by Harlow et al.), or DP-1ΔHEP (DP-1 mutant lacking heptad repeat hypothesized to be required for interaction with Trip-Br1) (see Figure 12 and previous sections).
Figure 13: Reporter assays showing that transactivation of 6xE2F-Luc is dependent on Trip-Br1- and DP-1-hydrophobic heptad repeat domains. A. Trip-Br1/DP-1/E2F-1 transactivates 6xE2F-Luc by 40-fold. B. All three factors must be overexpressed to induce maximal promoter activity. Maximal activity induced by these factors is dependent on an intact DP-1 hydrophobic heptad repeat (C), and an intact Trip-Br1 hydrophobic heptad repeat (D). Average of at least 3 experiments +standard deviation.
Overexpression of wild type DP-1, DP-1ΔHEP or DP-1ΔDBD with E2F-1 results in approximately 15-fold activation of the 6xE2F promoter (see figure 13C, compare lanes 1, 2, 3, and 4). These results are not surprising since DP-1 does not contribute to transactivation alone. Overexpression of DP-1ΔHEP or DP-1ΔDBD with E2F-1 and Trip-Br1 results in approximately 15-fold activation of the 6xE2F promoter (see figure 13C, compare lanes 1, 6 and 7). This is approximately one fourth of the activity that was obtained using the wild-type DP-1 construct in similar assays (Figure 13C, compare lanes 1, 5, 6, and 7). These results are consistent with published results showing that the function of Trip-Br1 is mediated through DP-family transcription factors (Gupta et al., 2003; Hsu et al., 2001).

To address the dependence of maximal Trip-Br1-mediated expression of 6xE2F on the Trip-Br1 heptad repeat, a series of assays were performed using wild-type DP-1 and E2F-1 and wild type and mutant Trip-Br1 constructs. Overexpression of wild-type Trip-Br1, Trip-Br1ΔHEP, Trip-Br1ΔTHD2, or Trip-Br1ΔAcD with E2F-1 and DP1 resulted in about 49, 14, 45, and 47-fold activation of the 6xE2F promoter respectively (see Figure 13D, compare lanes 3, 4, 5, and 6), demonstrating that the transactivation of the promoter is dependent on an intact Trip-Br1 heptad repeat region.

To test for the requirement of overexpression of functional E2F-1 for maximal transactivation of 6xE2F-Luc by Trip-Br1, I conducted a series of assays overexpressing E132 in place of E2F-1. E132 is a DNA-binding-deficient E2F-1 mutant, kindly provided by Dr. Ed Harlow. Overexpression of E132/ DP1 /Trip-Br1 resulted in about 12-fold induction of 6xE2F, less than one fourth of the activation of 6xE2F by Trip-Br1
overexpression in the presence of wild-type E2F-1 and DP-1. Overexpression of E132 and DP-1 transactivates 6xE2F by about 6-fold (Figure 14). These data suggest that full transactivation of the 6xE2F-Luc requires a functional E2F-1 (Figure 14, overall trend). This is not surprising since E2F-1 has intrinsic transactivation activity and is required for promoter transactivation by DP-1.

I and others have demonstrated that overexpression of Trip-Br1 activates p21 expression from the native locus (Watanabe-Fukunaga et al. 2005). To determine whether this activation is dependent on the Trip-Br1 heptad repeat, I compared the effects of overexpression of Trip-Br1 and TRIP1ΔHEP on the expression of a reporter construct containing 1200 bases of the human p21 promoter (p21(1200)-Luc). Figure 15A shows E2F- and Sp-binding elements in the proximal region of the p21 promoter. Since I am particularly interested in identifying p53-independent mechanisms that regulate the p21
promoter, I conducted these assays in the PG8 (p53 knock-down) HeLa derivative cell line. I found that overexpression from transfection of 800 ng of Trip-Br1, or two other Trip-Br1 mutants (TRIP1ΔTHD2 and TRIP1ΔAcD) efficiently transactivates the promoter construct by approximately 5-fold (Figure 15B, compare lanes 1, 2, 4, and 5) whereas TRIP1ΔHEP is less efficient at the promoter transactivation (Figure 15B, compare lanes 1 and 3).

![Figure 15: Transactivation of p21(1200) by Trip-Br1 is dependent on the Trip-Br1 hydrophobic heptad repeat domain. A. Segment of p21 promoter showing E2F- and Sp- binding elements. B. Effects of overexpression of Trip-Br1 and Trip-Br1 mutants on p21(1200)-Luc. TRIP1ΔHEP is less efficient at transactivating the promoter than constructs with this domain intact. Average of three experiments ±standard deviation.](image-url)
Since transactivation of this promoter construct is dependent on an intact Trip-Br1 heptad repeat domain, these data suggest that p21 is regulated either directly or indirectly by Trip-Br1 association with DP-1.

### 3.3.3. Conclusions based on Heptad Repeat Analyses

Trip-Br1 interacts with DP-1 and the heptad repeat domains of each protein are required for this interaction. Trip-Br1 transactivates a minimal E2F-responsive promoter and well as a reporter containing 1200 bases of the p21 promoter. Transactivation of each of these constructs is dependent on the hydrophobic heptad repeat domain of Trip-Br1.

### 3.4. Introduction to Trip-Br1 acidic transactivation (PHD bromo-interacting) domain

To expand our understanding of how Trip-Br1 contributes to transcriptional regulation, I have initiated detailed characterization of the Trip-Br1 acidic transactivation domain (PHD-bromo-interacting domain). The Trip-Br1 acidic transactivation domain was identified based on homology to MDM2 and has been shown to be important in Trip-Br-mediated transcriptional regulation (Hsu et al., 2001; Martin et al., 1995). Yeast-2 hybrid and GST-pull-down assays have shown that Trip-Br1 interacts with the PHD-bromodomain-containing transcription factor KRIP-1 (Hsu et al. 2001). Interestingly, while there is significant homology between the MDM2 and Trip-Br acidic transactivation domains, the MDM2 domain does not interact with the KRIP-1 PHD-bromodomain, suggesting that these interactions are regulated and highly specific (Hsu et al., 2001).
The PHD-bromodomain occurs in several classes of proteins and is involved in DNA and RNA binding, mediating protein–protein interaction and in recognition of differentially modified histone tails (Ragvin et al., 2004). A number of transcription factors contain PHD-bromodomains, including Mi-2 (a component of the NuRD histone deacetylase complex) (Woodage et al., 1997), the ACF1/WSTF protein family (subunits of chromatin remodelling complexes) (Langst and Becker, 2001); and p300/CBP (transcriptional co-adaptor proteins) (Lundblad et al., 1995). Human p300 has acetyl-transferase (HAT) activity and a PHD-bromodomain (Ragvin et al., 2004) (Figure 16). Trip-Br1 is hypothesized to interact with the p300 PHD-bromodomain via the Trip-Br1 PHD bromo-interacting domain. (Hsu et al., 2001).

Figure 16: Schematic of PHD-bromodomain and acetyltransferase activity domains of p300. Re-drawn from Ragvin et al. (2004).

P300 and the related transcriptional coactivator, CBP [cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB)-binding protein], have HAT activity, are expressed in most tissues, and interact with numerous transcription factors to contribute to transcriptional coactivation and repression. Interactions with components of the general transcriptional machinery, such as TFIID,
TFIIB, and the RNA polymerase II holoenzyme (RNAPII) have also been suggested to contribute to p300 and CBP function (Blobel, 2002). In addition, binding of p300 and CBP to transcription factors may facilitate HAT activity near specific nucleosomes in target gene promoter regions (reviewed in (Vo and Goodman, 2001)).

It is well documented that p21 is transcriptionally regulated by p300 through its association with Sp-family transcription factors (Xiao et al., 2000). Sp-factors are also known to physically interact with and function in conjunction with E2F-family members (Lin et al., 1996). Since I and others have shown that Trip-Br1 expression affects transcription from the p21 promoter I hypothesize that Trip-Br1 may contribute to Sp-mediated transcription (Watanabe-Fukunaga et al. 2005). I hypothesize that this phenomenon may occur via one or both of the following mechanisms: As I have shown previously, Trip-Br1 interacts with E2F-1/DP-1 and may be recruited to Sp-binding elements through E2F-1/DP-1-interaction with Sp1. Alternatively, since Sp1 collaborates with p300 to regulate p21 transcription, (Xiao et al., 2000), and Trip-Br1 may associate with p300 to contribute to Sp-mediated transactivation, Trip-Br1 may be recruited to Sp-binding elements independently of E2F-1/DP-1 complexes.

To begin to address these questions, I first tested the ability of Trip-Br1 to interact with p300. The PHD bromo-interacting domain of Trip-Br1 is hypothesized to be necessary for p300-Trip-Br1 interaction, although this has not been confirmed experimentally (Hsu et al., 2001). I have constructed mutant Trip-Br1 proteins that lack the acidic transactivation domain (PHD-bromo-interacting domain) (TRIP∆AcD) and have tested its ability to interact with p300 using GST-pull-down assays. To address the
question of the role of Trip-Br1 on transactivation of p21, we then tested the activity of a minimal p21 promoter with overexpression of Trip-Br1, TRIP1ΔAcD, E2F-1, DP-1, p300, and Sp1.

3.4.1. Results

3.4.1.1. Trip-Br1 interacts with p300 independently of the Trip-Br1 acidic transactivation domain.

To initiate more detailed analyses of the mechanism by which the PHD-bromodomain-interacting region of Trip-Br1 is involved in transcriptional regulation, I constructed a mutant Trip-Br1 that lacks the 3’ acidic transactivation domain. The acidic transactivation domain of Trip-Br1 protein and the alignment of the homologous region in MDM2 are illustrated in Figure 17.

Figure 17: Alignment of Trip-Br1 and mdm2 acidic transactivation domain. Shaded residues are regions of homology. References are indicated in the figure.
To determine whether the interaction between p300 and Trip-Br1 is dependent on
the acidic transactivation regions of Trip-Br1, I conducted a series of GST-pull-down
assays to test for protein-protein interaction between wild-type and mutant Trip-Br1
proteins and the PHD-bromodomain of p300 (Figure 18). GST-pull-down assays show
that wild-type and mutant Trip-Br1 proteins interact with the PHD-bromodomain of p300
(Figure 18, compare output in lanes 2, 3, and 4) and Trip-Br1 does not interact with the
GST tag (Figure 18, lane 1). These data indicate that the interaction between Trip-Br1
and p300 is not dependent on the acidic transactivation domain of Trip-Br1.

Figure 18: GST-pull down assays showing that the interaction between Trip-Br1
and p300 PHD-bromodomain is independent of the acidic transactivation domain of
Trip-Br1. Myc-Trip-Br1, myc-TRIP1Δ17, myc-TRIP1ΔAcD were combined with GST-
p300(aa744-1540). As a control, myc-Trip-Br1 was combined with GST tag only (first
column). Rows 1 and 2 show expression of input proteins. Myc-Trip-Br1, myc-
TRIP1Δ17, and myc-TRIP1ΔAcD were pulled down by GST-p300(aa744-1540) whereas
the GST tag did not interact with Trip-Br1 (see row 3).
3.4.1.2. Trip-Br1 limits Sp1/p300-mediated transactivation of p21 independently of the Trip-Br1 acidic transactivation domain.

To test the function of the PHD bromo-interacting domain of Trip-Br1 in transcriptional regulation of Sp-responsive promoters, I conducted a series of luciferase assays to test the effects of overexpression of E2F-1, DP-1, Sp1, p300 and Trip-Br1 and TRIP1∆AcD on the transcriptional activity of a promoter construct containing a segment of the p21 promoter harboring Sp1 binding elements. To attempt to eliminate confounding effects of contribution of E2F to the regulation of the promoter, I chose to use a portion of the p21 promoter that contains three Sp-family binding elements and a mutation in a downstream E2F-binding element (p21mut1-Luc) for these assays (see Figure 19A). Unexpectedly, in my hands, p21mut1-Luc is E2F-responsive where overexpression of E2F-1/DP-1 transactivate the promoter by 2.5-fold (Figure 19B, compare lanes 1 and 2). Even more surprisingly, overexpression of either Trip-Br1 or TRIP1∆AcD limited the transactivation of the promoter by E2F-1/DP-1, suggesting that Trip-Br1 may act as a repressor in some promoter contexts and this activity is independent of the acidic transactivation domain (Figure 19B, compare lanes 1, 2, 3, and 4). To determine whether transactivation of p21mut1 by Sp-associated factors is affected by the Trip-Br1 acidic transactivation domain, I tested overexpression of combinations of E2F-1, DP-1, Sp-1, and p300 on the minimal promoter and compared these data with transactivation by these factors with addition of Trip-Br1 or TRIP1∆AcD (Figure 19B, C, D, and E and Figure 20B and C). Unexpectedly, transactivation of p21mut1 by E2F1/DP1, E2F1/DP1/p300, E2F1/DP1/Sp1, Sp1/p300, and E2F1/DP1/Sp1/p300 was
limited by overexpression of Trip-Br1 or TRIPΔAcD (Figure 19B, compare lanes 2, 3, and 4; Figure 19C, compare lanes 3, 4, and 5; Figure 19D, compare lanes 3, 4, and 5; and Figure 20A, compare lanes 3, 4, and 5). These data underscore the notion that Trip-Br1 can act as a repressor in some promoter contexts and that this activity is independent of the acidic transactivation domain.
Figure 19: Trip-Br1 negatively regulates E2F-1/DP-1 and Sp1/p300-mediated transactivation of p21mut1-Luc. A. Sequence of promoter region of p21mut1-Luc. Note that E2F-binding element is mutated (Hiyama et al. 1998; Gartel et al. 1998; Gartel and Tyner 2002). B-E. Results of overexpression of Trip-Br1 or TRIP1ΔAcD on Sp1/p300- and/or E2F-1/DP-1-mediated transactivation of p21mut1-Luc expression. In all cases, Trip-Br1 and TRIP1ΔAcD limit transactivation of p21mut1-Luc by these factors. Results are from a single experiment.
To address the possibility that transactivation of p21mut1 by Sp1 and associated factors is limited by Trip-Br1 due to the mutation in the downstream E2F-binding element, I performed a series of parallel assays to those described above using a p21 promoter construct that is identical to p21mut1 except that the downstream E2F-binding element is intact (p21(100)-Luc) (Figure 20 B). Overexpression of E2F-1/DP-1/Sp1/p300 induces about 5-fold activation of both p21mut1-Luc and p21(100)-Luc. Overexpression of either Trip-Br1 or TRIPΔAcD in either promoter context limits this transactivation (Figure 20A, compare lanes 2, 3, and 4; Figure 20C, compare lanes 2, 3, and 4). These assays demonstrate that the effects of Trip-Br1 are independent of the downstream E2F-binding element where, like assays performed using p21mut1, Trip-Br1 and TRIPΔAcD limit the transactivation of the wild-type promoter by E2F1/DP1/Sp1/p300.

Given that I and others have shown that overexpression of Trip-Br1 activates p21 from the native locus (see previous sections), it was unexpected that Trip-Br1 would limit p21 promoter activity in these reporter assays. To test whether this effect was due to the short promoter used in these assays, I performed a series of assays using longer p21 promoter constructs (Figure 21).
Figure 20: Trip-Br1 negatively regulates E2F-1/DP-1/Sp1/p300-mediated transactivation of p21mut1-Luc and p21(100)-Luc. A. Trip-Br1 and TRIP1ΔAcD limit E2F-1/DP-1/Sp1/p300-mediated transactivation of p21mut1-Luc. B. Sequence of promoter region in p21(100)-Luc. Note that E2F-binding element is intact (Hiyama et al. 1998; Gartel et al. 1998; Gartel and Tyner 2002). C. Results of overexpression of Trip-Br1 or TRIP1ΔAcD on Sp1/p300/E2F-1/DP-1-mediated transactivation of p21(100)-Luc expression. In both promoter contexts, Trip-Br1 and TRIP1ΔAcD limit transactivation of p21mut1-Luc by E2F-1/DP-1/Sp1/p300. Results are average of 2 experiments ± standard deviation.
To determine whether the unexpected behavior of Trip-Br1 in the contexts of p21(mut1)-Luc and p21(100)-Luc are promoter-specific I tested the effects of overexpression of E2F-1/DP-1/Sp1/p300 on promoter constructs containing 800 (p21(800)-Luc) and 1200 (p21(1200)-Luc) bases of the p21 promoter. Figure 21A shows the sequence of the proximal region of the p21 promoter. Overexpression of E2F-1/DP-1/Sp1/p300 result in 4- and 2.5-fold activation of p21(800)-Luc and p21(1200)-Luc respectively (Figure 21B, compare lanes 1 and 2 and 21C, compare lanes 1 and 2). Like assays performed using the shorter promoter constructs, overexpression of Trip-Br1 or TRIP1∆AcD moderately limited this transactivation (Figure 21B, compare lanes 2, 3, and 4 and Figure 21C, compare lanes 2, 3, and 4). To further address the discrepancy between reporter assay and realtime PCR results described earlier, I transfected increasing amounts of Trip-Br1 plasmid onto the p21(800) promoter and tested for activity (Figure 21D). I determined that overexpression of lower doses of Trip-Br1 by transfection of lower amounts of plasmid limits transactivation of the p21 promoter, whereas with overexpression from transfection of upwards of 1 µg of Trip-Br1 plasmid, the p21 promoter can be transactivated (Figure 21D, overall trend). These data suggest that the response of a promoter to Trip-Br1 may be dependent on Trip-Br1 expression, where lower expression limits p21 expression whereas extreme Trip-Br1 overexpression induces p21. I speculate that this may be due to a general apoptotic response resulting from protein overexpression. Further investigations are certainly required to work out the technical and biological implications of these observations.
Figure 21: Overexpression of Trip-Br1 limits E2F-1/DP-1/Sp1/p300-mediated transactivation of p21(800)-Luc and p21(1200)-Luc promoter constructs. A. Partial sequence of p21 promoter region of p21(800)-Luc and p21(1200)-Luc. B and C. Effects of overexpression of Trip-Br1 or TRIP1ΔAcD on E2F-1/DP-1/Sp1/p300-mediated transactivation of (B) p21(800)-Luc and (C) p21(1200)-Luc. D. Transfection of 100 ng-400 ng of Trip-Br1 limits E2F-1/DP-1/Sp1/p300-mediated transactivation of p21(800)-Luc whereas transfection of over 800 ng of Trip-Br1 plasmid contributes to transactivation by these factors. Results are from a single experiment.
3.4.2. Conclusions based on PHD-bromo-interacting domain

The Trip-Br1 acidic transactivation domain (PHD-bromo-interacting domain) is hypothesized to interact with p300. Using GST-pull-down assays, I confirmed that Trip-Br1 and p300 interact, although this interaction is not dependent on the PHD-bromo-interacting domain. A minimal p21 promoter is transactivated by p300/Sp1/E2F-1/DP-1 and this transactivation is limited by overexpression of both Trip-Br1 and TRIP1∆AcD, suggesting that Trip-Br1 activity on the p21 promoter is independent of the acidic transactivation domain.

3.5. Discussion

I have shown that Trip-Br1 interacts with DP-1 and this interaction is dependent on the hydrophobic heptad repeat regions of Trip-Br1 and DP-1. I have also shown that Trip-Br1 interacts with the PHD-bromodomain of p300, although this interaction is not dependent on the acidic transactivation domain of Trip-Br1. These results suggest that either another Trip-Br1 protein domain is required to mediate this interaction, or there are other proteins that act as tethers to bind Trip-Br1 and p300. Recall that E2F-1 and Sp-1 both functionally and physically interact (Lin et al., 1996). I hypothesize that DP-1 may tether Trip-Br1 to a complex containing E2F-1, DP-1, Trip-Br1, p300, and Sp-1 as well as other factors. Further investigations are required to determine whether TRIP1∆HEP is pulled down by p300. If the trip-Br1 mutant that lacks the heptad repeat does not associate with p300, these data demonstrate that the heptad repeat is required for Trip-Br1-p300 interaction and these data would implicate DP-1 as an intermediary in the complex.
Intact DP-1 and Trip-Br1 heptad repeats are required for efficient transactivation of 6xE2F-Luc. The p21 promoter is known to be E2F-responsive and overexpression of Trip-Br1 alone contributes p21 transactivation in a heptad-repeat-dependent manner. Given these results, I anticipated that Trip-Br1 would potentiate the transactivation of p21 by other factors that are known to up-regulate its expression (E2F-1, Sp-1, and p300). It was therefore unexpected that in my hands, the transactivation of p21 promoter constructs by E2F-1/DP-1, E2F-1/DP-1/p300, E2F-1/DP-1/p300/Sp1, and E2F-1/DP-1/Sp1 is limited by overexpression of Trip-Br1 and TRIP1ΔAcD. A simple explanation for this activity is that Trip-Br1 is sequestering these transcription factors away from the promoter so that the activating transcription factors are not efficiently transactivating the promoter. Also, since Sp1 is E2F-responsive (Nicolas et al., 2003), overexpression of E2F-1 may be increasing expression of Sp1 and with overexpression of Trip-Br1, Trip-Br1 is sequestering Sp-1 away from the promoter.

I determined that overexpression of lower doses of Trip-Br1 plasmid limits transactivation of the p21 promoter by E2F-1/DP-1/Trip-Br1/Sp1/p300, whereas with overexpression of upwards of 1 µg of Trip-Br1 plasmid, the p21 promoter can be transactivated. These data suggest that the behavior of the p21 promoter may be influenced by the degree to which Trip-Br1 is overexpressed.

This phenomenon of differential regulation of p21 depending on the experimental context is described in other systems (reviewed in (Gartel and Radhakrishnan, 2005)). For example, while Sp1 is known as an activator of p21 transcription (Gartel and Tyner, 2002), in smooth muscle cells, Sp1 has been found to repress p21, leading to decrease in
cyclinD/cdk4-p21 complex formation, ultimately resulting in growth inhibition (Kavurma and Khachigian, 2003). Intriguingly, this effect was mediated through the Sp1-binding sites in the proximal p21 promoter (Kavurma 2004). Based on our results, it is tempting to speculate that Trip-Br1 may be involved in integrating some of these signals.

Also, it is generally accepted that Notch signaling positively regulates p21 levels, although it has been shown that in endothelial cells, Notch activation leads to p21 repression and to cell cycle arrest where Notch-mediated p21 repression reduces cyclin D/cdk4 complex formation, inhibiting phosphorylation of RB and induction of cell cycle arrest (Noseda et al., 2004). These observations demonstrate that p21 regulation is not straightforward and further studies are required.
4. TRIP-BR1 REGULATES THE TUMOR SUPPRESSOR P14/ARF

4.1 Introduction

I am particularly interested in investigating novel mechanisms by which Trip-Br1 contributes to reduced cell viability and apoptosis. I have alluded to seemingly incongruent reports where Trip-Br1 has in some accounts, been reported to induce cell proliferation and in other contexts, reported to give rise to reduced cell viability. For example, the oncogenic potential of Trip-Br1 was demonstrated in NIH3T3 mouse fibroblasts where ectopic expression of Trip-Br1 gave rise to anchorage-independent growth and tumor formation in nude mice (Tang et al. 2005), whereas, overexpression of Trip-Br1 in HeLa cells leads to cell growth arrest, evidently through accumulation of the tumor-suppressor p53 (Watanabe-Fukunaga et al. 2005). I hypothesize that a possible reason for the differences in Trip-Br1 function may be the status of the E2F-responsive tumor suppressor p19ARF in the cell lines used in the studies. I anticipate that in p19ARF null NIH3T3 fibroblasts, the general stimulation of E2F-responsive promoters by Trip-Br1 in the absence of a functional ARF locus stimulates cell proliferation. However, if transcription of the ARF locus is stimulated in HeLa cells by Trip-Br1, then ARF-induced signaling cascades would result in cell growth arrest.

P14/ARF is a well characterized tumor suppressor. Among other mechanisms, ARF functions as a tumor suppressor by sequestering MDM2 to the nucleolus, limiting MDM2-mediated p53 degradation (Kamijo et al., 1998; Tao and Levine, 1999). The ARF promoter contains E2F-family protein binding sites and is activated by E2F-family transcription factors. ARF expression is known to be up-regulated with ectopic
expression of E2F-1, E2F-2, and E2F-3, contributing to ARF-induced apoptosis (Ginsberg 2002; Kowalik et al. 1995; Parisi et al. 2002). I hypothesize that if Trip-Br1 stimulates transcription from the p14/ARF promoter through these elements in an analogous fashion to its regulation of b-myb, 6xE2F-Luc, and p21(1200)-Luc, then cell arrest mediated by p53 and p53 independent pathways may ensue. There are a number of pathways that give rise to ARF-dependent apoptosis. As listed before, p53 stabilization leads to the up-regulation of downstream targets like p21 (see previous sections). In addition, ARF has been implicated in mitochondrial-dependent apoptosis, involving increased expression of two proapoptotic Bcl-2 family proteins, BAX and BIM, with subsequent cytochrome c release from mitochondria, decreased mitochondrial membrane potential, and activation of procaspase-9 to induce apoptosis (Nakazawa et al., 2003; Suzuki et al. 2003). Based on these reports, we tested the ability of Trip-Br1 to potentiate the transcription of the E2F-responsive, pro-apoptotic p14ARF and tested the effects of overexpression of Trip-Br1 on potential downstream targets BAX and BIM.
4.2. Results

4.2.1. Trip-Br1 regulates the p14/ARF promoter and transactivation is dependent on intact Trip-Br1 and DP-1 heptad repeats and functional E2F-1.

To begin the characterization of the effects of Trip-Br1 on p14/ARF transcription, 642 bases of the human ARF promoter were cloned into the pGL3-luciferase basic vector (Figure 22A) (kindly provided by Dr. Sang-Woo Kim).

![Diagram of p14ARF promoter-luciferase constructs used in transient transfection assays.](image)

The p14/ARF promoter contains 4 putative E2F-binding and 6 putative Sp-binding sites within the region 642 bases upstream from the start of transcription (Figure 22A). To investigate the relative contributions of the E2F-binding sites to gene regulation, a truncated promoter was constructed where two E2F-binding sites are removed (Figure 22B).
Transient transfection assays using PG8 cells (p53 knock-down HeLa derivatives) show that overexpression of E2F-1 alone, E2F-1/DP-1, and E2F-1/DP-1/Trip-Br1 transactivate ARF642-Luc by approximately 10, 14, and 40-fold respectively (Figure 23, grey bars, lanes 3, 4, & 5). Overexpression of E2F-1/DP-1 has little effect on the shorter promoter (ARF(151)-Luc) with E2F-1/DP-1/Trip-Br1 inducing about 4-fold activation (Figure 23, white bars, lanes 4 and 5). ARF(151)-Luc lacks 2 E2F-binding elements present in ARF(642)-Luc, providing a plausible explanation for reduction in E2F-1 activation. Previous analyses have shown that simple deletions of putative E2F-binding sites in the ARF promoter are insufficient to eliminate p14/ARF promoter transactivation by E2F. To explain these data, it was hypothesized that E2F-family transcription factors may be tethered to the promoter through the flanking Sp-1 binding sites in the ARF promoter (Parisi et al. 2002). Alternatively, E2F-transcription factors may be recruited to non-consensus E2F-binding sites in the p14/ARF promoter.

Trip-Br1 activates ARF642-Luc by about ten-fold in the absence of overexpression of E2F-1 and DP-1 (Figure 23, see lane 6). Overexpression of DP-1/Trip-Br1 and E2F-1/Trip-Br1 activate the promoter by about 7- and 15-fold, respectively (Figure 23, lanes 7 and 11 respectively), implying that all three proteins must be present for maximal induction. According to the current model, Trip-Br1 is recruited to E2F-responsive promoters by physical interaction with DP-1. It is therefore likely that, in the absence of overexpression of E2F-1 and/or DP-1, Trip-Br1 activity is mediated through endogenous E2F- and DP-family proteins.
To address the dependence of maximal Trip-Br1-mediated expression of ARF on DP-1 family members, a series of assays were performed using wild-type DP-1, DP1ΔDBD or DP1ΔHEP (see previous sections). Overexpression of DP1ΔDBD or DP1ΔHEP with E2F-1 and Trip-Br1 results in approximately 27-fold and 15-fold activation of the ARF promoter (see Figure 23, lane 9 and 10), approximately one-half and one-fourth of the activity that was obtained using the wild-type DP-1 construct in
similar assays (see Figure 23, compare lanes 5, 9, and 10). Note, however that the 27-fold activation of the promoter is approximately twice the activity of that obtained when both wild-type DP-1 and DP-1 mutants are omitted (see Figure 23, compare lanes 9 and 11), suggesting that the DP-1ΔDBD is partially functional in mediating Trip-Br1 transactivation activity. The deletion in this mutant renders it unable to bind DNA but able to bind E2F-1, likely providing sufficient tethering of Trip-Br1 to the promoter to affect expression. Overexpression of DP-1ΔHEP with E2F-1 and Trip-Br1 is similar to omitting DP-1 altogether, suggesting that Trip-Br1 is not recruited to the promoter at all. These results are consistent with published accounts (Hsu et al. 2001) and my data presented in previous sections showing that the function of Trip-Br1 is mediated through DP-family transcription factors.

I established in previous sections that Trip-Br-transactivation of a minimal E2F-responsive promoter and 1200 bases of the p21 promoter is dependent on an intact Trip-Br1 heptad repeat. To elucidate the dependence of the transactivation of ARF on Trip-Br1 protein domains, I performed a series of luciferase assays using Trip-Br1 mutants constructed and described in previous sections (Figure 24).
Figure 24: Maximum Trip-Br1-mediated transactivation of ARF(642)-Luc is dependent on the Trip-Br1 hydrophobic heptad repeat domain.  

A. Transactivation of ARF(642)-Luc by Trip-Br1 and Trip-Br1 mutants.  

B. Transactivation of ARF(642)-Luc by Trip-Br1 and Trip-Br1 mutants with E2F-1 and DP-1. Results are averages ± standard deviation.
To address the dependence of maximal Trip-Br1-mediated expression of ARF on the Trip-Br1 heptad repeat, a series of assays were performed using wild-type DP-1 and E2F-1 and wild type and mutant Trip-Br1 constructs. Overexpression of Trip-Br1, TRIPΔTHD2, TRIPΔHEP, or TRIPΔAcD resulted in about 15, 13, 7, and 10-fold activation of the ARF642-Luc promoter respectively (see figure 24A), demonstrating that the transactivation of the promoter is dependent on an intact Trip-Br1 heptad repeat region. This effect is more pronounced when Trip-Br1 is overexpressed with it binding partners, E2F-1 and DP-1. Overexpression of Trip-Br1, TRIPΔTHD2, TRIPΔHEP, or TRIP1ΔAcD with E2F-1 and DP-1 resulted in about 44, 41, 10, and 47-fold activation of the ARF promoter respectively (see figure 24B), demonstrating that the transactivation of the promoter is dependent on an intact Trip-Br1 heptad repeat region.

It has been shown previously that p14/ARF is transactivated by the “activator E2Fs” (E2F-1, E2F-2, and E2F-3) and unaffected (or maintained inactive) by overexpression of the “repressor E2Fs” (E2F-4 and E2F-5) (Parisi et al. 2002). I was interested in determining whether overexpression of the repressor E2Fs would affect Trip-Br1-mediated transactivation of ARF. To this end, to be consistent with other analyses of the p14/ARF promoter, I conducted a series of reporter assays in PG8 cell to address this question (Figure 25).
As reported in the literature (Parisi et al., 2002; Robertson and Jones, 1998), E2F-1 with overexpression of DP-1 transactivates p14/ARF and overexpression of E2F-4 or E2F-5 with DP-1 does not affect the promoter (Figure 25, compare lanes 1, 2, 3, and 4). Overexpression of E2F-1 with DP-1 and Trip-Br1 transactivate ARF by about 40-fold (Figure 25, lane 5). Interestingly, overexpression of E2F-4 or E2F-5 with DP1 and Trip-Br1 transactivate the ARF(642)-Luc by approximately 30-fold (Figure 25, compare lanes 1, 5, 6, and 7). These data show that while overexpression of the repressor E2Fs limits
Trip-Br1 transactivation of the promoter compared to similar assays using E2F-1, the repressor E2Fs do not completely inhibit Trip-Br1-mediated transactivation of the promoter. It is possible that Trip-Br1 is still interacting with DP-1 to transactivate the promoter and recruiting endogenous E2F-1 and overexpression from transfection from 10 ng of repressor E2F-expression plasmids is insufficient to overcome these associations.

Remember that ARF is both E2F-responsive and has putative Sp-binding elements. Given the unexpected behavior of Trip-Br1 in the context of the p21 promoter, we wanted to test the effects of overexpression of Trip-Br1 with Sp1 and p300 as well as E2F-1 and DP-1 in the context of the ARF promoter (Figure 26).

Figure 26: Trip-Br1 does not contribute to Sp1/p300-mediated transactivation of ARF(642)-Luc. Co-overexpression of Sp1/p300 transactivate ARF(642)-Luc by about 75-fold and addition of Trip-Br1 and its binding partners does not contribute to additional transactivation of the promoter in this context (compare lanes 1, 5, 6, 7, 8). Results are average of 2 experiments ± standard deviation.
Overexpression of Sp1 and p300 transactivate the promoter efficiently (Figure 26, lane 5). Overexpression of Trip-Br1 and E2F-1 and DP-1 do not affect transactivation of ARF by Sp1 and p300 (Figure 26, compare lanes 5, 6, 7, and 8).

To determine whether overexpression of Trip-Br1 affects ARF mRNA levels, I performed realtime PCR to measure ARF expression in HAM5-1, HAM4-8, PG8, and vector cells induced with 0, 0.5, and 1.0 ug/ml DOX. Ectopic expression of Trip-Br1 induced by DOX did not affect ARF mRNA expression (Figure 27A, note similar ARF mRNA expression in all treatments in all cell lines).

**Figure 27:** Overexpression of Trip-Br1 alone does not induce increased ARF mRNA expression whereas expression of Trip-Br1 with its binding partners, E2F-1 and DP-1 enhances ARF mRNA expression in HeLa cells. A. Ectopic expression of Trip-Br1 does not affect ARF mRNA levels in HAM derivatives. Legend indicates [DOX] used to induce Trip-Br1 expression. B. Overexpression of Trip-Br1; E2F-1/DP-1; and Trip-Br1-1/E2F-1/DP-1 result in 1.4, 2.4, and 4.6-fold ARF mRNA. Results are average of 3 experiments ± standard deviation.
Realtime PCR analyses probing for ARF mRNA in cells transfected with Trip-Br1 or Trip-Br1 and binding partners E2F-1 and DP-1 demonstrated that cells expressing E2F1/DP1, Trip-Br1, and Trip-Br1/E2F1/DP1 show 2.5, 1.4, and 4-fold induction of ARF (Figure 27B, compare ARF mRNA expression in all cases). These data show that while overexpression of Trip-Br1 alone is insufficient to induce ARF expression from the native locus, overexpression of Trip-Br1 and its binding partners E2F-1 and DP-1 can induce ARF mRNA.

4.2.2. Ectopic expression of Trip-Br1 induces increased BAX expression independently of ARF.

I have established that overexpression of Trip-Br1 with its binding partners E2F-1 and DP-1 affect transcription from the ARF promoter. Studies show that Trip-Br1 induces up-regulation of p53 and p21, and the apparent regulation of ARF by Trip-Br1 may contribute to these phenomena (see previous sections). Since ARF has been implicated in inducing pro-apoptotic BAX expression (see introduction), I investigated whether Trip-Br1 contributes to up-regulation of BAX and whether this effect is p53- and/or ARF-dependent (Figure 28). To test whether overexpression of Trip-Br1 induces increase in BAX protein, I induced HAM4-8 and HAM5-1 cells with 0.5 ug/ml DOX for 12, 24, and 48 hours.
Figure 28: Trip-Br1 contributes to p53-dependent, ARF-independent up-regulation of pro-apoptotic BAX. A. Western blot showing DOX-induced expression of Trip-Br1 in HAM4-8 cells results in increased BAX protein at 24- and 48-hours post-induction compared to HAM5-1 cells. B. Western blot showing that BAX protein levels do not increase in H1299 cells (p53-/-) in response to overexpression of Trip-Br1. C. Western blot showing that BAX protein levels increase in U2OS cells (ARF-/-) in response to overexpression of Trip-Br1. D and E. Realtime PCR results showing that Trip-Br1 induces moderate up-regulation of BAX mRNA (average of three experiments ± standard deviation).
HAM4-8 cells overexpress Trip-Br1 in response to DOX treatment, whereas HAM5-1 cells express endogenous levels of Trip-Br1. I compared BAX protein expression over time in these cells and found that in HAM4-8 cells, there is an increase in BAX expression relative to that in HAM5-1 cells after 24 and 48 hours of DOX treatment (Figure 28A, compare BAX expression in HAM4-8 and HAM5-1 cells at 24 and 48 hours). These data suggest that in response to ectopic expression of Trip-Br1, BAX protein expression is up-regulated.

To determine whether up-regulation of BAX protein is dependent on p53, we overexpressed Trip-Br1 in p53-null H1299 cells and measured BAX protein after 48 hours. BAX levels were not increased in experiments performed in p53 null H1299 cells (Figure 28B, compare BAX protein in H1299 cells expressing endogenous (right) and ectopic (left) Trip-Br1). These data show that BAX induction by Trip-Br1 likely requires p53.

To determine whether the observed Trip-Br1-induced up-regulation of BAX is dependent on ARF, we tested the response of BAX to overexpression of Trip-Br1 in derivatives of U2OS cells. U2OS cells have a mutation at the ARF locus, making this cell line functionally ARF -/- (Russo et al. 2006). The U2OS derivative cell line, U4-5, overexpresses Trip-Br1 in response to DOX treatment, whereas U5-1 expresses endogenous levels of Trip-Br1 (see table 1 in section 2). I induced U2OS cell derivative U5-1 and U4-5 cells with 0.5 ug/ml DOX for 24, and 48 hours. I compared BAX protein expression over time in these cells and found that in U4-5 cells, there is an increase in BAX expression relative to U5-1 cells after 24 and 48 hours of DOX treatment (Figure
compare BAX expression in U4-5 and U5-1 cells at 24 and 48 hours). These data suggest that in response to ectopic expression of Trip-Br1, BAX expression is up-regulated in an ARF-independent manner.

To determine whether the Trip-Br1-mediated up-regulation of BAX is at the level of transcription, I used realtime PCR to quantify BAX mRNA in cells expressing endogenous and ectopic Trip-Br1 (Figure 28D). I measured BAX expression in HAM5-1, HAM4-8, PG8, and vector cells induced with 0, 0.5, and 1.0 ug/ml DOX. Ectopic expression of Trip-Br1 by DOX induced 1-, 2-, and 3-fold induction of BAX in p53 knock-down PG8 cells; and 1-, 1.5-, and 3.75-fold induction of BAX in p53 “normal” HAM4-8 cells (Figure 28D, compare BAX mRNA expression in all treatments, in all cell lines). Since overexpression of Trip-Br1 in cells that express p53 induce a slightly more pronounced increase in BAX mRNA relative to expression in p53 knock-down cells, these data suggest that p53 enhances Trip-Br1-mediated induction of BAX mRNA. Realtime PCR analyses probing for BAX mRNA in HeLa cells transfected with Trip-Br1 or Trip-Br1 and binding partners E2F-1 and DP-1 demonstrate that cells expressing E2F1/DP1, Trip-Br1, and Trip-Br1/E2F1/DP1 show 5, 7, and 19-fold induction of BAX, suggesting that BAX can be significantly affected by ectopic expression of Trip-Br1 and its binding partners (Figure 28E). There are a number of factors that could be contributing to this effect.

It has been reported that in addition to BAX, ARF-induction can also contribute to pro-apoptotic BIM up-regulation (Nakazawa et al., 2003). Interestingly, BIM also contains E2F-binding elements in its promoter (Hershko and Ginsberg, 2004).
determine whether Trip-Br1 may contribute to BIM transcriptional regulation, we performed a series of luciferase assays using a reporter construct containing 346 bases of the BIM promoter (Figure 29A). Overexpression of E2F1/DP1 and E2F1/DP1/Trip-Br1 transactivate the BIM promoter by 1.9- and 14-fold respectively (Figure 29A, compare lanes 2 and 3). To determine whether overexpression of Trip-Br1 affects BIM mRNA levels, I performed realtime PCR to measure BIM expression in HAM5-1, HAM4-8, PG8, and vector cells induced with 0, 0.5, and 1.0 ug/ml DOX. Ectopic expression of Trip-Br1 induced by DOX did not affect BIM mRNA expression (Figure 29B).

**Figure 29: Effects of overexpression of Trip-Br1 on BIM transcription.** Trip-Br1 moderately up-regulated BIM-Luc (A, average of 3 experiments ± standard deviation) but does not induce BIM transcription (B, preliminary data from single experiment).

### 4.3. Conclusions

Using reporter assays, we have determined that Trip-Br1 can contribute to E2F- and DP- mediated transactivation of p14 ARF and this transactivation is dependent on an intact heptad repeat domain. ARF up-regulation from the native locus is only accomplished with overexpression of Trip-Br1 with its binding partners E2F-1 and DP-1.
Overexpression of Trip-Br1 also induces up-regulation of BAX, and this increase appears to be dependent on p53 and independent of ARF. Overexpression of Trip-Br1 also gives rise to moderate induction of a BIM reporter construct, although this effect is not apparent at the BIM native locus.

4.4 Discussion

ARF is a key tumor suppressor that is both E2F- and Sp- responsive. It has been demonstrated by us and others (Hsu et al. 2001) that Trip-Br1 functions at E2F-responsive promoters through its heptad repeat domain. Trip-Br1 may also contribute indirectly to Sp-mediated transcriptional regulation through its heptad repeat. I therefore investigated the possibility that Trip-Br1 may regulate ARF. Trip-Br1 with its binding partners E2F-1 and DP-1 transactivate the ARF promoter and induce ARF mRNA expression. These data suggest that the Trip-Br1-induced reduction in cell viability may be attributable to ARF expression. ARF expression can induce a number of pro-cell-cycle arrest pathways and is also involved in p53-dependent cell death, although it has been implicated in p53-independent pathways (see introduction). I discovered that Trip-Br1 induces BAX protein and mRNA expression and based on our analyses, this expression is p53-dependent and ARF-independent. Since BAX may also be induced directly as BAX is known to be regulated by Sp1 and Trip-Br1 may be contributing directly to Sp-mediated regulation through the E2F-1/DP-1 complex (Schmidt et al., 1999). This possibility warrants further investigation. Pro-apoptotic BIM is also E2F-responsive and therefore I investigated whether Trip-Br1 may contribute to its regulation. Preliminary data show that Trip-Br1 may contribute to BIM regulation.
5. TRIP-BR1 AND CYCLINS

5.1. Introduction

It has previously been demonstrated that viral and cellular cyclins interact with Trip-Br1 and it is hypothesized that the putative cyclin A binding domain is required for this interaction, although this has not been confirmed experimentally (Yuan, unpublished data). The putative Trip-Br1 cyclin A binding domain was identified based on homology to the E2F-1 cyclin binding domain (Hsu et al., 2001). The significance of the E2F-1-cyclin A interaction in gene regulation has been characterized. When cyclin A/cdk2 forms a stable complex with E2F-1, the E2F-1/DP-1 complex is phosphorylated, rendering it unable to bind DNA, and unable to act as a transcriptional activator complex. Cyclin A/cdk2-mediated E2F-1 inactivation down-regulates E2F-responsive genes in late S-phase (Krek et al., 1994; Xu et al., 1994). In addition to E2F-1, RB (Adams et al., 1996), p53 (Luciana et al., 2000), p21, p27 (Adams et al., 1996; Chen et al., 2000) and MDM2 (Zhang and Prives, 2001) harbor consensus cyclin A/cdk2 binding domains (RXLYY', where X, Y, and Y' are any hydrophobic amino acids) (Canela et al., 2006). Cyclin B/cdk1 complexes also phosphorylate a wide range of targets, triggering signaling cascades, activating downstream protein kinases as well as directly regulating proteins important in metabolic and structural changes (Furukawa et al., 2000). While several proteins contain the consensus sequence, other proteins such as lamins (Peter et al., 1990), nonmuscle caldesmon (Yamashiro et al., 1995) and p60src (Morgan et al., 1989; Shenoy et al., 1989) contain both consensus and non-consensus cdk2-cyclin B phosphorylation sequences (Colgan et al., 1998). Binding assays show that Trip-Br1
interacts with cyclins and may also be phosphorylated and regulated by cyclin/cdk complexes. Interestingly, I have identified a consensus phosphorylation sequence for cyclinB/cdk1 in the Trip-Br1 polypeptide (S/TPXR/K (Nigg, 1991; Songyang, 1994)). I anticipate that Trip-Br1 may interact with cyclin B-cdk1 and phosphorylation by these complexes may regulate Trip-Br1 activity.

Since Trip-Br1 interacts with cyclins, it is also possible that Trip-Br1 may modify cyclin activity in a cdk-independent fashion. This phenomenon has been described in other contexts where, for example, D-type cyclins bind to the estrogen receptor to enhance its transcriptional activity in an estrogen- and cdk-independent manner (Neuman et al. 1997). Also, cyclin D2 interacts with the myb-like protein, DMP1 (Hirai and Sherr, 1996). Independent of cdk4 and RB phosphorylation by cyclin D/cdk4, cyclin D2 interferes with DMP1-mediated transcriptional activity (Inoue and Sherr, 1998). These results indicated that D-type cyclins can be involved in processes that do not depend on cdks (Neuman et al., 1997; Zwijsen et al., 1997), and by analogy, Trip-Br1 may also contribute to cdk-independent, cyclin-dependent regulation of target genes.

Using GST-pull-down and co-immunoprecipitation assays, I show that Trip-Br1 interacts with cellular cyclin B. These interactions are potentially important because Trip-Br1 is implicated in the G1-S transition and potentially in other cellular processes. To characterize the relevance of the Trip-Br1-cyclin interaction in the context of transcriptional regulation, I have performed a series of reporter assays to determine how cyclins and Trip-Br1 contribute to the regulation of 6xE2F-Luc, ARF642-Luc, and BIM-Luc. Since reporter assays using the p21 promoter have yielded complicated results, I
have deferred investigations of cyclins in this context to later efforts. In addition, I have identified a cyclin B/cdk1 consensus phosphorylation sites in the Trip-Br1 sequences (identified using KinasePhos software). I have performed mutational analyses to investigate whether Trip-Br1 may be phosphorylated and if so whether it may affect Trip-Br1 function.

5.2 Results

5.2.1. Trip-Br1 interacts with cyclin B.

Trip-Br1 interacts with the WDSV rv-cyclin and cellular cyclins A and D in yeast-2-hybrid and GST-pull-down assays (Yuan unpublished results). Since I identified a putative cyclin B/cdk1 phosphorylation sequence in Trip-Br1, I wanted to test whether Trip-Br1 interacts with cyclin B and to test for these interactions, I performed co-immunoprecipitation assays (Figure 30). Myc-tagged cyclin B was co-overexpressed with HA-tagged Trip-Br1 in 293T cells. Co-immunoprecipitation assays demonstrate that HA-Trip-Br1 co-immunoprecipitates with myc-cyclin B (Figure 30, lane 6). I included WDSV in these assays as a positive control.

Figure 30: Co-immuno-precipitation assays show that Cyclin B (CycB) interacts with Trip-Br1. Empty CMV-myc expression vector, myc-WDSV, and myc-CycB were co-overexpressed with HA-Trip-Br1. Left side of figure shows expression of input proteins. HA-Trip-Br1 co-immunoprecipitates with myc-WDSV and myc-CycB, but not with myc epitope (lower panel of right side of figure).
Once I established that Trip-Br1 interacts with cyclin B, I determined whether the cyclin B-Trip-Br1 interaction is dependent on the putative Trip-Br1 cyclin binding domain. To this end, I constructed a mutant Trip-Br1 that lacks the amino-terminal putative cyclin binding domain (designated TRIP1Δ17). The putative cyclin binding domain of Trip-Br1 protein and the alignment of the homologous region in E2F-family proteins are illustrated in Figure 31.

![Figure 31: Alignment of Trip-Br1 and E2F1 cyclin binding domain.](image)

Regions of homology are shaded. References are indicated in the figure.

To determine whether the interaction between cyclins and Trip-Br1 is dependent on the putative cyclin binding regions of Trip-Br1, I conducted GST-pull-down assays to test for interaction between wild-type and mutant proteins (Figure 32). GST-pull-down assays show that wild-type Trip-Br1 and cyclin B interact whereas cyclin B does not interact with TRIP1Δ17 (Figure 32 compare lanes 1 and 3). These data show that the
interaction between Trip-Br1 and cyclin B is dependent on the putative cyclin binding
domain of Trip-Br1. I anticipate that cyclin A interacts with Trip-Br1 in an analogous
fashion.

![Figure 32: Cyclin B interacts with Trip-Br1 in a Trip-Br1 cyclin-binding-domain-dependent manner.](image)

Purified GST-CycB and GST were combined with either myc-Trip-Br1 or myc-TRIP1Δ17. Row 1 shows coomassie stain of input GST-proteins. Row 2 shows expression of input myc-Trip-Br1 and myc-TRIP1Δ17. Row 3 shows that only myc-Trip-Br1 (not mycTRIP1Δ17) is pulled down by GST-CycB.

5.2.2. Trip-Br1 and cyclins A and B in transcriptional regulation

To begin to characterize the significance of the interaction between Trip-Br1 and
cyclins in transcriptional regulation, I performed a series of luciferase assays to determine
whether cyclins affect 6xE2F, ARF, and BIM expression and whether they affect the
function of Trip-Br1 in these contexts. I have analyzed cyclin A because Trip-Br1
activity may be affected by the cyclin A/cdk2 phosphorylation of E2F-1 in the E2F-1/DP-
1 complex (Krek et al. 1994). I have analyzed cyclin B because we show here that Trip-
Br1 interacts with cyclin B.
5.2.3. Cyclins and 6xE2F-Luc

To determine the effects of cyclins on Trip-Br1-mediated transactivation of a minimal E2F-responsive promoter, I conducted a series of luciferase assays using 6xE2F-Luc to address these questions (Figure 33). Transient transfection assays in HAM3 cells show that overexpression of cyclin A alone does not affect transactivation of 6xE2F1-luc (Figure 33A, compare lanes 1 and 2). Overexpression of cyclin B alone transactivates 6xE2F-Luc by 4-fold (Figure 33A, compare lanes 1 and 3). Overexpression of E2F-1/DP-1 transactivate 6xE2F-Luc by 15-fold (Figure 33B, lane 2). Overexpression of cyclin A or cyclin B with E2F-1/DP-1 transactivate 6xE2F-Luc by 10-and 21-fold, respectively, demonstrating that cyclin A limits the transactivation of 6xE2F-Luc by E2F-1/DP-1, while cyclin B adds to the transactivation of the promoter by E2F-1/DP-1 (Figure 33B, lanes 3 and 4).

To elucidate the effects of overexpression of cyclin A or B on the transactivation of 6xE2F-Luc by Trip-Br1 and its binding partners, E2F-1 and DP-1, and to test whether the cyclin effect is dependent on the Trip-Br1 cyclin-binding domain, I conducted a series of reporter assays. Overexpression of E2F-1/DP-1/Trip-Br1 transactivate 6xE2F-Luc by about 45-fold (Figure 33C, lane 2). Overexpression of cyclin A or cyclin B with E2F-1/DP-1/Trip-Br1 transactivate 6xE2F-Luc by 15-and 65-fold respectively, demonstrating again that cyclin A limits the transactivation of 6xE2F-Luc by E2F-1/DP-1/Trip-Br1, while cyclin B adds to the transactivation (Figure 33C, lanes 3 and 4).
Figure 33: Effects of cyclins A and B on Trip-Br1-mediated transactivation of 6xE2F-Luc. Overexpression of cyclin B transactivates 6xE2F-Luc in all contexts (see cyclin B overexpression in A-D), whereas overexpression of cyclin A limits E2F-1/DP-1 and E2F-1/DP-1/Trip-Br1-mediated transactivation of 6xE2F-Luc (see cyclin A overexpression in B-D). Results are averages of three experiments ± standard deviation.
To determine whether these effects are dependent on the Trip-Br1 cyclin-binding domain, I overexpressed E2F-1/DP-1/TRIP1Δ17 to transactivate 6xE2F-Luc by 45-fold (Figure 33D lane 2). These data demonstrate that TRIP1Δ17 is as efficient as the wild-type protein at transactivating the promoter. Overexpression of cyclin A or cyclin B with E2F-1/DP-1/TRIP1Δ17 transactivate 6xE2F-Luc by about 29-and 60-fold respectively (Figure 33D, lanes 3 and 4). Note that overexpression of E2F-1/DP-1/Trip-Br1/cyclinA transactivates 6xE2F-Luc by an average of 15-fold, whereas overexpression of E2F-1/DP-1/TRIP1Δ17/cyclinA transactivates the promoter by an average of 30-fold (compare Figure 33C, lane 3 to Figure 33D, lane 3). To further elucidate whether the difference between the behavior of cyclin A in the presence of Trip-Br1 or TRIP1Δ17 is significant and perhaps biologically relevant, we repeated these assays 7 times. These assays show that there is no statistical difference between the effects of cyclin A in the presence of Trip-Br1 or TRIP1Δ17 (Figure 34).
I therefore anticipate that the repression of the maximal promoter activity by cyclin A is mediated by phosphorylation of E2F-1 through mechanisms that have already been described. Further investigations will require construction of a cyclin-binding-deficient E2F-1 to test whether the repressive effect by cyclin A is mediated via the E2F-1 cyclin binding site.

5.2.4. ARF promoter and cyclins

I investigated the effects of overexpression of cyclins A and B on the E2F-1/DP-1/Trip-Br1-mediated transactivation of the p14/ARF promoter (ARF642-Luc) (Figure 35). To determine the optimal cyclin plasmid concentration for transfection for assaying...
cyclin effect on ARF(642)-Luc, I transfected either 20 or 200 ng of either CMV-cyclin A or CMV-cyclin B into HAM3 cells with ARF642-Luc and combinations of Trip-Br1 and its binding partners. Transient transfection assays show that overexpression from transfection of 200 ng of cyclin A or 20 or 200 ng of cyclin B induce ARF642-Luc by 5-fold for cyclin A, or 4- and 16-fold, respectively, for cyclin B. Overexpression from transfection of 20 ng of CMV-cyclinA inhibits promoter activity by 45%, demonstrating that at lower expression, cyclin A reduces promoter activity. Like 6xE2F-Luc, cyclin B activates ARF642-Luc alone (Figure 35A, compare lanes 1, 2, and 3 for cyclin A and lanes 1, 4, and 5 for cyclin B). Overexpression E2F-1/DP-1 transactivates ARF642-Luc by 15-fold and overexpression from transfection of 20 or 200 ng of cyclin A with E2F1/DP1 transactivate ARF642-Luc by 5-and 50-fold, respectively, demonstrating that moderate overexpression of cyclin A limits the transactivation of ARF642-Luc by E2F-1/DP-1 (Figure 35B, compare lanes 1, 2, 3, and 4).
Figure 35: Effects of transfection of 20 and 200 ng of cyclin A or 20 and 200 ng of cyclin B on Trip-Br1-mediated transactivation of ARF(642)-Luc. Overexpression of cyclin B transactivates ARF(642)-Luc in all contexts (see cyclin B overexpression in A-D), whereas overexpression from transfection of 20 ng of cyclin A limits E2F-1/DP-1 and E2F-1/DP-1/Trip-Br1-mediated transactivation of ARF(642)-Luc (see cyclin A(20ng) overexpression in B-D). Results are averages of at least 2 experiments ± standard deviation.
Overexpression from transfection of 20 or 200 ng of cyclin B with E2F1/DP1 transactivate ARF642-Luc by 15-and 80-fold respectively, demonstrating that overexpression of cyclin B can enhance ARF transactivation by E2F1/DP1 (Figure 34B, compare lanes 1, 2, 5, and 6). Overexpression of E2F-1/DP-1/Trip-Br1 or E2F-1/DP-1/Trip-Br1\(\Delta\)17 transactivates ARF642-Luc by approximately 45-fold (Figure 35 C and D, lanes 2 in each case). Overexpression from transfection of 20 or 200 ng of cyclin A or 20 or 200 ng of cyclin B with E2F-1/DP-1/Trip-Br1 transactivate ARF642-Luc by 18- and 65-fold, respectively, for cyclin A and 50- and 100-fold, respectively, for cyclin B, demonstrating again that moderate overexpression of cyclin A limits the transactivation of ARF642-Luc by E2F-1/DP-1/Trip-Br1, while cyclin B potentiates the transactivation (Figure 35C, lanes 1, 2, 3, 4 for cyclin A and lanes 1, 2, 5, 6 for cyclin B). Cyclin effects on E2F-1/DP-1/TRIP1\(\Delta\)17-mediated transactivation are similar to those for Trip-Br1 (Figure 35 D). Since Trip-Br1 and TRIP1\(\Delta\)17 behave similarly in these assays, these results demonstrate that cyclin activity is independent of the putative Trip-Br1 cyclin binding domain.

5.2.5. Cyclins and Bim promoter

To investigate the effects of overexpression of cyclins A and B on the E2F-1/DP-1/Trip-Br1-mediated transactivation of the BIM promoter I conducted a series of reporter assays testing activity from the BIM promoter (Figure 36).
Figure 36: Overexpression of cyclins A or B does not affect Trip-Br1-mediated transactivation of BIM-Luc. Results are averages of 2 experiments ± standard deviation.
Unlike cyclin overexpression in the contexts of 6xE2F-Luc and ARF642-Luc, in the context of the BIM promoter, overexpression of 20 ng of cyclin A or B did not affect expression of BIM. These data suggest that the effects of cyclins are promoter-specific.

5.3. Conclusions based on cyclins and reporter assays

I have confirmed that Trip-Br1-cyclin B binding is dependent on an intact Trip-Br1 cyclin binding domain. Reporter assays testing the effects of overexpression of cyclins on a series of promoters have shown that moderate overexpression of cyclin A limits transactivation of Trip-Br1/E2F-1/DP-1-mediated transactivation of 6xE2F-Luc and ARF(642)-Luc and this effect is independent of the Trip-Br1 cyclin binding domain. These promoters are transactivated by overexpression of cyclin B alone. I anticipate that this effect is due to a general stimulation of pro-cell-cycle arrest pathways due to excessive overexpression of cyclins. Therefore, rather than pursuing this approach to characterizing the significance of the interaction between cyclins and Trip-Br1, I decided to determine whether Trip-Br1 may be post-translationally modified by cyclin/cdk complexes.

5.4. Trip-Br1 is phosphorylated

I have shown that Trip-Br1 interacts with cyclin B. To further investigate the significance of this interaction, I investigated whether Trip-Br1 is phosphorylated and whether these modifications may involve cyclins and cdks. Supporting this notion, I have observed that Trip-Br1 migrates on SDS-PAGE gel as a doublet. To investigate whether the slower-migrating band is a phosphorylated version of Trip-Br1, I conducted phosphatase assays. Treatment of immobilized Trip-Br1 with lambda protein
phosphatase completely eliminated the slower-migrating band (Figure 37, compare banding pattern in phosphatase-treated to untreated Trip-Br1), showing that Trip-Br1 is phosphorylated.

![Image](image.png)

**Figure 37: Trip-Br1 is phosphorylated.** Treatment with lambda phosphatase eliminates slower migrating Trip-Br1 band.

To identify kinases that may phosphorylate Trip-Br1, I analyzed the Trip-Br1 sequence using KinasePhos software to probe for consensus phosphorylation sites. Interestingly, we identified a putative cyclinB/cdk1 phosphorylation consensus sequence with the phosphor-acceptor at serine 92 (Ser-92). Figure 38 shows alignment between consensus cyclin B/cdk1 phosphorylation sequence and putative site identified at position 92 in the Trip-Br1 amino acid sequence.

<table>
<thead>
<tr>
<th>Trip-Br1 sequence</th>
<th>88-PVP S-P-P-AAP-98</th>
</tr>
</thead>
<tbody>
<tr>
<td>p34cdc2 consensus</td>
<td>S/T-P-X-R/K</td>
</tr>
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Phosphoacceptor-change to alanine and to asparic acid


**Figure 38: Trip-Br1 consensus cyclin B-cdk1 phosphorylation site at serine 92 (Ser-92).**
To investigate the significance of the putative Trip-Br1 phosphorylation site, we constructed two mutant Trip-Br1 proteins that have an alanine Trip-Br1(S→A) or an aspartic acid Trip-Br1(S→D) in the place of Ser-92. We decided to change Ser-92 to alanine because alanine is a small amino acid that should not impact the structure of Trip-Br1 except at residue-92. We changed Ser-92 to aspartic acid because aspartic acid at this residue should mimic constitutive phosphorylation at Ser-92. To determine whether the putative cyclin B/cdk phosphorylation site affects the migration of Trip-Br1 on an SDS-PAGE gel and whether removal of the site affects Trip-Br1 function, we compared migration of wild-type Trip-Br1 and mutant phospho-Trip-Br1 proteins on an SDS-PAGE gel. Trip-Br1(S→A) migrates slower than the presumably unphosphorylated wild-type Trip-Br1 protein (Figure 39, compare wild-type Trip-Br1 in lane 1 to Trip-Br1(S→A) in lane 2). An upper band does not appear, indicating that the phosphorylation of this form of Trip-Br1 is eliminated with mutation of the residue. The lower band of Trip-Br1(S→D) migrates at the same rate as Trip-Br1(S→A), although Trip-Br1(S→D) shows a second band that migrates slightly faster than the wild-type, unphosphorylated Trip-Br1 protein (Figure 39, lane 3). The plasmids used to generate these proteins have been sequenced and they do not contain any additional mutations that would account for the differences in migration between the wild-type and phospho-mutants. The variability in migration rates may be due to conformational changes in the proteins that are not denatured in the standard SDS-PAGE preparation processes. We are investigating these observations further.
To determine whether phosphorylation of Ser-92 has an affect on the ability of Trip-Br1 to transactivate 6xE2F-Luc, I performed a series of luciferase assays to address this question (Figure 40). Transient transfection assays in HAM3 cells show that overexpression from transfection of 200 ng of TripBr1(S→A) or Trip-Br1(S→D) (with E2F-1 and DP-1) transactivates 6xE2F-Luc by 49-fold (Figure 40, lane 5). This value is comparable to the activity of wild-type Trip-Br1 in this context (see previous sections). These data demonstrate that mutations of Ser-92 in Trip-Br1 do not affect the activity of Trip-Br1 in the context of the 6xE2F-Luc promoter. We are investigating the behavior of these mutants with overexpression of cyclin, with overexpression of cdk dominant negative constructs and in the contexts of the p21 and ARF promoters to characterize the significance of the putative cyclinB-cdk1 phosphorylation site further.
5.4.1. Conclusions

We have demonstrated that Trip-Br1 is phosphorylated and have generated preliminary investigations of a putative cyclin B/cdk phosphorylation site at Trip-Br1 Ser-92. Mutant proteins migrate differently from wild-type proteins on SDS-PAGE gels, but are functionally similar in the context of the 6xE2F-Luc promoter.

5.5. Discussion

Co-immunoprecipitation and GST-pull-down assays show that cyclin B interacts with Trip-Br1. Mutational analyses demonstrate that the Trip-Br1-cyclin B interaction is
dependent on the Trip-Br1 cyclin binding domain. I anticipate that cyclin A interacts
with Trip-Br1 in an analogous fashion. In reporter assays, the Trip-Br1 mutant that lacks
the cyclin binding domain is as efficient at transactivating promoters as the wild-type.
The effects of overexpression of cyclins A and B on Trip-Br1-mediated promoter
transactivation is independent of the cyclin binding domain. We will investigate the role
of cyclinA/cdk2 phosphorylation of the E2F-1/DP-1 (Trip-Br1) complex on promoter
transactivation by constructing a mutant E2F-1 protein that lacks the cyclin A binding
site. Finally, I discovered that Trip-Br1 is phosphorylated and the functional significance
of this phosphorylation is to be determined.
6. SUMMARY OF MICROARRAY ANALYSES

Microarray analyses were performed to identify genes that are differentially expressed in cells with normal, relative to ectopic Trip-Br1 expression. I performed comparative microarray analyses in HAM4-8 (with inducible Trip-Br1) to HAM5-1 (without inducible Trip-Br1) cells induced with 0.5 µg/ml DOX for 48 hours. These analyses showed that 1767 genes were up-regulated 2-fold or more in cells overexpressing Trip-Br1 relative to cells with normal expression of Trip-Br1 and 1397 genes were down-regulated in response to overexpression of Trip-Br1. Figure 41 summarizes microarray analyses showing the number of genes up-regulated 20 to 100 fold (22 genes), 10 to 20 fold (133 genes), 5 to 10 fold (402 genes), and 2 to 5 fold (1210 genes) and down-regulated 2 to 5 fold (1012 genes), 5-10 fold (289 genes), 10 to 20 (76 genes), and 20 to 50 fold (20 genes) in response to overexpression of Trip-Br1.

![Figure 41: Summary of microarray data. 22 genes were up-regulated 20 to 100 fold, 133 genes 10 to 20 fold, 402 genes 5 to 10 fold and 1210 genes 2 to 5 fold. 1012 genes were down-regulated 2 to 5 fold, 289 genes 5 to 10 fold, 76 genes 10 to 20, and 20 genes 20 to 50 fold in response to overexpression of Trip-Br1.](image-url)
Of these genes, it was noted that Trip-Br1 up-regulates the pro-apoptotic Bcl2 family member BIK. BIK is an intriguing candidate to investigate further since it is involved in mitochondrial-dependent apoptotic pathways. BIK is induced in response to genotoxic stress (radiation; doxorubicin) and in response to over-expression of E1A or p53. BIK appears to recruit another Bcl2 family member, BAK, to the endoplasmic reticulum (ER) and mediates release of calcium from the ER to coordinate mitochondrial apoptosis in a BAX-dependent manner (Mathai et al. 2005). I therefore speculate that since BAX protein increase in response to Trip-Br1 overexpression, the up-regulation of BIK mRNA that is evident in the microarray analyses may contribute to Trip-Br1-induced, BIK-, and BAX-dependent mitochondrial apoptotic pathways. Further investigations are required to confirm expression patterns of BIK in response to Trip-Br1 expression and to elucidate the biological significance of this response.

Based on microarray analyses, p53, ARF, BIM and BAX are not transcriptionally regulated by Trip-Br1. These data are not surprising since we did not find transcriptional regulation from the native locus of p53 or BIM in our analyses described here. Also, ARF mRNA was only up-regulated (4-fold) in cells overexpressing Trip-Br1 and its binding partners E2F-1 and DP-1, and not in cells overexpressing Trip-Br1 alone. Unexpectedly, microarray data show that with ectopic expression of Trip-Br1 (induced by 0.5 ug/ml DOX for 48 hours), p21 mRNA is down-regulated by 13-fold relative to cells expressing endogenous Trip-Br1. These data were not anticipated given that both we and Watanabe-Fukunaga et al. 2005 reported Trip-Br1-mediated up-regulation of p21 mRNA in induced HAM4-8 cells relative to HAM5-1 cells. Recall, however, that a
series of our reporter assays show distinct down-regulation of Sp1/p300-induced transactivation of p21, suggesting that the Trip-Br1-mediated regulation of p21 is complicated and multi-faceted.
7. GENERAL DISCUSSION

I have conducted a thorough investigation into the function of Trip-Br1 and have identified it as a complicated and multifunctional integrator of cellular signals. Trip-Br1 has been reported to induce inappropriate cell division in some contexts and to reduce cell viability in other cell lines (Tang et al 2005; Watanabe-Fukunaga et al. 2005). I was intrigued by the putative tumor-suppressor functions of Trip-Br1 and pursued further investigations of these pathways. Watanabe-Fukunaga et al. (2005) attributed Trip-Br1-induced reduction in cell viability to accumulation of p53 protein and attributed this effect to a post-transcriptional mechanism. In agreement with these results, I have shown that Trip-Br1 does not affect the p53 promoter or p53 expression from the native locus, however, p53 protein was found to accumulate in p53 +/- cells in response to Trip-Br1. I have demonstrated that overexpression of Trip-Br1 induces increased p21 mRNA and protein in some cellular contexts and this effect is not dependent on p53. There are a number of mechanisms by which p21 is regulated transcriptionally, independently of p53 and we investigated details of the functional domains of the Trip-Br1 protein that are required for p21 regulation and other promoters involved in pro-cell-cycle arrest pathways. Of three potential mechanisms by which Trip-Br1 could feasibly contribute to the transcriptional regulation, I identified the heptad repeat as being critical in the regulation of an artificial E2F-responsive promoter, p21, and ARF. I also discovered that Trip-Br1 is phosphorylated and this may be critical in Trip-Br1 regulation. Further analyses are required in this area.
The importance of Trip-Br1 in cellular processes is highlighted by the number of genes that are affected when it is overexpressed as demonstrated by microarray analyses. The complexity of Trip-Br1 function is also underscored in the variability in reports in the literature regarding the effects of Trip-Br1 overexpression on cell proliferation. Table 2 summarizes these published results. For example, there are cell lines listed in Table 2 that are both p53 +/+ and p21 +/+ , and among these, are cases where overexpression of Trip-Br1 leads to cell death and others where overexpression of Trip-Br1 lends to proliferation (compare human HeLa to WI-38 cell lines). Additionally, WI-38 cells that are positive for both p16 and ARF, reportedly proliferate inappropriately with overexpression of Trip-Br1 (Sim et al. 2006). Also, U2OS cells which are defective at these loci proliferate longer in culture that HeLa derivatives overexpressing Trip-Br1, but still lose viability after prolonged Trip-Br1 induction (Watanabe-Fukunaga et al. 2005). WI-38 cells express functional Bcl2 family members BIM and BAX and they still appear to proliferate inappropriately in response to Trip-Br1 overexpression. The compendium of these data suggests that thorough understanding of Trip-Br1 function and its targets in terms of cell regulation are yet to be elucidated. I will make full use of microarray data generated in these investigations to gain insight into novel mechanism by which Trip-Br1 contributes to cell cycle regulation.

**Table 2: Outcome of overexpression of Trip-Br1 in cell lines tested to date.** + indicates wild-type, - indicates no function, NF indicates no reference found to determine gene status in cell line.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53</th>
<th>p21</th>
<th>p16</th>
<th>ARF</th>
<th>BIM</th>
<th>BAX</th>
<th>RB</th>
<th>Effect of ectopic expression of Trip-Br1</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HeLa</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Cell death (5 days)</td>
<td>Watanabe-Fukunaga et al. 2005; ATCC catalogue</td>
</tr>
<tr>
<td>2. U2OS</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Cell death (10 days)</td>
<td>Watanabe-Fukunaga et al. 2005; ATCC catalogue</td>
</tr>
<tr>
<td>3. NIH3T3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NF</td>
<td>NF</td>
<td>NF</td>
<td>Proliferation</td>
<td>Tang et al. 2005; Shibue 2006</td>
</tr>
<tr>
<td>4. 3Y1</td>
<td>Mut</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NF</td>
<td>NF</td>
<td>+</td>
<td>Proliferation</td>
<td>Sugimoto et al. 1999; Nakajima et al. 1998; Hui et al. 2004; Abe et al. 2002 Nakajima et al. 1992</td>
</tr>
</tbody>
</table>
Table 2 highlights the general notion that the function of Trip-Br1 and the outcome of Trip-Br1 overexpression are dependent on the cell context. Figure 41 summarizes this hypothesis. In cells that are normal for Trip-Br1 targets, overexpression of Trip-Br1 will give rise to cell death due to the appropriate signals from these downstream proteins (Figure 42, left panel). In cells where one or more of Trip-Br1 pro-cell-cycle arrest targets are mutated or deleted, up-regulation of Trip-Br1 will inappropriately signal for proliferation (Figure 42, right panel).

Figure 42: Schematic of general Trip-Br1 function in cell proliferation.
8. MATERIALS AND METHODS

8.1 Cell culture and growth assays

HeLa and HeLa and U2OS derivatives, 293T, and H1299 cells were maintained in 60mm x 15mm tissue culture dishes (Corning) in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) containing 10% Fetal Bovine Serum (FBS) and 0.5% penicillin/streptomycin/L-glutamine at 37°C with 5% CO₂. Cells were maintained by splitting 1:4 every two days into fresh media. DOX induction of HeLa and U2OS derivatives was performed by addition 0.5 or 1.0 ug/ml DOX in ddH₂O to media 12 hours after splitting cells.

For colony formation assays, approximately 1x10⁵ HAM4-8, PG8, HAM4-1, and vector cells were seeded into twelve well tissue culture plates. Cells were allowed to adhere for 12 hours at which time, 0.5 ug/ml of DOX was added to the culture medium. A set of replicate plates were left untreated and used as negative controls for the effect of DOX in each cell line. DOX media and DOX-free media were changed every 48 hours. Ninety-six hours after induction, adherent cells were stained with crystal violet stain (0.5% crystal violet in methanol) and the number the number of colonies formed in each case was recorded.

MTT cell viability assays were performed following manufacturer’s suggestions (ATCC). Cells were prepared as described above for colony formation assays. At 12, 48, 96, and 120 hours post-induction, HAM4-8, PG8, HAM5-1, and PG8 cells were treated with MTT reagent, and reduction reactions allowed to proceed for four hours at which time a detergent cell lysis buffer was added. Absorbance of cell extracts was
measured on a Biomate 3 Spectrophotometer (ThermoSpectronic) at 570nm. Mean absorbances at each time point were analyzed using a one-way ANOVA using SPSS software.

8.2 Transient transfection

Cells were split and diluted 24 and 48 hours prior to transfection to ensure that cells are actively dividing at the time of transfection and to minimize experiment to experiment variability. Forty-eight hours before transfection, cells were trypsinized, triterated thoroughly, and split and diluted 1:3 into fresh media. Twenty-four hours before transfection, cells were split and diluted from 1:4 to 1:6 into antibiotic-free media. Transfections were done at about 70% confluency and carried out following manufacturer’s specifications (Invitrogen Lipofectamine transfection reagents). Briefly, DNA was diluted into 200 ul of Opti-MEM low-serum medium (Gibco) and combined with 8 ul of PLUS reagent. After 15 minutes incubation at room temperature, 200 ul of Opti-MEM with 10 ul of Lipofectamine reagent was added to each reaction. Reactions were incubated at room temperature for 15 minutes and during this time, the antibiotic-free media was removed from cells and replaced with 1.8 ml of fresh Opti-MEM. After the 15 minute incubation, DNA solutions were added to the cells drop-wise. Cell mixtures were incubated at 37°C in 5% CO₂ for 3 hours at which time, the 2 ml reaction mixture was removed from the cells and replaced with 4 ml of DMEM with antibiotics.

8.3 Preparing cell extracts

For immunoblotting and luciferase assays, media was removed from cultured cells and cells were placed on ice and washed twice with cold PBS. Cold Passive Lysis
Buffer (Promega) was added and cells were incubated on ice for 15 minutes. Cell lysates were removed from culture dishes and placed into cold, 1.5 ml eppendorf tubes. Cell lysates were vortexed and for luciferase assays, centrifuged for 30 seconds and 300 ul of supernatant transferred to a new tube and stored at -70°C until assayed. For immunoblotting, 200 ul of cell extract was transferred to an new tube and combined with 200 ul of 2X SDS PAGE buffer (100mM TrisHCL pH 6.8, 200mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and boiled for 5 minutes, and DNA sheared by passing lysate through syringe and samples stored at -70°C.

**8.4. Western Blot**

Approximately 2x10^6 cells were lysed with 400 ul of lysis buffer (Invitrogen) and combined with 2X SDS PAGE buffer and boiled as described above. Ten to 30 ul of sample was loaded onto a 12% SDS-PAGE gel and electrophoresed for 1.5 to 2 hours at 150 volts in Tris-Glycine buffer. Gels, filter paper and PVDF membrane were equilibrated in transfer buffer (48mM Tris, 39mM glycine, 15% methanol) for 15 minutes prior to transfer. Protein transfer was accomplished using the Transblot Semi-Dry transfer cell (Biorad) following manufacturer’s guidelines (30 minutes at 10 V).

After transfer, membranes were blocked with 10% nonfat milk in PBS or 1% BSA in Tris containing 0.1% Tween 20 for 1 hour at room temperature with gentle agitation. Membranes were incubated with the appropriate primary antibodies (see Table 2 for dilution and incubation profiles). Secondary antibodies included HPR-conjugated antirabbit, antimouse, or antigoat and blots detected using enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).
Table 3: List of antibodies used in these investigations.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution reagent</th>
<th>Working dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-myc epitope (mouse, monoclonal)</td>
<td>1% dry milk in PBST</td>
<td>1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-p53 (mouse, monoclonal)</td>
<td>1% dry milk in PBST</td>
<td>1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-p21 (rabbit, polyclonal)</td>
<td>1% dry milk in PBST</td>
<td>1:750</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti β-tubulin (mouse, monoclonal)</td>
<td>1% dry milk in PBST</td>
<td>1:750</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-Trip-Br1 (goat, polyclonal)</td>
<td>1% dry milk in PBST</td>
<td>1:500</td>
<td>Imagenex</td>
</tr>
<tr>
<td>anti-DP1 (mouse, “power”-clonal)</td>
<td>1% dry milk in PBST</td>
<td>1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-BAX (rabbit, monoclonal)</td>
<td>1% dry milk in PBST</td>
<td>1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-BIM</td>
<td>1% dry milk in PBST</td>
<td>1:500-1:1500</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>anti-p300 (mouse, monoclonal)</td>
<td>1% dry milk in PBST</td>
<td>1:20 000</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-PHOS (mouse, monoclonal)</td>
<td>BSA in Tris</td>
<td>1:1500</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-E2F1 (mouse, monoclonal)</td>
<td>1% dry milk in PBST</td>
<td>1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Anti-Sp1 (mouse, monoclonal)</td>
<td>1% dry milk in PBST</td>
<td>1:1000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Goat anti-rabbit HRP</td>
<td>1% dry milk in PBST</td>
<td>1:2000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mouse anti-goat HRP</td>
<td>1% dry milk in PBST</td>
<td>1:2000</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Sheep anti-mouse HRP</td>
<td>1% dry milk in PBST</td>
<td>1:2000</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>
8.5. Plasmid construction

The CMV-myc and pBluescriptSK- vectors were obtained from Clontech. CMV-myc-hTripBr1 was constructed by PCR amplifying the full human Trip-Br1 cDNA from a human Trip-Br1 cDNA (provided by Rikiro Fukunaga) and ligating the fragment into CMV-myc EcoRI to Acc65I. CMV-myc-TRIP1Δ17 and CMV-myc-TRIP1ΔAcD were constructed by PCR-amplifying bases 51-701 and 1-501, respectively, of the Trip-Br1 sequences and ligating them into CMV-myc EcoRI to Acc65I. Sequences of amplification primers are provided in table 4.

pRL-TK renilla (Promega) was used as a transfection efficiency control for luciferase assays. The p53 promoter region (-426 to +106) (genbank gi189463) was amplified from a sample of human genomic DNA and cloned into the pGL3 Basic vector (Promega) Acc65I to NcoI. Dr Susan Evans provided a cDNA clone of the human p53 gene (pC53NN). 6xE2F-Luc, CMV-E2F-1, and CMV-DP1 were provided by Stephen I-Hong Hsu and Joseph V. Bonventre. GST-DP1 was constructed by PCR-amplifying the full DP-1 cDNA from CMV-DP1 and cloning the fragment into pGEX6P3 (provided by Dr. Mark Berryman) EcoRI to XhoI. pCGN-CMV-Sp1, GST-p300aa744-1540 and pCMV-p300 were obtained from Michael Lairmore. p21mut1, p21(100), p21(800), and p21(1200) were obtained from Xiao-Fan Wang. pGL3-ARF(642)-luc, pGL3-ARF(151)-luc and cyclin A and B expression vectors were provided by Sang-Woo Kim. pGL2-BIM-Luc was provided by Subhas Biswas.
Table 4: List of PCR amplification primers used in these investigations.

<table>
<thead>
<tr>
<th>Primers for amplification</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trip-Br1</td>
<td>SE11ECOR15'-TTTTTTGAATTCGATGAGCAAGGGTCTGA&lt;br&gt;SE11ACC65I3'-TTTTTTGGTACCGCCCTGAGCCCCG</td>
</tr>
<tr>
<td>TRIP1Δ17</td>
<td>SE11DELTA175'-TTTTTTGAATTCGATGAGCAAGGGTCTGA&lt;br&gt;SE11NLSDELTA175'-&lt;br&gt;TTTTTTGAATTCGATGAGCAAGGGTCTGA&lt;br&gt;SE11ACC65I3'-TTTTTTGGTACCGCCCTGAGCCCCG</td>
</tr>
<tr>
<td>TRIP1ΔAcD</td>
<td>SE11ECOR15'-TTTTTTGAATTCGATGAGCAAGGGTCTGA&lt;br&gt;SE11DELTAacD3'-TTTTTTGAATTCGATGAGCAAGGGTCTGA</td>
</tr>
<tr>
<td>GST-DP-1</td>
<td>DP1GST5'-TTTTTTGAATTCGATGAGCAAGGGTCTGA&lt;br&gt;DP1GST3'-TTTTTTGATGTCATGCATGCATGCATGCATGCC</td>
</tr>
</tbody>
</table>

8.6. Site-Directed Mutagenesis

Trip-Br1 mutants, TRIP1ΔHEP, TRIP1ΔTHD2, Trip-Br1(S→A) and Trip-Br1(S→D) and DP-1 mutant DP-1ΔHEP were constructed using the Kunkle method of site-directed mutagenesis (Kunkle 1985; Kunkle et al. 1987). For construction of Trip-Br1 mutants, full-length Trip-Br1 was subcloned EcoRI to Acc65I from CMV-myc-Trip-Br1 into pBluescriptSK- to construct pBSK-Trip-Br1. pBSK-Trip-Br1 was transformed into CJ236 [E.coli strain that lacks dUTPase (dut-) and uracil deglycosidase (ung-) and incorporates dUTP into DNA]. CJ236 harboring pBSK-Trip-Br1 was superinfected with bacteriophage M13K07 (NEB Biolabs). Packaged ssDNA plasmid was isolated from virions using the Biolab 101 ssDNA isolation kit. In-vitro primer extension reactions were performed by combining the U-containing pBSK-Trip-Br1 with the appropriate mutagenic oligonucleotide (see Table 4 for primers used in mutagenesis reactions) with Buffer A (0.2 M Tris-HCl (pH 7.5), 0.1 M MgCl₂, 0.5 M NaCl, 0.01 M dithiothreitol...
Reactions were heated to 65°C and cooled slowly to room temperature. The primer extension reaction was performed by incubating the annealing reaction with dNTPs (BioRad), Klenow polymerase, DNA ligase (NEB Biolabs), ATP (Sigma) and Buffer B (0.2 M Tris-HCL (pH 7.5), 0.1M MgCl₂, 0.1 M DTT). Primer extensions reaction were incubated at 14°C overnight and transformed into DH5α. Colonies were screened for desired mutation. Colonies putatively harboring plasmids with the desired mutation were grown in liquid culture and plasmids isolated using the Wizard® (Promega) plasmid mini-prep kit. Fragments carrying mutation were subcloned from pBluescriptSK- to CMV-myc EcoRI to Acc65I for expression in mammalian cells. For construction of DP-1ΔHEP, DP-1 was PCR-amplified from CMV-DP-1 and ligated EcoRI to XhoI into pBluescriptSK-. After mutagenesis reaction, mutant DP-1 fragment was subcloned into pGEX-6P3 for expression in BL-21 for protein overexpression. All plasmid constructed were sequenced at the Ohio University Genomics Facility.

Table 5: List of mutagenic primers used in these analyses.

<table>
<thead>
<tr>
<th>Mutagenic Primers:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delta HEPTAD-Phos-GCACCCCCGCCCCGTGGCCTCTAGATCTATCCAGGCCTGTCATG</td>
</tr>
<tr>
<td>Delta THD-2-Phos-AGTTGGCGCTGACAATTACTGGCAAGATCTGAGCGCCAGCTG</td>
</tr>
<tr>
<td>DP1MUT-Phos-GGTTGGCCGGCTGGCCTGGGTACCCAGACCAATCCACTTGATCTC</td>
</tr>
<tr>
<td>DELTADC2-Phos-CCACCTGTCCTGGCCACCTGCAGCCCCAGTGGTGCTCAGAACCTTACTGC</td>
</tr>
<tr>
<td>DETACDC2(2)-Phos-GAGGACCTCAGCCACATTGAGCTCCTGGCTAGGGCTCCCAACCTTGCTGCA</td>
</tr>
</tbody>
</table>
8.7. GST-pull down assays

GST-pull-down assays were performed using the ProFound™ Pull-Down GST Protein:Protein Interaction Kit (Pierce). GST-tagged proteins were expressed using BL21 Star™ (De3)pLysS competent cells (Invitrogen). pGEX6P3 plasmids were transformed into BL21 and 50-300 ml of culture grown to log phase and induced with 100mM IPTG to promote GST-fusion protein expression. BL21 cells were collected by centrifugation and GST fusion proteins isolated according to manufacturer’s specifications (Pierce). Briefly, 5-15 ml of bacterial cells expressing GST-fusion proteins were collected and lysed into 200-600 ul of ProFound® lysis buffer. 50 ul of Glutathione agarose beads was added to 300 ul of cleared cell lysate and incubated at 4°C for four hours. Glutathione agarose beads were collected and washed 7-10 times with cold TBST. Myc-tagged “prey” proteins were overexpressed in HAM3 cells. Transfected cells were lysed into 400 ml of ProFound® lysis buffer and 300 ul of cleared HAM3 cell lysate added to GST-tagged fusion proteins immobilized on glutathione beads. “Bait”-“prey” reactions were incubated with gentle agitation at 4°C for 4-12 hours. Beads were washed 7-15 times with TBST and 200 ul of SDS-PAGE loading buffer added to immobilized protein complexes. Samples were heated to 95°C for 5 minutes and electrophoresed on 12% gel and proteins detected by western blot using appropriate antibodies.

8.8. Co-Immunoprecipitation

1 ug of empty c-myc vector, myc-cyclin B or myc-WDSV was co-transfected with 1 ug of HA-Trip-Brl into 293T cells. Whole cell extracts were prepared by brief sonication and cell debris cleared by centrifugation at 4°C for 10 min. Anti-C-myc
monoclonal antibody agarose beads (Clontech) were added to 500 ul of cell extract and reactions incubated overnight with gentle agitation. Agarose beads were collected by centrifugation and washed five times with 1 ml TBST buffer. 200 ul of SDS-PAGE loading buffer added to immobilized protein complexes. Samples were heated to 95°C for 5 minutes and electrophoresed on 12% gel and proteins detected by western blot using appropriate antibodies.

8.9. Phosphatase assays

Phosphatase assays were performed by overexpressing 1 ug myc-Trip-Br1 or myc-TRIPΔ17 in HAM3 cells, immunoprecipitating myc-tagged proteins and treating immobilized proteins with lambda protein phosphatase. Myc-Trip-Br1 and myc-TRIP1Δ17 were immunoprecipitated from solution using the Anti-c-Myc Immunoprecipitation Kit (Sigma). Cells were lysed into 400 ul of lysis buffer following manufacturer's specifications. Twenty ul of mouse anti-c-Myc agarose beads were added to cell lysates and mixtures incubated at 4°C for four hours with gentle agitation. Agarose beads were collected into IP-spin columns and washed five times with 700 ul of 1xIP buffer followed by two washes with 700 ul of 0.1xIP buffer. Immobilized proteins were collected on the IP-spin columns and 2 units of lambda protein phosphatase (λ-PP) in 50 ul buffer (NEB biolabs) added to immobilized proteins (negative controls were exposed to phosphatase buffer only, no phosphatase) for 2 hours at 30°C. λ-PP and buffer were removed and immobilized myc-tagged proteins washed in 0.1xIP buffer and 100 ul SDS-PAGE buffer added. λ-PP-treated and untreated immobilized proteins were
boiled for 5 minutes and electrophoresed on a 12% SDS-PAGE and proteins detected using anti-c-myc antibody.

8.10. Luciferase assays

Luciferase assays were performed 48 h after transfection using Dual Luciferase Assay System (Promega) following manufacturer’s instructions. The activity of the firefly luciferase was measured using a Turner TD-20/20 luminometer. Luciferase activity was standardized against the activity of the control Renilla luciferase reporter, pRL-CMV (10 ng). The data were normalized by dividing the luciferase units by the renilla units (luciferase units/renilla units). Data reported are the average of at least two independent experiments and average deviation reported.

8.11. PCR

PCR amplification was performed using the iTaq™ DNA polymerase kit (BioRad).

8.12. Realtime PCR

Cells were lysed and RNA extracted using the AURUM Total RNA Isolation Mini kit (Bio-Rad). RNA concentration was measured using the 260nm/280nm method on a Biomate 3 Spectophotometer (ThermoSpectronic). cDNA for realtime PCR was constructed from 1ug total RNA using the cDNA archive cDNA synthesis system (ABI). Realtime PCR was performed using the TAQMAN system (ABI) and analyzed using the iCycler iQ thermal cycler (BioRad). Relative mRNA levels were normalized to GAPDH mRNA levels using the geNorm system of mRNA normalization based on the delta-delta ct method (Vandesompele et al. 2002). Table 6 is a list of probes used in these analyses.
Table 6: Taqman probe sets used for realtime PCR analyses.

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Taqman probe ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>p14</td>
<td>Hs00172983_m1</td>
</tr>
<tr>
<td>p21</td>
<td>Hs00233365_m1</td>
</tr>
<tr>
<td>p53</td>
<td>Hs00153349_m1</td>
</tr>
<tr>
<td>BAX</td>
<td>Hs00180269_ml</td>
</tr>
<tr>
<td>BIM</td>
<td>Hs00197982_m1</td>
</tr>
<tr>
<td>Trip-Br1</td>
<td>Hs00203547_m1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>4333764T</td>
</tr>
</tbody>
</table>

8.13. Microarray

Two plates each of approximately 1x10⁶ HAM4-8 and HAM5-1 were seeded into 60 mm tissue culture dishes and allowed to adhere for 12 hours at which time they were induced with 0.5 ug/ml DOX. Forty-eight hours after induction, RNA was extracted using the AURUM total RNA extraction kit (BioRad). RNA quality and microarray analyses were performed at the Ohio State University Comprehensive Cancer Center Core Facility (OSUCCCF). RNA quality was analyzed using an Agilent Bioanalyzer 2100. Microarray analyses were performed using the Human Genome U133 Plus 2.0 Array which is a whole human genome expression array.
9. REFERENCES


