RhoBTB2 (DBC2) Is a Mitotic E2F1 Target Gene with a Novel Role in Apoptosis*

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We have identified the RhoBTB2 putative tumor suppressor gene as a direct target of the E2F1 transcription factor. Overexpression of E2F1 led to up-regulation of RhoBTB2 at the level of mRNA and protein. This also occurred during the induction of E2F1 activity in the presence of cycloheximide, thus indicating that RhoBTB2 is a direct target. RNAi-mediated knockdown of E2F1 resulted in decreased RhoBTB2 protein expression, demonstrating that RhoBTB2 is a physiological target of E2F1. Because E2F1 primarily serves to transcribe genes involved in cell cycle progression and apoptosis, we explored whether RhoBTB2 played roles in either of these processes. We found RhoBTB2 expression highly up-regulated during mitosis, which was partially dependent on the presence of E2F1. Furthermore, overexpression of RhoBTB2 induced a short term increase in cell cycle progression and proliferation, while long term expression had a negative effect on these processes. We similarly found RhoBTB2 up-regulated during drug-induced apoptosis, with this being primarily dependent on E2F1. Finally, we observed that knockdown of RhoBTB2 levels via siRNA delayed the onset of drug-induced apoptosis. Collectively, we describe RhoBTB2 as a novel direct target of E2F1 with roles in cell cycle and apoptosis.

The Rb-E2F pathway is a critical regulator of molecular mechanisms governing various aspects of cell proliferation, differentiation, and survival (for review, see Refs. 1–5). Indeed, the Rb-E2F pathway is aberrantly regulated in some fashion in almost every instance of human malignancy (for review, see Refs. 6). One result of Rb–E2F pathway deregulation is unstrained activation of E2F target genes, which can contribute to oncogenic transformation (3). Likewise, many identified E2F target genes play direct roles in the biological effects associated with deregulation of the Rb-E2F pathway (7, 8). Yet while many crucial E2F targets associated with this biological phenotype have been identified, many more remain to be characterized.

Nine E2F family members have been identified thus far (E2F1–8), with E2F3 having two variants (E2F3A and E2F3B) (9–26). The E2F family members can be loosely divided into three different classes based on structure and function, although recent reports have weakened this simplistic view of the E2F family when basing classification solely on function (2). E2F1 is a member of the first class of E2Fs, which consists of E2F1, -2, and -3A, and are commonly referred to as the growth promoting E2Fs. In a simplified view, these E2Fs primarily serve to activate the transcription of genes necessary for DNA replication and cell cycle progression. However, E2F1 is somewhat unique within the family in that it can also promote apoptosis (27–29) by directly inducing the transcription of proapoptotic genes, as well as contributing to the active repression of antiapoptotic genes (for review, see Ref. 8). Recent studies have also demonstrated roles for E2F1 in the DNA damage response and checkpoint control (for review, see Ref. 30). Given the importance of E2F1 in proliferation, DNA damage/checkpoint control and apoptosis, it is crucial to identify target genes that mediate these effects.

RhoBTB2, or Deleted in Breast Cancer 2 (DBC2), is a putative tumor suppressor gene whose activity has been found to be altered in human malignancy by means of deletion or loss of heterozygosity (31–37), down-regulation (38), or point mutation (38, 39). RhoBTB2 is an atypical Rho GTPase, with a conserved Rho GTPase domain at the N terminus, followed by two BTB domains, which are presumably involved in protein–protein interactions. Biological studies of RhoBTB2 activity have demonstrated that overexpression of RhoBTB2 can lead to growth inhibition in breast cancer cell lines, whereas point mutants derived from primary tumors have lost this ability (38). Further studies employed a microarray-based network analysis approach and found that alteration of RhoBTB2 levels influences pathways responsible for cell cycle, apoptosis, cytoskeleton, and membrane-trafficking (40).

While the role of RhoBTB2 as an inhibitor of proliferation and putative tumor suppressor is clear, its mechanism of action is not. An elegant study by Wilkins et al. (39) identified RhoBTB2 as a substrate for the Cul3 ubiquitin ligase complex, and that point mutants of RhoBTB2 derived from human malignancy were unable to bind Cul3, therefore elevating RhoBTB2 expression due to decreased degradation. A more recent study looking into the biochemistry behind the growth inhibitory effect of RhoBTB2 in breast cancer samples identified cyclin D1 as being down-regulated following RhoBTB2
overexpression, suggesting a molecular target for the growth inhibitory effect of RhoBT2 (41). Whether cyclin D1 is a direct or indirect target of RhoBT2/Cul3 remains unclear.

While deletion and point mutations account for a small fraction of disruptions of RhoBT2 function in malignancy, to date no other molecular regulators of RhoBT2 besides Cul3 have been identified. Given the importance of RhoBT2 with its association to carcinogenesis, it is of great benefit to identify mechanisms that regulate its expression. In this report, we identify RhoBT2 as a novel transcriptional target of E2F1. We demonstrate that overexpression of E2F1 directly activates RhoBT2 expression, and that knockdown of E2F1 decreases the expression of RhoBT2, thus indicating that E2F1-mediated activation of RhoBT2 is a physiologically relevant event. Furthermore we show that RhoBT2 is up-regulated during mitosis, as well as during drug-induced apoptosis, and that this activation is partially and primarily dependent of E2F1, respectively. Finally, we demonstrate that RhoBT2 has active roles in E2F-mediated processes of cell cycle progression and apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**—The H1299 cell line was a gift from Dr. Jiandong Chen (Moffitt Cancer Center, Tampa, FL) and cultured in DMEM supplemented with 2 mM L-glutamine, 5% fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S). The MCF7 and MCF10A mammary fibrocytic cell lines were a gift from Dr. Richard Jove (City of Hope, Duarte, CA) and were cultured in DMEM-F12 supplemented with 2 mM L-glutamine, 10% FBS and 1% P/S. The T98G glioblastoma cell line was a gift from Dr. Joseph Nevins (Duke University, Durham, NC) and grown in DMEM supplemented with 2 mM L-glutamine, 10% FBS, and 1% P/S. The H1299-pBS/U6 and H1299-shE2F1 cell lines were constructed and cultured as previously described (42–44). The H1299-ER-E2F1 cell line was constructed and cultured as previously described (42–45).

**Adenovirus**—The Ad-GFP and Ad-E2F1 adenovirus were kind gifts from Dr. Timothy Kowalik (University of Massachusetts, Worchester, MA) (46, 47). The Ad-E2F1 (1–283) adenovirus was constructed as previously described (48). Both the Ad-E2F1 and Ad-E2F1 (1–283) adenovirus express GFP from an independent CMV promoter. The Ad-RhoBT2 adenovirus was constructed using a cDNA construct of RhoBT2 with an N-terminal 3×FLAG sequence and a C-terminal Myc tag. The entire double-tagged sequence was used for virus construction with the Stratagene AdEasy™ Adenoviral Vector System using the pShuttle-ires-hrGFP-1 vector following the manufacturer’s protocol. Titering was conducted using the Stratagene AdEasy™ Viral Titer Kit.

**Real-Time PCR**—Total cell RNA was harvested using the RNeasy Mini Kit (Qiagen) using the optional DNase treatment. Reverse transcriptase (RT) reactions were random hexamer-primed using Applied Biosystems’ (Foster City, CA) High Capacity cDNA Archive kit. Standard curves were constructed using serial dilutions of pooled sample RNA (50, 10, 2, 0.8, 0.4, and 0.08 ng) per reverse transcriptase reaction. One “no reverse transcriptase” control was included for the standard curve and for each sample.

**TaqMan® Gene Expression Assays (Applied Biosystems)** were used. The assay primer and probe sequences are proprietary. TaqMan® probe Hs01598093_g1 was used for RhoBT2. Real-time quantitative PCR analyses were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All standards and samples were tested in triplicate wells. The no template control (H2O), no RT controls, no amplification control (Bluescript plasmid), and no RNA control were tested in duplicate wells. PCR was carried out with the Taqman® Universal PCR Master Mix (Applied Biosystems) using 2 μl of cDNA and 1× primers and probe in a 20-μl final reaction mixture. After a 2-min incubation at 50 °C, AmpliTaq Gold was activated by a 10-min incubation at 95 °C, followed by 40 PCR cycles consisting of 15 s of denaturation at 95 °C and hybridization of probe and primers for 1 min at 60 °C.

Data were analyzed using SDS software version 2.2.2 and exported into an Excel spreadsheet. The 18 S data were used for normalizing the gene values, ng gene/ng 18 S per well.

**RhoBT2 Antibody Production**—Affinity-purified rabbit polyclonal antibody was generated toward a peptide corresponding to human RhoBT2 amino acids 673–687 (KEEDHYQRARKEREK) by Pacific Immunology (Ramona, CA). Specifically, a 16-amino acid peptide (CKEEDHYQRARKEREK) was conjugated (via an artificial N-terminal cysteine residue) to keyhole limpet hemocyanin and used to immunize rabbits. Serum was subjected to peptide column affinity purification prior to use in immunofluorescence. Antibody specificity was demonstrated using a previously described RhoBT2 siRNA (40).

**Plasmids, siRNA, and Transfections**—The RhoBT2 shRNA vector (V2H5-256373) and empty control pSM2C were purchased from Open Biosystems. RhoBT2 siRNA was custom made (Ambion) using a previously published RhoBT2 siRNA (DBC2-γ) sequence (40). siCONTROL non-targeting siRNA (Dharmacon) was used for all negative controls. The siRNA was transfected using Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s protocol. The pBB14 membrane GFP plasmid was a kind gift from Dr. L. W. Enquist (Princeton), constructed as previously described (49) and transfected with Lipofectamine™ 2000 following the manufacturer’s protocol.

**Immunofluorescent Microscopy**—Cells were grown on Lab-Tek® II Chamber Slides™ (Nunc), fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X, then blocked with 2% bovine serum albumin in PBS. The primary RhoBT2 antibody was used at a 1:40 concentration, and the secondary antibody was Alexa Fluor® 555 goat anti-rabbit Ig antibody (Molecular Probes) at a concentration of 1:2000. Cover slips were mounted using ProLong® Gold antifade reagent with DAPI (Molecular Probes). Samples were viewed with a fully automated, upright Zeiss Axio-ImagerZ.1 microscope with a ×40 or ×63/1.40NA oil immersion objective, and DAPI, FITC, and Rhodamine filter cubes. Equal exposure times were used for each sample. Images were produced and quantified using the AxiosCam MRm CCD camera and Axiovision version 4.5 software suite.

**Flow Cytometry**—Triplicate samples of cells were detached from culture plates via trypsin, washed twice with PBS, and then fixed in 70% ethanol. The fixed cells were washed twice with PBS and treated with RNase A and propidium iodide (PI). PI staining was used to measure for cell cycle status using a Becton-Dickinson FACScan instrument and Cell Quest software.
**BrdU Incorporation Assays**—For adenovirus-based experiments, cells were infected at the time of plating with equal amounts of adenovirus and BrdU was added 24 h prior to the experimental time point. BrdU incorporation assays were performed at the indicated time points using a Chemicon® BrdU Cell Proliferation Assay kit following the published protocol.

**MTS Assays**—For siRNA-based experiments, cells were first transfected as described under “Results,” trypsinized after 24 h, counted, then plated in triplicate in 96-well plates. The specific drug treatments were then administered 24 h later, and the MTS assays were conducted using a Promega CellTiter 96® AQuesus One Cell Proliferation Assay kit following the published protocol. For adenovirus-based experiments, cells were infected at the time of plating with equal amounts of adenovirus, with MTS assays being conducted as previously noted.

**RESULTS**

**E2F1 Overexpression Up-regulates RhoBTB2**—Using a microarray screen, we sought to identify novel targets of the E2F1 transcription factor. In this approach, we infected the H1299 cell line with adenovirus expressing either a green fluorescent protein control construct (Ad-GFP) or an E2F1 cDNA construct (Ad-E2F1). RNA was harvested at 24 and 48 h and processed for microarray analysis. Among the list of genes whose transcripts were found to be highly induced upon adenovirus-mediated overexpression of E2F1 was RhoBTB2.

To confirm the microarray results, we infected H1299s with either Ad-GFP, Ad-E2F1, or Ad-E2F1-(1–283), a deletion mutant of E2F1 that is lacking the pRb-binding/transactivation domain (50). Using real-time polymerase chain reaction (PCR) to quantify RhoBTB2 mRNA expression, we found that Ad-E2F1 infection does indeed induce RhoBTB2 transcript 5- and 20-fold compared with that of Ad-GFP infection at the 24- and 48-h time points, respectively (Fig. 1A). Lack of RhoBTB2 induction by Ad-E2F1-(1–283) infection confirms that up-regulation of RhoBTB2 by E2F1 is dependent on the E2F1 C-terminal transactivation domain.

Because the experiments conducted to this point employed the H1299 cell line, we sought to ensure that up-regulation of RhoBTB2 mRNA expression by E2F1 was not cell line-dependent. To this end, we infected the T98G and MCF7 cell lines with...
either Ad-GFP or Ad-E2F1 and conducted real-time PCR as in the prior experiment. We observed up-regulation of RhoBTB2 upon Ad-E2F1 infection similar to that observed in H1299s, thus confirming that up-regulation of RhoBTB2 expression by E2F1 is not cell line-specific (Fig. 1B).

To conduct protein-based studies of RhoBTB2, we raised a polyclonal antibody against a 15 amino acid peptide sequence located within the C terminus. While the antibody was very poor at recognizing endogenous RhoBTB2 protein in a denatured state by Western blot, we were able to visualize endogenous RhoBTB2 protein via immunofluorescent microscopy (IFM) (Fig. 1C, top). To verify that the observed signal was specific for RhoBTB2, we transiently knocked-down RhoBTB2 expression using siRNA (40) and stained for RhoBTB2 using IFM. We found that transfection of siRNA targeted toward RhoBTB2 diminished the observed RhoBTB2 signal by \( \frac{1}{2} \), providing evidence that the antibody is indeed specific for RhoBTB2 (Fig. 1C).

We additionally verified antibody specificity by Western blot to provide a second independent measure of specificity. Since the antibody is poor at recognizing endogenous RhoBTB2 via Western blot, we utilized transiently overexpressed RhoBTB2 as a proxy. As shown in the first lane, no RhoBTB2 signal was observed in non-transfected cells, however, a band corresponding to RhoBTB2 was clearly evident upon transient transfection of a RhoBTB2 expression vector (Fig. 1D). This signal was diminished upon co-transfection of increasing amounts of a short-hairpin inhibitory RNA vector targeted toward RhoBTB2. Collectively, these experiments demonstrate that our polyclonal antibody is specific for RhoBTB2.

Having an antibody functional for RhoBTB2 protein quantification, we sought to determine if the observed up-regulation of RhoBTB2 mRNA by E2F1 overexpression resulted in a corresponding increase of RhoBTB2 at the level of protein. To this end, an HA-tagged E2F1 expression vector (HA-E2F1), as well as a GFP-expression vector were co-transfected into RhoBTB2 expression. Upon staining for RhoBTB2, GFP-positive and -negative cells were used to select for transfected and non-transfected cells, respectively. We found that cells positive for GFP (transfected) expressed a substantially higher level of RhoBTB2 protein as compared with adjacent GFP-negative cells (Fig. 1E), thus confirming that E2F1 overexpression also results in increased expression of RhoBTB2 protein. Taken together, these results demonstrate that RhoBTB2 is up-regulated at the level of both mRNA and protein upon E2F1 overexpression.

**RhoBTB2 Is a Direct E2F1 Target Gene**

We considered the possibility that RhoBTB2 might be an indirect target of E2F1; to address the issue of direct versus indirect activation, we utilized a well-characterized H1299 cell line with an estrogen receptor-fused E2F1 expression vector stably integrated (H1299 ER-E2F1) (42–45). The result is an exogenous E2F1 protein that is transcriptionally inactive due to estrogen receptor-mediated cytoplasmic localization. Using this system, E2F1 activity can be rapidly induced through nuclear localization by addition of the estrogen receptor ligand 4-hydroxytamoxifen (4-OHT), while simultaneously blocking new protein synthesis by means of CHX treatment. Any transcripts found to be induced by 4-OHT in the presence of CHX can be considered direct E2F1 targets.

As shown in Fig. 2A, RhoBTB2 mRNA expression was relatively low in the untreated H1299 ER-E2F1 cell line, as well as after 8 and 24 h of treatment with CHX alone. As expected, up-regulation of RhoBTB2 was readily observed after 8 and 24 h of

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**FIGURE 2.** RhoBTB2 expression is directly and physiologically regulated by E2F1. A, real-time PCR analysis of RhoBTB2/18S in the H1299-ER-E2F1 cell line treated with CHX, 4-OHT or both at 8- and 24-hour time points. B, Western blot for E2F1 in the H1299-pBS/U6 and H1299-shE2F1 cell lines demonstrating efficient knockdown of E2F1 (top). IFM for RhoBTB2 with our rabbit polyclonal antibody conducted on the H1299-pBS/U6 and H1299-shE2F1 cell lines. DAPI, blue; RhoBTB2, red at \( \times 63 \) (bottom). Quantification of RhoBTB2 signal intensity per area of whole-field images is provided on the right.
E2F1 nuclear localization through treatment with 4-OHT. This activation of RhoBTB2 transcription by 4-OHT was not abrogated upon co-administration of CHX, thus providing evidence that RhoBTB2 is a direct transcriptional target of E2F1 (Fig. 2A).

Having shown the ability of artificially overexpressed E2F1 to directly upregulate RhoBTB2 expression, we next sought to determine if E2F1 plays a role in regulating physiological expression of RhoBTB2. To this end, we employed H1299 cell lines with a stably integrated short-hairpin inhibitory RNA targeted toward E2F1 (H1299-shE2F1) or an empty vector control (H1299-pBS/U6) (42–44). As previously reported, we observed significant knockdown of E2F1 in the H1299-shE2F1 cell line in comparison to that of the control H1299-pBS/U6 cell line (45) (Fig. 2B, top). We stained the cells for RhoBTB2 and compared expression levels between the two lines by means of IFM. The H1299-pBS/U6 control cell line with unaltered E2F1 expressed RhoBTB2 at levels comparable to that of the parental H1299 line (Fig. 2B, middle). In contrast, the H1299-shE2F1 cell line displayed greatly diminished expression of RhoBTB2 when compared with that observed in the H1299-pBS/U6 cell line (Fig. 2B, bottom). Given that knock-down of E2F1 diminishes RhoBTB2 expression, we conclude that E2F1 is indeed a physiological regulator of RhoBTB2.

RhoBTB2 Is Up-regulated during Mitosis, Which Is Partially Dependent on E2F1—One of the main functions of the growth promoting E2Fs is to activate the transcription of genes critical for cell cycle progression (2). Having identified RhoBTB2 as a direct E2F1 target gene, we postulated that RhoBTB2 expression might be regulated in a cell cycle stage-specific manner. To examine RhoBTB2 expression during interphase and various stages of mitosis, we stained asynchronously growing population of H1299s for RhoBTB2 and examined them for cells in the aforementioned mitotic stages via IFM. As shown in the top panel of Fig. 3A, H1299s in interphase expressed a relatively low level of RhoBTB2; however upon the initiation of prophase, RhoBTB2 levels increased dramatically. RhoBTB2 expression remained highly elevated through metaphase and anaphase, and did not begin to decrease until telophase/cytokinesis (Fig. 3A, top).

A vast majority of cancers exhibit aberrant regulation of the RB-E2F pathway, with the end result being unrestrained E2F activity. We considered the possibility that the observed mitotic up-regulation of RhoBTB2 may be an artifact of the highly transformed H1299 phenotype. To address this issue, we conducted identical experiments in the MCF10A cell line, a non-tumorigenic mammary fibrocystic cell line. In these experiments, we observed mitotic up-regulation of RhoBTB2 that parallels that observed in H1299s for RhoBTB2 and examined them in the aforementioned mitotic stages via IFM. As shown in the top panel of Fig. 3A, H1299s in interphase expressed a relatively low level of RhoBTB2; however upon the initiation of prophase, RhoBTB2 levels increased dramatically. RhoBTB2 expression remained highly elevated through metaphase and anaphase, and did not begin to decrease until telophase/cytokinesis (Fig. 3A, top).

FIGURE 3. RhoBTB2 is up-regulated during mitosis, which is partially dependent on E2F1. A, IFM for RhoBTB2 at X63 of cells in interphase, prophase, metaphase, anaphase, and telophase/cytokinesis on an asynchronously growing population of H1299s (top) and MCF10As (bottom). B, quantification of RhoBTB2 signal intensity per area of images provided in A. C, Western blot for RhoBTB2 in H1299s released from a double thymidine block, with accompanying DNA content analysis for cell cycle status as described under "Experimental Procedures." D, IFM as in A, but in the H1299-pBS/U6 and H1299-shE2F1 cell lines. E, quantification of RhoBTB2 signal intensity per area of images is provided in three-dimensions.
Because IFM experiments demonstrated that RhoBTB2 was highly up-regulated during mitosis, we postulated that this level of expression may be detectable in synchronized cells by Western blot, and thus may be utilized to provide a second independent measure of mitotic up-regulation of RhoBTB2. To examine this, we synchronized H1299s at the G1/S-phase boundary, collected samples for flow cytometric analysis of DNA content, and Western blot analysis of RhoBTB2 expression at various time points post-release. We noticed detectable RhoBTB2 beginning in late S-phase that persisted until the exit from G2/M (Fig. 3).

We postulated that E2F1 may be contributing to the observed mitotic up-regulation of RhoBTB2 and utilized the aforementioned E2F1-proficient and -knockdown cell lines, H1299-pBS/U6 and H1299-shE2F1, to compare mitotic up-regulation of RhoBTB2. Asynchronously growing populations of the two cell lines were stained for RhoBTB2 and examined for cells in interphase and various stages of mitosis as previously described. As expected, mitotic up-regulation of RhoBTB2 was readily observed in the H1299-pBS/U6 cell line; however, we noted an impaired mitotic up-regulation of RhoBTB2 in the H1299-shE2F1 cell line (Fig. 3, D and E). While there is an evident up-regulation of RhoBTB2 during prophase, it is significantly impaired when compared with that observed with the E2F1-proficient H1299-pBS/U6 cell line. This trend of diminished mitotic up-regulation of RhoBTB2 continued throughout all of the mitotic phases examined (Fig. 3, D and E). Taken together, these experiments demonstrate that RhoBTB2 is up-regulated during mitosis, and that E2F1 contributes to this regulation.

**Overexpression of RhoBTB2 Both Positively and Negatively Influences Cell Cycle Progression and Proliferation**

—Given the observation that RhoBTB2 is regulated in a cell cycle-dependent manner, we sought to determine if RhoBTB2 played a direct role in this process. To address this issue, we constructed adenovirus expressing either GFP (Ad-GFP) or RhoBTB2 (Ad-RhoBTB2). Asynchronously growing H1299s were then infected with equal amounts of either virus and harvested after 48 h for flow cytometric analysis of cell cycle status via propidium iodide (PI) staining. Cell cycle status percentage derivations were acquired using software analysis of DNA content as described under “Experimental Procedures.”

As shown in Fig. 4A, overexpression of RhoBTB2 altered the cell cycle status of H1299s by increasing the fraction of cells in S-phase, with 35% of cells infected with Ad-GFP in S-phase and 54% of cells infected with Ad-RhoBTB2 in S-phase. Because this assay could be interpreted to indicate either an increase in proliferation or an S-phase arrest, we conducted a BrdU incorporation assay to examine proliferation at the same time point. After 48 h of infection, cells overexpressing RhoBTB2 displayed increased BrdU incorporation relative to the Ad-GFP control.
indicating that the observed increase in the S-phase fraction was likely a manifestation of increased proliferation (Fig. 4B).

Having noted that overexpression of RhoBTB2 increased the S-phase fraction and the amount of DNA replication, we wanted to examine whether these indicators of increased proliferation manifested as an increase in total cell number. To test this hypothesis, we infected asynchronously growing H1299s with either Ad-GFP or Ad-RhoBTB2 and examined the increase in viable cells over 96 h via MTS assay. Interestingly, cells infected with Ad-RhoBTB2 displayed an increase in the number of viable cells that was less than that observed for cells infected with the Ad-GFP control virus, with Ad-RhoBTB2 and Ad-GFP increasing their MTS emission intensity ~1.5- and 1.75-fold, respectively (Fig. 4C). This indicated that although overexpression of RhoBTB2 displayed characteristics of increased proliferation at 48 h post-infection (Fig. 4A and B), this did not result in an increase in the total number of viable cells over an extended time course.

We interpreted this result as indicating that cells infected with Ad-RhoBTB2 were either being lost due to apoptosis or arresting/slowing proliferation at a time point subsequent to 48 h post-infection, with the latter being most probable since we did not observe an increase in sub-G1 content upon RhoBTB2 overexpression via flow cytometry (Fig. 4B). To address this, we infected H1299s with either Ad-GFP or Ad-RhoBTB2 and analyzed BrdU incorporation at 24, 48, 72, and 96 h post-infection. Intriguingly, while overexpression of RhoBTB2 increased BrdU incorporation relative to GFP at the 24- and 48-h time points, this increased uptake decreased at 72 h and was negative by 96 h (Fig. 4D). Taken together, these results suggest that overexpression of RhoBTB2 leads to a short-term positive influence on proliferation and a subsequent long-term negative proliferative influence.

RhoBTB2 Is Up-regulated during Drug-induced Apoptosis, Which Is Primarily Dependent on E2F1—

E2F1 is somewhat unique among other E2F family members in that it not only has the ability to transactivate genes critical for cell cycle progression, but is also a potent inducer of apoptosis through promoting the transcription of proapoptotic genes (for review, see Ref. 8). Given this fact, we investigated whether RhoBTB2 expression was affected by drug-induced apoptosis. To determine whether RhoBTB2 is regulated by apoptotic insults, we treated H1299s with cisplatin, flavopiridol or etoposide, chemotherapeutic agents where E2F1 is known to be a critical mediator, and conducted IFM to determine whether these cytotoxic insults had any effect on RhoBTB2 expression. As shown in Fig. 5A, we observed that administration of all of the chemotherapeutic agents tested resulted in increased RhoBTB2 protein expression.

While we observed up-regulation of RhoBTB2 during cytotoxic insult, we wanted to determine if E2F1 was responsible for this up-regulation. To examine this issue, we utilized the previously described E2F1 proficient and knockdown cell lines H1299-pBS/U6 and H1299-shE2F1 and conducted IFM on cells treated with the aforementioned apoptotic stimuli. As previously observed, RhoBTB2 expression was diminished in the
untreated H1299-shE2F1 cell line compared with the control H1299-pBS/U6 cell line (Fig. 4B). Upon the induction of apoptosis, the control H1299-pBS/U6 cell line behaved similar to that of the parental H1299s, with up-regulation of RhoBTB2 being clearly evident after 24 h (Fig. 4B). In stark contrast, we observed very little up-regulation of RhoBTB2 in the H1299-shE2F1 cell line. Fig. 4C displays E2F1 protein levels at 24-h post-treatment, demonstrating that E2F1 up-regulation does not occur in the H1299-shE2F1 cell line even in the presence of cytotoxic insult. It should be noted that in the presence of flavopiridol, we observe up-regulation of E2F1 to be highest shortly after treatment (around 6 h) and diminished by 24 h, which explains the seemingly diminished E2F1 expression as compared with the no treatment control. Taken together, these results demonstrate that RhoBTB2 is up-regulated during drug-induced apoptosis, and that this up-regulation is primarily dependent on the presence of E2F1.

siRNA-mediated Knockdown of RhoBTB2 Impairs the Induction of Drug-induced Apoptosis—Previous experiments demonstrated that RhoBTB2 is up-regulated during drug-induced apoptosis in an E2F1-dependent manner; we therefore wanted to explore whether RhoBTB2 was playing an active role in the apoptotic process. To address this question, we transiently depleted RhoBTB2 via transfection of a RhoBTB2-specific siRNA, induced apoptosis utilizing the previously employed cytotoxic drug treatments, and measured the relative number of viable cells throughout the time course by means of an MTS assay. As evident in Fig. 6, A, B, and C, treatment with cisplatin, flavopiridol, or etoposide led to a decrease in the number of viable cells in those transfected with either siControl or siRhoBTB2; however, cells transfected with siRhoBTB2 did not begin to lose viable cells until a later time point.

Because an MTS assay measures the relative number of viable cells, it is not a direct measurement of apoptosis per se. To more directly examine the induction of apoptosis, we conducted identical transfections and drug treatments and utilized Western blot analysis of cleaved PARP to measure the induction of apoptosis. In both cisplatin and flavopiridol treated cells, PARP cleavage was evident 16 h prior in cells transfected with the control siRNA in comparison to those transfected with the RhoBTB2-specific siRNA. However in the case of etoposide, there was no evident delay in PARP cleavage. We are uncertain as to why depletion of RhoBTB2 abrogated the effect of etoposide on the number of viable cells, yet did not effect the induction of apoptosis as measured by PARP cleavage; however given our previous observations, we speculate that depletion of RhoBTB2 under this context may affect positively affect proliferation. We interpret this data as indicating that under the context of certain cytotoxic drug treatments; RhoBTB2 acts as a positive contributor to apoptosis.

DISCUSSION

E2F is perhaps best known for its ability to promote the transcription of genes involved in the G1/S-phase transition; however an increasing amount of evidence implicates a role for E2F in the regulation of genes with mitotic functions. Overexpression of E2F1 or E2F2 induces a subset of genes with mitotic functions, and E2F1 can be found at the promoters of genes with mitotic functions (51–55). Furthermore, targets of E2F1 and E2F2 tend to be physiologically regulated temporally at two distinct cell cycle stages: G1/S and G2, implicating a role for
E2F-mediated transcription long after E2F is thought to be inactive (51).

While a number of mitotic E2F targets have been identified, few have been characterized. In this work, we demonstrate that RhoBTB2 is a direct target of E2F1 that is physiologically up-regulated during mitosis, and although more work needs to be done, RhoBTB2 appears to associate with the spindle apparatus during mitosis. We further show that mitotic up-regulation of RhoBTB2 is partially dependent of E2F1, as knockdown of E2F1 expression via shRNA abrogates mitotic up-regulation of RhoBTB2. It is possible that the remaining mitotic up-regulation of RhoBTB2 in the absence of E2F1 is dependent on E2F2 or E2F3a; however, we have not pursued this hypothesis.

In addition to being a mitotic target of E2F1, we also find that RhoBTB2 is an apoptotic target of E2F1 as well. RhoBTB2 is up-regulated upon treatment with chemotherapeutic drugs, which is primarily independent on E2F1 as knockdown of E2F1 with shRNA abrogates this effect as well. We see a greater dependence on E2F1 for apoptosis-induced up-regulation as opposed to mitotic up-regulation, and this may be due to an inability of E2F2 or E2F3a to compensate, as E2F1 is the primary inducer of apoptosis among the activating E2Fs.

To further explore the significance of E2F-mediated regulation of RhoBTB2, we examined a functional role for RhoBTB2 in either of these processes. Short term overexpression of RhoBTB2 resulted in characteristics of increased proliferation, while long term expression was found to be growth inhibitory. In the case of apoptosis, we find that depletion of RhoBTB2 by siRNA slows the induction of drug-induced apoptosis. Both of these findings are consistent with its putative role as a tumor suppressor gene. While deciphering mechanisms by which RhoBTB2 influences cell cycle and proliferation and the induction of apoptosis was beyond the scope of this study, published reports on RhoBTB2 have led to some intriguing hypotheses.

In agreement with our observations, RhoBTB2 was shown to inhibit cell proliferation in a breast cancer cell line deficient for RhoBTB2 (38). Further studies asserted that RhoBTB2-mediated down-regulation of cyclin D1 was obligatory for this effect (41). Another study utilizing pathway-based analysis of gene expression patterns found RhoBTB2 to effect the expression of genes associated with cell cycle, apoptosis, cytoskeleton and membrane-trafficking pathways (40). But perhaps the most intriguing study found that RhoBTB2 directly bound and was a substrate of the Cul3 ubiquitin ligase (39). The authors proposed a hypothesis in which RhoBTB2 served as a scaffold that recruited proteins to the Cul3 complex to be targeted for degradation. This seems quite rational, as other BTB/POZ domain-containing proteins have similar functions (39, 56–59).

Given the previously mentioned studies, coupled with our own observations, we believe that the functional significance of E2F1-mediated up-regulation of RhoBTB2 could be directly related to the ability of RhoBTB2 to recruit proteins to the Cul3 complex to be targeted for degradation. We propose a model similar to that proposed by Wilkins et al., in which the physiological role of RhoBTB2 in mitosis and apoptosis is to recruit regulatory proteins to the Cul3 complex to be targeted for degradation (39), and that the cell cycle effects observed during overexpression may be a non-physiological response from RhoBTB2 targeting proteins to Cul3 in phases of the cell cycle where RhoBTB2 would not normally be present. Given the published effect of RhoBTB2 expression of cyclin D1, it would seem like an attractive candidate to mediate this effect. While the mechanisms behind the biological functions of RhoBTB2 are yet to be determined, it is clear that RhoBTB2 is indeed a physiologically relevant direct target of E2F1.

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REFERENCES

1. Bracken, A. P., Ciro, M., Cocito, A., and Helin, K. (2004) Trends Biochem. Sci. 29, 409–417
2. DeGregori, J., and Johnson, D. G. (2006) Curr. Mol. Med. 6, 739–748
3. Johnson, D. G., and DeGregori, J. (2006) Curr. Mol. Med. 6, 731–738
4. Cam, H., and Dynlacht, B. D. (2003) Cancer Cell 3, 311–316
5. Harbour, J. W., and Dean, D. C. (2000) Genes Dev. 14, 2393–2409
6. Nevins, J. R. (2001) Hum. Mol. Genet. 10, 699–703
7. DeGregori, J. (2002) Biochim. Biophys. Acta 1602, 131–150
8. Dimova, D. K., and Dyson, N. J. (2005) Oncogene 24, 2810–2826
9. Yee, A. S., Reichel, R., Kovessi, I., and Nevins, J. R. (1987) The EMBO J. 6, 2061–2068
10. Ivey-Hoque, M., Conroy, R., Huber, H. E., Goodhart, P. J., Oliff, A., and Heinbrook, D. C. (1993) Mol. Cell. Biol. 13, 7802–7812
11. Lees, J. A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N., and Helin, K. (1993) Mol. Cell. Biol. 13, 7813–7825
12. Leone, G., Nuckolls, F., Ishida, S., Adams, M., Sears, R., Jakoi, L., Miron, A., and Nevins, J. R. (2000) Mol. Cell. Biol. 20, 3626–3632
13. He, Y., Armanious, M. K., Thomas, M. J., and Cress, W. D. (2000) Oncogene 19, 3422–3433
14. Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z. X., Xu, G., Wydner, K. L., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994) Genes Dev. 8, 2665–2679
15. Beijersbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlee, L., Voorhoeve, P. M., and Bernards, R. (1994) Genes Dev. 8, 2680–2690
16. Buck, V., Allen, K. E., Sorensen, T., Bybee, A., Hjimans, E. M., Voorhoeve, P. M., Bernards, R., and La Thangue, N. B. (1995) Oncology 11, 31–38
17. Itoh, A., Levinson, S. F., Morita, T., Kourembanas, S., Brody, J. S., and Mitsialis, S. A. (1995) Cell Mol. Biol. Res. 409–417
18. Trimarchi, J. M., Fairchid, B., Verona, R., Moberg, K., Andon, N., and Lees, J. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2850–2855
19. Cartwright, P., Muller, H., Wagener, C., Holm, K., and Helin, K. (1998) Oncogene 17, 611–623
20. Gaubatz, S., Wood, J. G., and Livingston, D. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9190–9195
21. de Bruin, A., Maiti, B., Jakoi, L., Timmers, C., Buerki, R., and Leone, G. (2003) J. Biol. Chem. 278, 42041–42049
22. Di Stefano, L., Jensen, M. R., and Helin, K. (2003) The EMBO J. 22, 6289–6298
23. Logan, N., Delavaine, L., Graham, A., Reilly, C., Wilson, J., Brummelkamp, T. R., Hjimans, E. M., Bernards, R., and La Thangue, N. B. (2004) Oncogene 23, 5138–5150
24. Logan, N., Graham, A., Zhao, X., Fisher, R., Maiti, B., Levine, G., and La Thangue, N. B. (2005) Oncogene 24, 5000–5004
25. Maiti, B., Li, J., de Bruin, A., Gordon, F., Timmers, C., Opavsky, R., Patil, K., Tuttle, J., Cleghorn, W., and Levine, G. (2005) J. Biol. Chem. 280, 18211–18220
26. Christensen, J., Cloos, P., Toffegaard, U., Klinkenberg, D., Bracken, A. P., Trinh, E., Heeran, M., Di Stefano, L., and Helin, K. (2005) Nucleic Acids Res. 33, 5458–5470
27. Wu, X., and Levine, A. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3602–3606
28. Qin, X. Q., Livingston, D. M., Kaelin, W. G., Jr., and Adams, P. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10918–10922
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29. Shan, B., and Lee, W. H. (1994) *Mol. Cell. Biol.* 14, 8166–8173
30. Stevens, C., and La Thangue, N. B. (2004) *DNA Repair (Amst)* 3, 1071–1079
31. Lundgren, R., Mandahl, N., Heim, S., Limon, J., Henriksson, H., and Mitelman, F. (1992) *Genes, Chromosomes Cancer* 4, 16–24
32. Emi, M., Fujiwara, Y., Nakajima, T., Tsuchiya, E., Tsuda, H., Hirohashi, S., Maeda, Y., Tsuruta, K., Miyaki, M., and Nakamura, Y. (1992) *Cancer Res.* 52, 5368–5372
33. Bova, G. S., Carter, B. S., Bussemakers, M. J., Emi, M., Fujiwara, Y., Kyprianou, N., Jacobs, S. C., Robinson, J. C., Epstein, J. I., and Walsh, P. C. (1993) *Cancer Res.* 53, 3869–3873
34. Fujiwara, Y., Emi, M., Ohata, H., Kato, Y., Nakajima, T., Mori, T., and Nakamura, Y. (1993) *Cancer Res.* 53, 1172–1174
35. Sunwoo, J. B., Holt, M. S., Radford, D. M., Deeker, C., and Scholnick, S. B. (1996) *Genes, Chromosomes Cancer* 16, 164–169
36. Brown, M. R., Chuaqui, R., Vocke, C. D., Berchuck, A., Middleton, L. P., Emmert-Buck, M. R., and Kohn, E. C. (1999) *Gynecologic Oncology* 74, 98–102
37. Wistuba, II, Behrens, C., Virmani, A. K., Milchgrub, S., Syed, S., Lam, S., Mackay, B., Minna, J. D., and Gazdar, A. F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 13647–13652
38. Wilkins, A., Ping, Q., and Carpenter, C. L. (2004) *Genes Dev.* 18, 856–861
39. Siripurapu, V., Meth, J., Kobayashi, N., and Hamaguchi, M. (2005) *Cell. Biol.* 346, 83–89
40. Yashihara, T., Collado, D., and Hamaguchi, M. (2007) *Biochem. Biophys. Res. Commun.* 358, 1076–1079
41. Ma, Y., and Cress, W. D. (2007) *Oncogene* 26, 3532–3540
42. Wang, C., Chen, L., Hou, X., Li, Z., Kabra, N., Ma, Y., Nemoto, S., Finkel, T., Gu, W., Cress, W. D., and Chen, J. (2006) *Nat. Cell Biol.* 8, 1025–1031
43. Ma, Y., Cress, W. D., and Haura, E. B. (2003) *Mol. Cancer Ther.* 2, 73–81
44. Kowalik, T. F., DeGregori, J., Schwarz, J. K., and Nevins, J. R. (1995) *J. Virol.* 69, 2491–2500
45. DeGregori, J., Kowalik, T., and Nevins, J. R. (1995) *Mol. Cell. Biol.* 15, 4215–4224
46. Frame, F. M., Rogoff, H. A., Pickering, M. T., Cress, W. D., and Kowalik, T. F. (2006) *Oncogene* 25, 3258–3266
47. Kalejta, R. F., Shenk, T., and Beavis, A. I. (1997) *Cytoometry* 29, 286–291
48. Cress, W. D., and Nevins, J. R. (1994) *J. Virol.* 68, 4213–4219
49. Ishida, S., Huang, E., Zuzan, H., Spang, R., Leone, G., West, M., and Nevins, J. R. (2001) *Mol. Cell. Biol.* 21, 4684–4699
50. Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R. A., and Dynlacht, B. D. (2002) *Genes Dev.* 16, 245–256
51. Weinmann, A. S., Yan, P. S., Oberley, M. J., Huang, T. H., and Farnham, P. J. (2002) *Genes Dev.* 16, 235–244
52. Zhu, W., Giangrande, P. H., and Nevins, J. R. (2004) *EMBO J.* 23, 4615–4626
53. Hernando, E., Nahle, Z., Juan, G., Diaz-Rodriguez, E., Alaminos, M., Hemann, M., Michel, L., Mittal, V., Gerald, W., Benezra, R., Lowe, S. W., and Cordon-Cardo, C. (2004) *Nature* 430, 797–802
54. Furukawa, M., He, Y. J., Borchers, C., and Xiong, Y. (2003) *Nat. Cell Biol.* 5, 1001–1007
55. Geyer, R., Wee, S., Anderson, S., Yates, J., and Wolf, D. A. (2003) *Mol. Cell* 12, 783–790
56. Pintard, L., Willis, J. H., Willems, A., Johnson, J. L., Srayko, M., Kurz, T., Glaser, S., Mains, P. E., Tyers, M., Bowerman, B., and Peter, M. (2003) *Nature* 425, 311–316
57. Xu, L., Wei, Y., Reboul, J., Vaglio, P., Shin, T. H., Vidal, M., Elledge, S. J., and Harper, J. W. (2003) *Nature* 425, 316–321
58. Polager, S., Kalma, Y., Berkovich, E., and Ginsberg, D. (2002) *Oncogene* 21, 437–446
59. Carrassa, L., Broggiini, M., Vikhanskaya, F., and Damia, G. (2003) *Cell Cycle* (Georgetown, TX) 2, 604–609