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RHOBTB2 (DBC2) IS A MITOTIC E2F1 TARGET GENE WITH A NOVEL ROLE IN APOPTOSIS

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Abstract

We have identified the *RhoBTB2* putative tumor suppressor gene as a direct target of the E2F1 transcription factor. Overexpression of E2F1 led to upregulation of RhoBTB2 at the level of mRNA and protein. This also occurred during the induction of E2F1 activity in the presence of cyclohexamide, 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. Since 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 upregulated 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 upregulated 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.

INTRODUCTION

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 ref. (6)). One result of Rb-E2F pathway deregulation is unrestrained 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).

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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 and colleagues 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 (39). 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 RhoBTB2's growth inhibitory effect (41). Whether Cyclin D1 is a direct or indirect target of RhoBTB2/Cul3 remains unclear.

While deletion and point mutations account for a small fraction of disruptions of RhoBTB2 function in malignancy, to date no other molecular regulators of RhoBTB2 besides Cul3 have been identified. Given the importance of RhoBTB2 with its association to carcinogenesis, it is of great benefit to identify mechanisms that regulate its expression. In this report, we identify RhoBTB2 as a novel transcriptional target of E2F1. We demonstrate that overexpression of E2F1 directly activates RhoBTB2 expression, and that knockdown of E2F1 decreases the expression of RhoBTB2, thus indicating that E2F1-mediated activation of RhoBTB2 is a physiologically relevant event. Furthermore we show that RhoBTB2 is upregulated 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 RhoBTB2 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 fibrocystic 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-RhoBTB2 adenovirus was constructed using a cDNA construct of RhoBTB2 with an N-terminal 3XFlag 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 RhoBTB2. 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 (H_2O), 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 1X 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 18s data were used for normalizing the gene values - ng gene/ng 18s per well.

RhoBTB2 antibody production

Affinity-purified rabbit polyclonal antibody was generated toward a peptide corresponding to human RhoBTB2 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 RhoBTB2 siRNA (40).

Plasmids, siRNA and transfections

RhoBTB2 siRNA was custom made (Ambion) using a previously published RhoBTB2 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% BSA in PBS. The primary RhoBTB2 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 40x or 63x / 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 AxioCam 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 hours prior to the experimental time point. BrdU incorporation assays were performed at the indicated time-points using a Chemicon ® BrdU Cell Proliferation Asssay Kit following the published protocol.

MTS assays

For siRNA-based experiments, cells were first transfected as described in results, trypisinized after 24 hours, counted, then plated in triplicate in 96-well plates. The specific drug treatments were then administered 24 hours later and the MTS assays were conducted using a Promega CellTiter 96® AQ_{ueous} 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 upregulates RhoBTB2 expression

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 hours 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 approximately 5- and 20-fold compared to that of Ad-GFP infection at the 24- and 48-hour time points, respectively (Fig. 1A). Lack of RhoBTB2 induction by Ad-E2F1(1–283) infection

confirms that upregulation of RhoBTB2 by E2F1 is dependent on E2F1's C-terminal transactivation domain.

Since the experiments conducted to this point employed the H1299 cell line, we sought to ensure that upregulation 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 upregulation of RhoBTB2 upon Ad-E2F1 infection similar to that observed in H1299s, thus confirming that upregulation of RhoBTB2 expression by E2F1 is not cell line specific (Fig. 1B).

In order 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 immunofluorescenct microscopy (IFM) (Fig. 1C, top). To verify that the observed signal was specific for RhoBTB2, we transiently knocked-down RhoBTB2 expression using a previously described siRNA and stained for RhoBTB2 expression using IFM. We found that transfection of siRNA targeted towards RhoBTB2 diminishes the observed RhoBTB2 signal by approximately 50%, 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 towards 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 upregulation 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 H1299s and assayed for 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 to 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 upregulated at the level of both mRNA and protein upon E2F1 overexpression.

RhoBTB2 expression is directly and physiologically regulated by E2F1

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 overexpressed 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 cyclohexamide (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 Figure 2A, RhoBTB2 mRNA expression was relatively low in the untreated H1299 ER-E2F1 cell line, as well as after 8 and 24 hours of treatment of CHX alone. As expected, upregulation of RhoBTB2 was readily observed after 8 and 24 hours of 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 to 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 upregulated 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 manner. To examine RhoBTB2 expression during interphase and various stages of mitosis, we stained an 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 Figure 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 upregulation 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 upregulation of RhoBTB2 that parallels that observed in H1299s (Fig. 3A, bottom), confirming that upregulation of RhoBTB2 during mitosis is not due to the highly transformed nature of H1299s. Figure 3B provides quantification of the observed RhoBTB2 signal intensity.

Since IFM experiments demonstrated that RhoBTB2 was highly upregulated 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 upregulation of RhoBTB2. To examine this, we synchronized H1299s at the G_1/S-phase boundary, and 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 G_2/M (Fig. 3C)

We postulated that E2F1 may be contributing to the observed mitotic upregulation of RhoBTB2 and utilized the aforementioned E2F1-proficient and -knockdown cell lines, H1299-pBS/U6 and H1299-shE2F1, to compare mitotic upregulation of RhoBTB2 in cells with diminished

expression of E2F1. 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 upregulation of RhoBTB2 was readily observed in the H1299-pBS/U6 cell line; however, we noted an impaired mitotic upregulation of RhoBTB2 in the H1299-shE2F1 cell line (Fig. 3D, E). While there is an evident upregulation of RhoBTB2 during prophase, it is significantly impaired when compared to that observed with the E2F1 proficient H1299-pBS/U6 cell line. This trend of diminished mitotic upregulation of RhoBTB2 continued throughout all of the mitotic phases examined (Fig. 3D, E). Taken together, these experiments demonstrate that RhoBTB2 is upregulated 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 hours 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 in experimental procedures.

As shown in Figure 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. Since 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 hours 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 hours 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 approximately 1.5 and 1.75 fold, respectively (Fig. 4C). This indicated that although overexpression of RhoBTB2 displayed characteristics of increased proliferation at 48 hours post-infection (Fig. 4A, 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 hours post-infection—with the latter being most probable since we did not observe an increase in sub-G_1 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 hours post-infection. Intriguingly, while overexpression of RhoBTB2 increased BrdU incorporation relative to GFP at the 24- and 48-hour time points, this increased uptake decreased at 72 hours and was negative by 96 hours (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 upregulated 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 effected 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 Figure 5A, we observed that administration of all of the chemotherapeutic agents tested resulted in increased RhoBTB2 protein expression.

While we observed upregulation of RhoBTB2 during cytotoxic insult, we wanted to determine if E2F1 was responsible for this upregulation. 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 to 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 upregulation of RhoBTB2 being clearly evident after 24 hours (Fig. 4B). In stark contrast, we observed very little upregulation of RhoBTB2 in the H1299-shE2F1 cell line. Figure 4C displays E2F1 protein levels at 24 hours post treatment, demonstrating that E2F1 upregulation 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 upregulation of E2F1 to be highest shortly after treatment (around 6 hours) and diminished by 24 hours, which explains the seemingly diminished E2F1 expression as compared to the no treatment control. Taken together, these results demonstrate that RhoBTB2 is upregulated during drug-induced apoptosis, and that this upregulation 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 upregulated 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 Figures 6A, 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.

Since 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 approximately 8 hours 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 upregulated 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 upregulation of RhoBTB2 is partially dependent of E2F1, as knockdown of E2F1 expression via shRNA abrogates mitotic upregulation of RhoBTB2. It is possible that the remaining mitotic upregulation 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 upregulated 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 upregulation as opposed to mitotic upregulation, 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.

In order 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 downregulation 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 upregulation 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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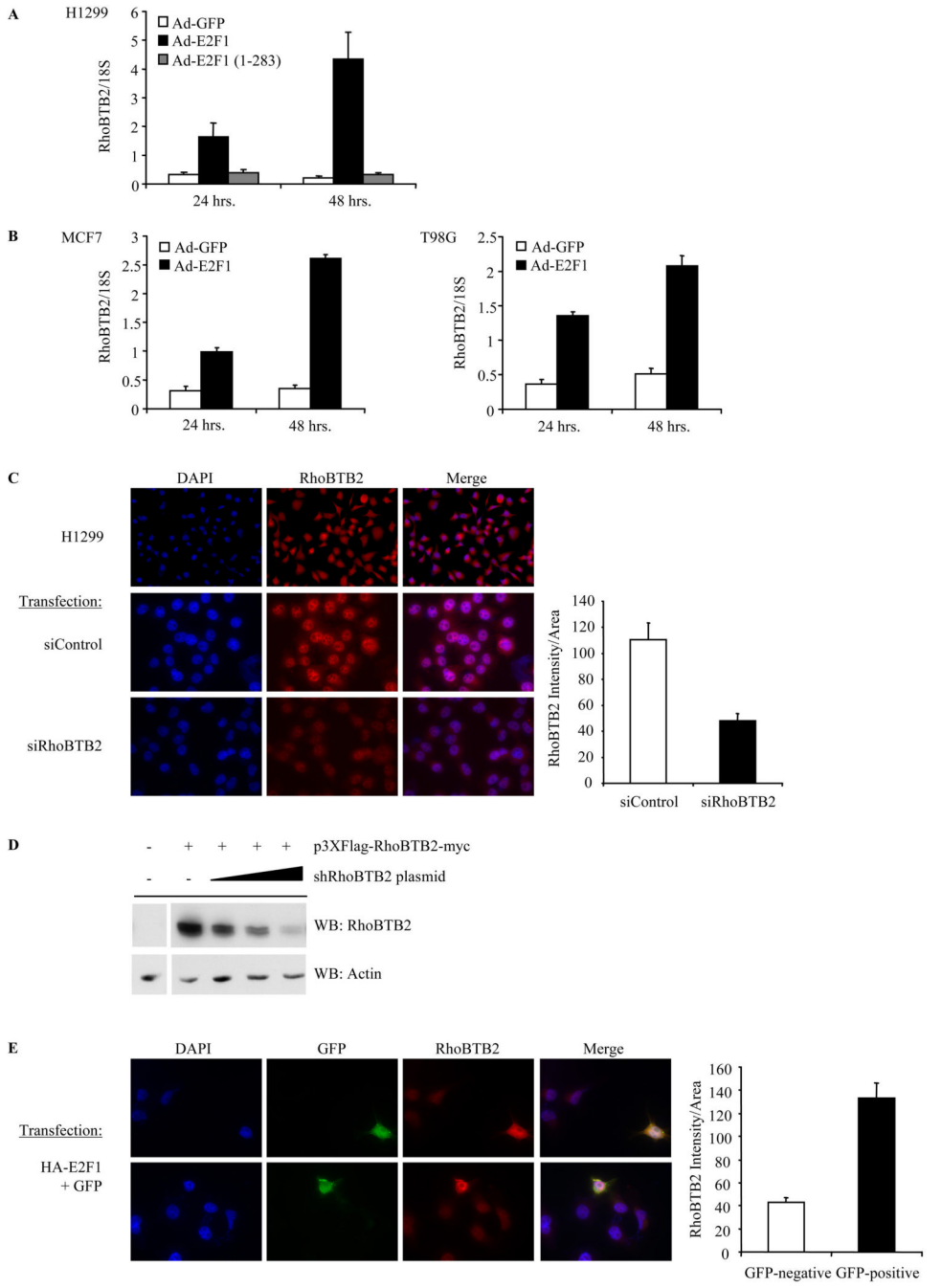

Fig. 1. E2F1 overexpression upregulates RhoBTB2

A: H1299s treated with equal amounts of Ad-GFP, Ad-E2F1 or Ad-E2F1(1–283) adenovirus with subsequent real-time PCR analysis of RhoBTB2/18S at 24- and 48-hour time points **B:** MCF7s or T98Gs were treated with either Ad-GFP or Ad-E2F1 with subsequent real-time PCR analysis for RhoBTB2/18S at 24- and 48 hour time points. **C:** Immunofluorescent microscopy (IFM) for RhoBTB2 in either non-trasfected H1299s at 40x (top), H1299s transfected with a control siRNA at 63x (middle) or transfected with siRNA specific to RhoBTB2 at 63x (bottom) —DAPI: blue; RhoBTB2: red. Quantification of RhoBTB2 signal intensity per area of whole-field images is provided on the right. **D:** A western blot for RhoBTB2 in either non-transfected, transfected with p3XFlag-RhoBTB2-myc or co-transfected with p3XFlag-RhoBTB2-myc and

increasing amounts of a shRNA targeted toward RhoBTB2 (shRhoBTB2) in H1299s. **E.** IFM at 63x of two different fields of H1299s transiently cotransfected with pcDNA3-HA-E2F1 and pBB14, a membrane GFP plasmid—DAPI: blue; GFP (transfected cells): green; RhoBTB2: red. Quantification of RhoBTB2 signal intensity per area of whole-field images is provided on the right.

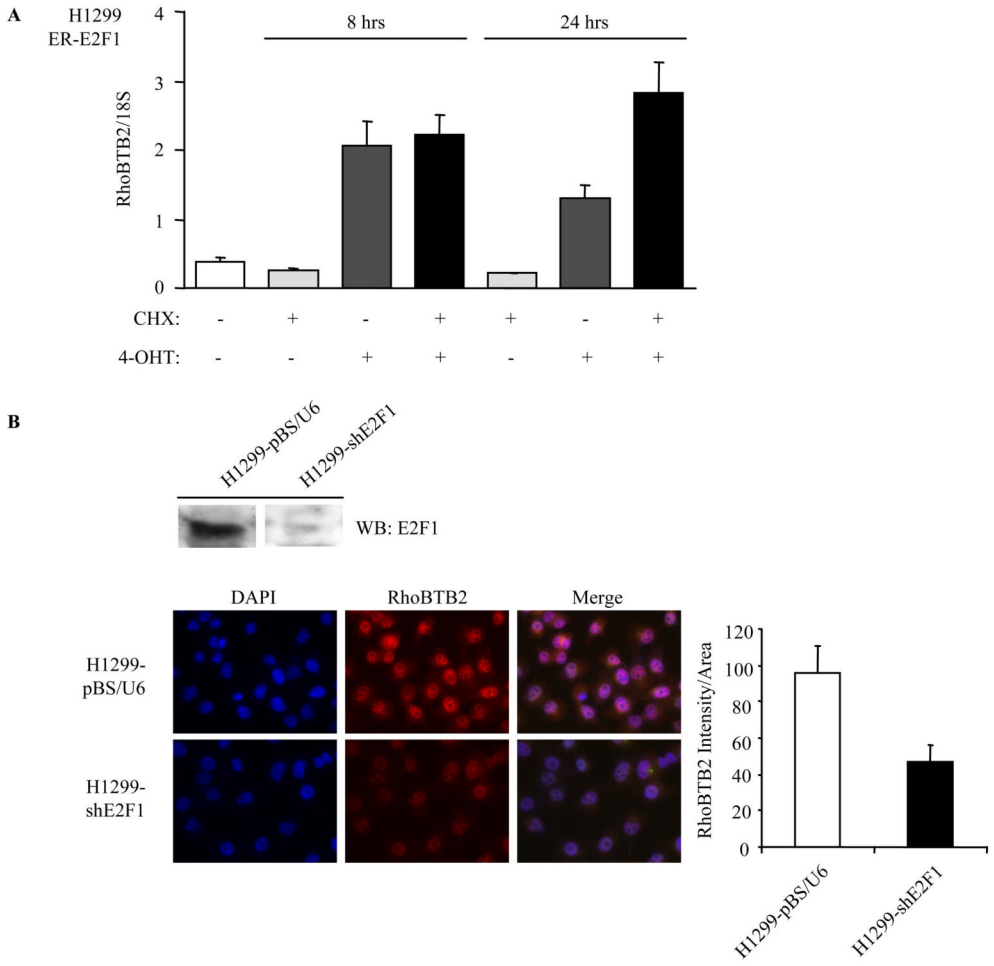

Fig. 2. RhoBTB2 expression is directly and physiologically regulated by E2F1

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:** A 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 63x (bottom). Quantification of RhoBTB2 signal intensity per area of whole-field images is provided on the right.

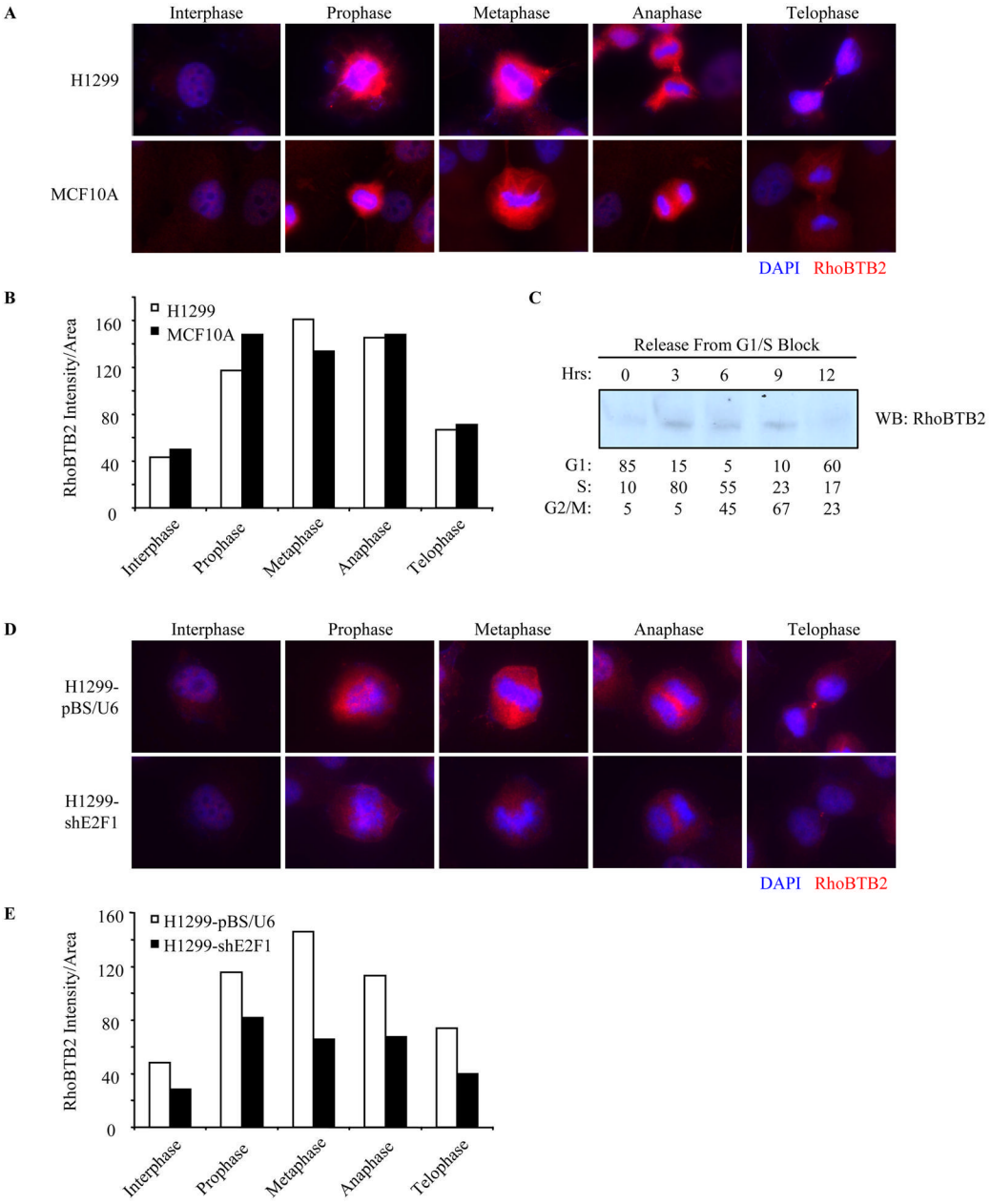

Fig. 3. RhoBTB2 is upregulated during mitosis, which is partially dependent on E2F1

A: IFM for RhoBTB2 at 63x 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 3A. **C.** Western blot for RhoBTB2 in H1299s released from a double thymidine block, with accompanying DNA content analysis for cell cycle status as described in experimental procedures. **D.** IFM as in 3A, but in the H1299-pBS/U6 and H1299-shE2F1 cell lines. **E.** Quantification of RhoBTB2 signal intensity per area of images is provided in 3D.

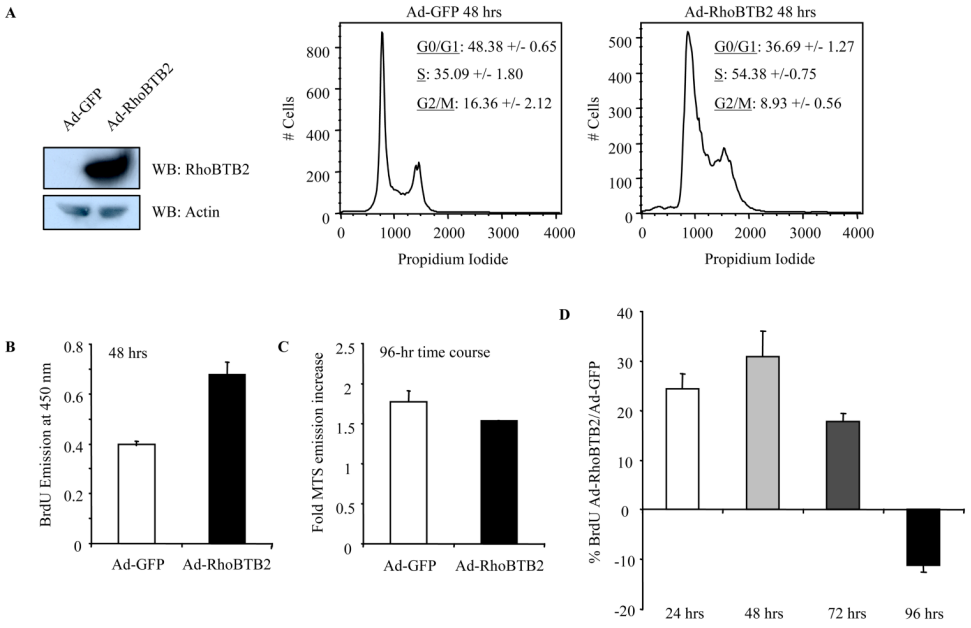

Fig. 4. Overexpression of RhoBTB2 both positively and negatively influences cell cycle progression and proliferation

A: A western blot for RhoBTB2 and actin upon infection with equal amounts of Ad-GFP or Ad-RhoBTB2 (left). DNA content and cell cycle status derivations of H1299s infected in triplicate with equal amounts of either the Ad-GFP or Ad-RhoBTB2 adenovirus via propidium iodide staining and flow cytometry. **B:** BrdU incorporation assay of H1299s infected in triplicate with equal amounts of either the Ad-GFP or Ad-RhoBTB2 adenovirus at 48 hours post infection. **C.** An MTS assay of the fold increase in MTS signal intensity over a 96-hour time course of triplicate samples of H1299s infected with either Ad-GFP or Ad-RhoBTB2. **D.** A BrdU incorporation assay as in 4B, but over a 96-hour time course. Data displayed reflects BrdU signal intensity percentage of Ad-RhoBTB2 infected cells to Ad-GFP infected cells.

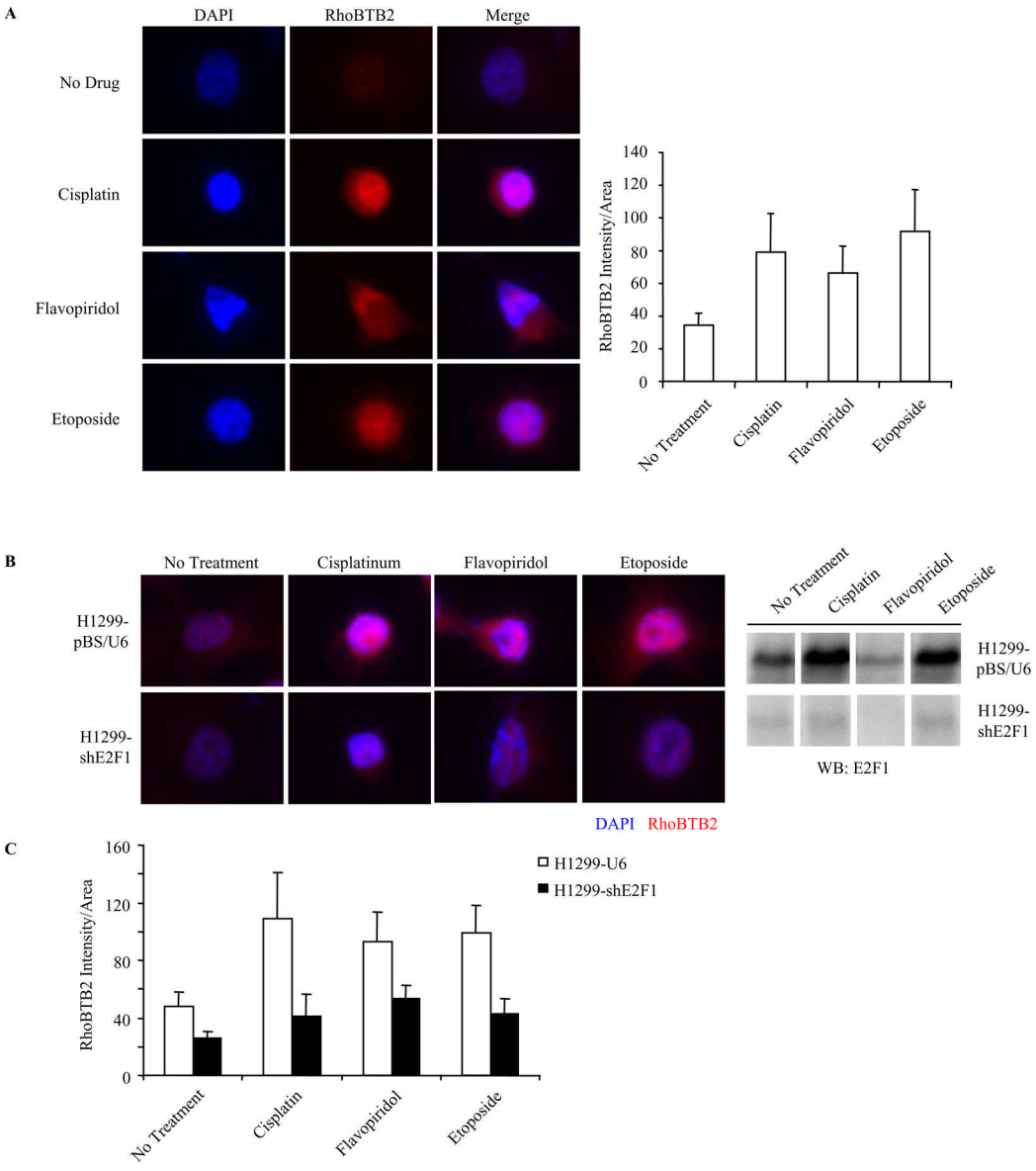

Fig. 5. RhoBTB2 is upregulated during drug induced apoptosis, which is primarily dependent on E2F1

A: IFM at 63x for RhoBTB2 in H1299s after 24 hours of treatment of either no treatment control, 20 uM cisplatin, 200 nM flavopiridol, or 20 uM etoposide. Quantification of RhoBTB2 signal intensity per area of images is provided on the right. **B:** IFM as in 5A, but with the H1299-pBS/U6 and H1299-shE2F1 cell lines --DAPI: blue; RhoBTB2: red (left). A western blot for E2F1 expression in H1299-pBS/U6 and H1299-shE2F1 cell lines for the drug treatments utilized in the left panel (right). **C.** Quantification of RhoBTB2 signal intensity per area of whole-field images of the treatments presented in 5B.

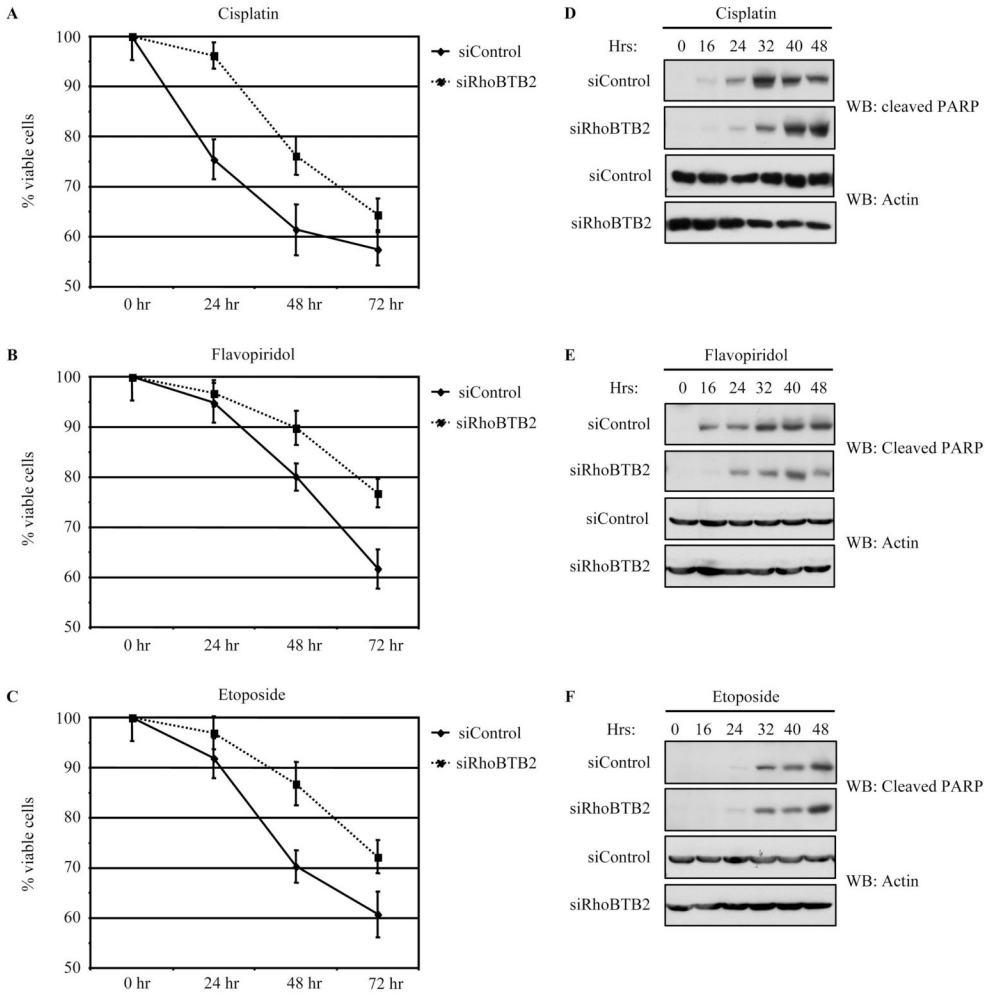

Fig. 6. siRNA-mediated knockdown of RhoBTB2 impairs the induction of drug-induced apoptosis

A-C: H1299s were transiently transfected with either a negative control siRNA, or siRNA against RhoBTB2, detached at 24 post-transfection, counted, and transferred to 96 well plates where an MTS assay was performed to analyze cell viability after 24, 48 and 72 hours. The cytotoxic drugs used were 20 uM cisplatin (A), 200 nM flavopiridol (B) and 20 uM etoposide (C). **D-E:** Western blots for cleaved PARP and actin control after 0, 16, 24, 32, 40 and 48 hours of treatment with either 20 uM cisplatinum (D), 200 nM flavopiridol (E) and 20 uM etoposide (F) in H1299s transfected with either a negative control siRNA (siControl) or siRNA against RhoBTB2 (siRhoBTB2).