The Nuclear Protein UHRF2 Is a Direct Target of the Transcription Factor E2F1 in the Induction of Apoptosis*

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Background: E2F1-induced apoptosis kills oncogene-stressed cells, and regulators of this process may act as tumor suppressors.

Results: We identified UHRF2 in a functional screen for mediators of E2F1 induced apoptosis.

Conclusion: UHRF2 binds directly to E2F1 and is required for E2F1 induction of apoptosis and expression of several important apoptosis inducers.

Significance: UHRF2 is a suspected tumor suppressor and our work suggests an anti-tumor mechanism.

The E2F1 transcription factor is active in many types of solid tumors and can function as either an oncogene or tumor suppressor in vivo. E2F1 activity is connected with a variety of cell fates including proliferation, apoptosis, senescence, differentiation, and autophagy, and these effects are mediated through differential target gene expression. E2F1-induced cell death is an innate anti-cancer mechanism to kill cells with a spontaneous oncogenic mutation that might otherwise form a cancer. Relatively little is known about the molecular circuitry that tips E2F1 balance toward proliferation during normal growth versus apoptosis during oncogenic stress, and which pathways mediate this decision. To further explore these mechanisms, we utilized an unbiased shRNA screen to identify candidate genes that mediate E2F1-induced cell death. We identified the ubiquitin-like with PHD and ring finger domains 2 (UHRF2) gene as an important mediator of E2F1-induced cell death. UHRF2 encodes a nuclear protein involved in cell-cycle regulation. Several of these domains have been shown to be essential for the regulation of cell proliferation, and UHRF2 has been implicated as an oncogene in some settings. Other reports have suggested that UHRF2 causes growth arrest, functions as a tumor suppressor, and is deleted in a variety of tumors. We show that UHRF2 is a transcriptional target of E2F, that it directly interacts with E2F1, and is required for E2F1 induction of apoptosis and transcription of a number of important apoptotic regulators.

Mammalian cell proliferation is generally dictated by extracellular signals that activate a cell division gene expression program. During the G1 phase of the cell cycle, this mitogenic stimulation and concomitant gene expression program initiate DNA synthesis (S phase), after which cells are committed to complete the cycle and divide. Passage through START coincides with activation of the E2F transcription factors, which induce expression of genes necessary for DNA replication, metabolism, and synthesis and are key transcriptional regulators of cell cycle progression during normal growth stimulation and cancer. The activity of E2Fs is inhibited by binding of the retinoblastoma (Rb) tumor suppressor gene. Rb binding to E2F proteins inhibits transcription by blocking the E2F transactivation region and also by actively recruiting histone deacetylases and other chromatin remodeling factors to repress gene expression (1, 2). Cdk phosphorylation of Rb during mitogenic stimulation, or loss of Rb in pre-neoplastic cells, prevents its inhibiting mechanism by which cells respond to different stimuli by triggering a program of cell death for the elimination of redundant, autoreactive, or pre-neoplastic cells. Thus, cells acquiring a pro-proliferatory mutation, such as loss of Rb, can be eliminated via apoptosis unless they carry or acquire another mutation that impairs the apoptotic process. DNA damage also induces E2F1 levels and leads to apoptosis, however, treatment with these agents leads to different post-translational modifications of E2F1 and does not mimic aberrant deregulation during cell cycle entry. E2F1 induces apoptosis in part through the transcription of proapoptotic targets, such as p19ARF, p73, APAFI, SIVA, CASP3, and CASP7 (5–11). Expression of some of these genes appears to be cell type specific and is not absolutely required for E2F1 apoptosis induction. E2F induces the expression of numerous pro-apoptotic Bcl-2 homology 3 (BH3)-only proteins including BIM, BNIP3, BOK, PUMA, and NOXA and represses the expression of the anti-apoptotic MCL1 gene (12–15). The mechanisms suppressing E2F induction of apoptosis during normal cell cycle entry but permitting,

2 The abbreviations used are: Rb, retinoblastoma; PHD, plant homeodomain; SRA, set- and ring-associated; m.o.i., multiplicity of infection; IP, immunoprecipitation; qPCR, quantitative PCR; nd, non-degradable; GO, gene annotation; MIA2, melanoma inhibitory activity 2; AZIN1, antizyme inhibitor 1; UHRF2, ubiquitin-like with PHD and ring finger domains 2.

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...or strengthening, cell death under conditions of E2F up-regulation that stem from the Rb pathway loss or other oncogenic mutations are not clearly understood.

To gain further insight into the mechanisms regulating E2F1-mediated cell death, we conducted an unbiased shRNA screen to identify regulators of E2F1 apoptosis induction. We identified the ubiquitin-like plant homeodomain (PHD) and ring finger domains 2 (UHRF2) gene as a mediator of E2F1-induced cell death. UHRF2 (also called NIRF) was previously identified as a ubiquitin ligase that interacts with the cyclin E-CDK2 complex and arrests cells in G1. UHRF2 contains a ubiquitin-like domain, PHD finger domain, a set- and ring-associated (SRA) domain, and a RING finger domain. Intriguingly, both UHRF2 and UHRF1 physically associate with the Rb tumor suppressor protein and appear to recognize DNA and histone methylation to control cell cycle progression and gene expression.

We demonstrate here that knockdown of UHRF2 impairs E2F1 activation of apoptosis and this effect is reverted by re-addition of a non-degradable form of UHRF2. Furthermore, E2F1 binds to the UHRF2 promoter and transcriptionally induces its expression. We also determined that UHRF2 mRNA and protein levels are induced in shUHRF1 knockdown cells, and conversely that UHRF1 mRNA is induced in shUHRF2 cells, suggesting potential compensatory regulation between these two proteins. We demonstrate that endogenous E2F1 and UHRF2 physically associate by co-immunoprecipitation assays. E2F1-induced gene expression profiles were determined in control and shUHRF2 cells. We found that the E2F1-induced target genes showing poor induction in shUHRF2 cells were significantly populated with apoptotic target genes like SIVA and BIM. These findings suggest a model whereby E2F1 induces expression of UHRF2, which then binds to E2F1 and positively regulates expression of pro-apoptotic target genes.

EXPERIMENTAL PROCEDURES

shRNA Screen—PLAT-A retrovirus packaging cells were previously obtained from T. Kitamura (22). An Open Biosystems human shRNAmir library was divided into 30 pools with 1000 shRNAs per pool (23). Pooled shRNA plasmids were packaged into retrovirus using PLAT-A cell lines and infected into the U2OS human osteosarcoma cell line (obtained from ATCC). Approximately 2 × 10⁷ U2OS cells were infected by library retroviral shRNAs at a multiplicity of infection of 0.5 and treated with puromycin to stably integrate and adherent cells 40 h post-infection and assayed by measuring anti-active caspase-3 by flow cytometry, or by staining using propidium iodide staining to measure the sub-G₁ DNA content (Sigma). For individual shRNA retesting, Open Biosystems shRNA constructs were obtained from the University of Minnesota RNAi core facility, packaged as lentivirus and stably integrated into U2OS cells followed by puromycin selection. The UHRF2 cDNA was kindly provided by Dr. Tsutomu Mori (Fukushima Medical University School of Nursing). To generate the non-degradable UHRF2 allele, we altered the UHRF2 coding cDNA to maintain amino acid coding sequence but prevent degradation by shRNA targeting molecules using the following primers: forward, 5’-CTCGAGCCATCCCATACTCTTTCCGTCGATGAAAGTATTATTAAAGC-3’ and reverse, 5’-CGCTTAAAGGAAAATCCATCGAGAAAGTAGAAGGATGGAATTCCAGG-3’. The UHRF2 promoter (wild-type and with two putative E2F binding sites mutated) was PCR amplified from human U2OS cells and cloned into pGL3 for luciferase assays, which were performed following the manufacturer’s instructions (Promega). A reverse primer was common to both fragments (5’-GACATGTACAACTTGAGAGCCACCAGGCGGCCTCG-3’) and was paired with either wild-type primer (5’-GTCGTA-GACGCTAGCGCTGGAGCTGCCGCCTCCG-3’) or 2x-mutant E2F binding sites (5’-GTCGTA-GACGCTAGCGCTGGAGCTGCCGCCTCAGCAGGAAAACCTCC-GGAAGTGGGTCGCGCCGGCGAATGAGTCCAGTGGCCGC-CAGGGCGCGCGCGTGTGTGCGAAGAGGAGGGCTGGAATTTCAGG-3’). RNA Isolation, Real-time PCR, and Microarray Analysis—We used Qiagen QIAshredder and RNaseasy Midi Kits to isolate cellular RNA and the QuantiTect SYBR Green RT-PCR kit from Qiagen for our quantitative real-time PCR. Each experimental condition used 200 ng of RNA for reverse transcription and RT-PCR and was performed in triplicate and normalized against GAPDH expression levels. Analysis was done with a StepOnePlus real-time PCR system (Applied Biosystem). For microarrays, RNA was harvested from infected cells and analyzed on Illumina Human WG-6 version 3 beadchips in duplicate. The GATHER (Gene Annotation Tool to Help Explain Relationships) website was used to perform GO and TRANSFAC analysis. The following primers were used for RT-PCR: UHRF2, forward, 5’-TTGCTGCTGATGAAAGACCTT-3’ and reverse, 5’-TTTCTGCATCAACTACCAATCC-3’; MIA2, forward, 5’-ATGGGGCTCCAGG-3’ and reverse, 5’-TTCCCACGGAAAACTACCA-3’; EIF5A2, forward, 5’-CTCCAAG-3’ and reverse, 5’-CTCCAAG-3’; CCNA2, forward, 5’-ICCGACGAGGACCCGGAGAAGGTAC-3’ and reverse, 5’-GCTGCCGTGCTGATGAAGACGTT-3’; AGGACCAGGAGAAGGTAC-3’; and reverse, 5’-ATGGGGCTCCAGG-3’; EIF5A2, forward, 5’-CTCCAAGGAAAACTACCA-3’ and identified the recovered shRNAs by sequence analysis. We sequenced a total of 2000 clones and listed the recurring clones in Fig. 1B. Around 26% of the entire library was screened in these analyses (8 of 30 pools total). Cell Culture and DNA Plasmids—U2OS (human osteosarcoma) cells from ATCC were cultured in DMEM with 10% fetal calf serum. Apoptotic cells were measured by harvesting floating and adherent cells 40 h post-infection and assayed by measuring anti-active caspase-3 by flow cytometry, or by staining using propidium iodide staining to measure the sub-G₁ DNA content (Sigma). For individual shRNA retesting, Open Biosystems shRNA constructs were obtained from the University of Minnesota RNAi core facility, packaged as lentivirus and stably integrated into U2OS cells followed by puromycin selection. The UHRF2 cDNA was kindly provided by Dr. Tsutomu Mori (Fukushima Medical University School of Nursing). To generate the non-degradable UHRF2 allele, we altered the UHRF2 coding cDNA to maintain amino acid coding sequence but prevent degradation by shRNA targeting molecules using the following primers: forward, 5’-GACATGTACAACTTGAGAGCCACCAGGCGGCCTCG-3’ and reverse, 5’-CGCTTAAAGGAAAATCCATCGAGAAAGTAGAAGGATGGAATTCCAGG-3’. 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RESULTS

shRNA Screen Identifies Genes That Mediate E2F1-induced Apoptosis—We utilized an unbiased shRNA screening approach to identify genes that mediate E2F1-induced apoptosis (Fig. 1). Our experimental design hypothesized that integration of shRNAs conferring resistance to E2F1-induced apoptosis should allow those cells to survive a dosage of E2F1 that efficiently kills control cells. We chose to activate cell death by ectopically expressing E2F1 because it more closely mimics the up-regulation of E2F1 seen following RB pathway mutation than that caused by DNA damage treatment, which leads to differential phosphorylation of E2F1. These cells could then be recovered and integrated shRNAs identified. We observed rare surviving cells following infection with E2F1 expressing adenovirus, and these cells were harvested so we could identify recovered shRNAs. We sequenced around 2000 clones and listed the top recovered genes in Table 1.

Frequently identified genes were individually knocked down in U2OS cells by shRNA and tested for their ability to inhibit E2F1-induced apoptosis. We analyzed each of these generated cell lines for resistance to E2F1-induced apoptosis. Each cell line was treated with 0.25% FBS containing media (serum dep-
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![Diagram of shRNA screening process](image)

**FIGURE 1.** A functional shRNA screen for regulators of E2F1-induced apoptosis. Approach for identifying shRNAs that confer resistance to E2F1-mediated apoptosis in U2OS cells. The Open Biosystems shRNA library was pooled, packaged into retrovirus, and stably selected in U2OS cells. shRNA carrying U2OS cells were infected with E2F1 sufficient to induce apoptosis in 100% of control cells. shRNA carrying cells that resisted E2F1-induced apoptosis were harvested, and genomic DNA was amplified by PCR and cloned to allow sequencing of the region containing the integrated shRNA. Approximately 2000 inserts were sequenced.

**TABLE 1**

List of gene targets identified by shRNA screening for regulators of E2F1-induced apoptosis. Ten recovered genes are listed with gene names and the number of times identified (Hits)

| Gene  | Full name                              | Hits |
|-------|----------------------------------------|------|
| EIF5A2| Eukaryotic translation initiation factor 5A2 | 9    |
| AZIN1 | Antizyme inhibitor                      | 4    |
| UHRF2 | Ubiquitin-like with PHD and ring finger domains 2 | 3    |
| MSH6  | E3 ubiquitin protein ligase             | 2    |
| ADAMTS3| ADAMTS-like 3                          | 2    |
| KTBD7 | Kelch repeat and BTB (POZ) domain containing | 2    |
| CCNA2 | Cyclin A2                               | 2    |
| GPR45 | G protein-coupled receptor 45           | 2    |
| WDFY3 | WD repeat and FYVE domain containing 3  | 2    |
| MIA2  | Melanoma inhibitory activity 2          | 2    |

**FIGURE 2.** Identification of mediators of E2F1-induced apoptosis. We targeted each of the genes recovered by our screening by shRNA to retest for prevention of E2F1-induced apoptosis. Individual shRNAs listed in Table 1 were stably integrated into U2OS cells. A, serum deprived control and shRNA target cells were infected with E2F1 expressing adenovirus and later measured for apoptosis by quantifying cleaved caspase-3 levels. The level of apoptosis induction by E2F1 compared with control in non-shRNA carrying cells was set to 100%. Three of the 10 shRNA lines (GPR45, MSH6, and ADAMTS3) did not display reduced E2F1-induced apoptosis. The other seven lines, however, recapitulated the findings of the screen and significantly reduced E2F1 induction of apoptosis from 25–55%. Student's t tests were performed and indicate that the reduced apoptosis seen in these seven cell lines was significant (p < 0.05). B, target gene knockdown was measured by qPCR, comparing levels in vector control to levels in shRNA knockdown lines. Results are reported as % remaining expression in target cells and are listed in the order presented in A.
6-fold increase in E2F1 binding compared to control IgG (Fig. 3B). We scanned the UHRF2 promoter for putative E2F recognition motifs. The analysis picked up a number of potential E2F binding sites, and we mutated two putative motifs that gave a high score by TRANSFAC. We sequenced the final construct to verify that the potential E2F binding sites were mutated (Fig. 3C). Our results indicated that E2F1 induced the wild-type construct 6-fold and the 2x mutant by 3-fold (Fig. 3D). Thus, mutating the two putative E2F sites reduced responsiveness to E2F1 activity by 50% ($p < 0.01$). E2F1 induces expression of UHRF2, binds to its promoter in vivo and in vitro, and that mutating the putative E2F motifs blocks induction and E2F1 binding in vitro.

Furthermore, UHRF2 and UHRF1 are structurally similar and it is not entirely clear to what extent these proteins share overlapping or distinct functions. We targeted UHRF1 for shRNA degradation in U2OS cells to gain a better understanding of the potential synergistic control of E2F1 induced apoptosis by UHRF1 with UHRF2. Surprisingly, we observed a greater than 2-fold increase in UHRF2 levels in shUHRF1 knockdown cells by qPCR, compared with levels in control cells (Fig. 4A). Furthermore, UHRF1 mRNA levels increased around 2.5-fold in the shUHRF2 cell lines, compared with control. We also generated U2OS cells carrying both shUHRF1 and shUHRF2. Levels of both UHRF1 and UHRF2 were reduced by 40 and 60%, respectively.

**FIGURE 3.** UHRF2 is an E2F1-induced target gene. A, serum deprived U2OS cells were infected with E2F1 expressing adenovirus and mRNA isolated 24 h post-infection for qPCR analysis. Each of the seven genes that significantly reduced E2F1-induced apoptosis in Fig. 2 were tested for transcriptional induction by E2F1. Three of the genes, EIF5A2, UHRF2, and CCNA2, were each induced around 5–8-fold by E2F1. AZIN1 was induced around 3-fold, and the other genes were uninduced. Results are shown as fold-induction compared with induction by control adenovirus in U2OS cells. Student's t tests were performed and indicate that the increased expression of EIF5A2, UHRF2, CCNA2, and AZIN1 was significant ($p < 0.05$). B, we performed chromatin immunoprecipitations to determine whether endogenous E2F1 binds directly to the native UHRF2 promoter. Chromatin was prepared from serum-deprived wild-type U2OS cells and immunoprecipitated with control IgG or anti-E2F1 antisera. qPCR was used to measure precipitated DNA. Units represent fold-induced increase in binding, comparing binding between anti-E2F1 and control IgG. ChIP analyses indicate that E2F1 binds directly to the endogenous UHRF2 promoter. C, mutations were introduced into two putative E2F sites in the human UHRF2 promoter. The first site, located between −520 and −527 relative to translation start, was mutated from “TTTCGG” to “TgcgcgG.” The second site, located between −451 and −458, was altered from “TTTCCGC” to “gcgcgCCG.” The wild-type and 2x mutant E2F UHRF2 promoters were PCR amplified and cloned upstream of luciferase in the pGL-3 vector. D, HEK293 cells were co-transfected with either wild-type or mutant luciferase constructs and harvested for ChIP assay 48 h later for immunoprecipitation with IgG or anti-E2F1 (C-20) antisera. We observe a 3.5-fold increased precipitation of the wild-type UHRF2 promoter by anti-E2F1 antisera compared with control IgG. Mutating two putative E2F binding sites significantly reduced pulldown by E2F1 compared with IgG, indicating that E2F1 binding has been perturbed by mutating the candidate E2F binding sites.
respectively, compared with levels in control cells. We asked whether the potential compensatory changes in UHRF2 mRNA levels also occurred at the protein level. We observed a decrease in UHRF2 protein levels in the shUHRF2 cell lines and also noted a 2–3-fold increase in UHRF1 protein in the shUHRF1 cells (Fig. 4B). We tested if E2F1-induced apoptosis is also affected by UHRF1 knockdown in Fig. 4C. Control, shUHRF1, shUHRF2, and combined shUHRF1/2 U2OS cells were infected with E2F1 expressing adenovirus and harvested later for quantification of levels of apoptotic cells. UHRF1 knockdown blocked E2F1 induction of apoptosis around 40% compared with control cells. As seen previously, E2F1-induced apoptosis was reduced around 50% in shUHRF2 cells. Combined knockdown of UHRF1 and UHRF2 in the same cell line led to a greater decrease in the number of apoptotic cells after E2F1 infection (70% reduction).

To confirm that the effect of shUHRF2 on E2F1-induced apoptosis was not due to off-target shRNA effects, we constructed a non-degradable (nd) allele of UHRF2 that maintained proper amino acid coding sequence but altered mRNA to prevent its degradation by the shRNA. This allele of UHRF2 was fused to an amino-terminal HA tag. The HA-ndUHRF2 or control plasmids were transfected into shUHRF2 U2OS cells and harvested 24 h post-transfection for anti-HA immunoblot analysis (Fig. 5A). We observed an accumulation of stabilized HA-UHRF2 following transfection of this construct. For apoptosis assays, control and shUHRF2 U2OS cells were transfected with control or HA-ndUHRF2 plasmids (46) and then infected 24 h later with control or E2F1-expressing adenovirus (Fig. 5B). Cells were harvested 48 h post-infection for measurement of apoptosis levels. E2F1 infection induced apoptosis in 65% of control cells, and shUHRF2 knockdown reduced the percent of apoptotic cells to 28%. Transfection of the HA-ndUHRF2 plasmid led to an E2F1-dependent restoration of apoptotic levels to 53%. These results indicate that E2F1-induced apoptosis is affected by UHRF2 degradation and not by off-target shRNA effects.

We showed that UHRF2 is an E2F1 transcriptional target and is bound by E2F1 at its promoter region. It is possible that UHRF2 facilitates apoptosis induction through E2F1 either by directly interacting with E2F1, or by performing other activities downstream of its induction by E2F1. We used a variety of co-immunoprecipitation assays to determine whether E2F1 and UHRF2 directly physically interact (Fig. 6). First, plasmids encoding HA-tagged E2F1 or Myc-tagged UHRF2 were co-transfected into HEK293 cells (Fig. 6A). Extracts were co-immunoprecipitated with control or anti-Myc monoclonal antisera (9E10) and immunoblotted with anti-HA antisera to detect precipitated E2F1. We observed a significant HA-E2F1 immunoprecipitation by Myc-UHRF2, compared with IgG control. In Fig. 6B, the reciprocal immunoprecipitation was performed. Extracts, prepared as in Fig. 6A, were immunoprecipitated with control IgG, anti-Myc, or anti-HA antisera.
U2OS cells were deprived of serum for 48 h and infected with adenovirus. RNA was harvested from infected cells and analyzed on Illumina Human WG-6 v3 beadchips. We identified E2F1-induced targets in U2OS cells by comparing gene induction levels between control or E2F1-infected cells. Genes that had been induced at least 2-fold by E2F1 were then further sorted based on the effect that UHRF2 knockdown had on E2F1-dependent expression.

Analysis on Illumina Human WG-6 v3 beadchips. Results were normalized using GeneSpring and analyzed for expression differences again using either GeneSpring software or Microsoft Excel.

Genes were initially sorted in Excel based on the strategy outlined in Fig. 7. To do this we compared gene expression in control cells infected with control or E2F1-expressing adenovirus. We focused on genes induced at least 2-fold by E2F1 in control U2OS cells, which totaled 2445 genes out of a total of 48,806, or roughly 5%. These E2F1-induced genes were then further sorted based on the effect that shUHRF2 had on their expression. We did this by comparing gene expression levels between E2F1-infected control versus shUHRF2 cells. Genes identified as being induced by E2F1 and that partially require UHRF2 for full expression are listed in Table 2. “F.I.” refers to fold-induction by E2F1, and “shUHRF2” is the ratio between the expression level of the gene in E2F1-infected shUHRF2 cells divided by the level in E2F1-infected control cells. The majority of E2F1-induced genes were unaffected by UHRF2 depletion. Around 6% of the E2F1-induced targets were reduced 50% or more following loss of UHRF2.

We used gene ontology (GO) annotations to better understand if the UHRF2-regulated E2F1 target genes could be grouped into functional categories. We selected the top 150 genes whose induction by E2F1 was most impaired by shUHRF2 and analyzed them by GO annotations using GATHER (Gene Annotation Tool to Help Explain Relationships). These genes showed a reduction in induction by E2F1 between 40 and 70% in shUHRF2 cells compared with control. GO analysis of these genes indicated that the functional category most highly represented in this group was apoptosis (GO: 0006915) followed by programmed cell death (GO:0012501) with p values of 0.01 for each category (Table 3). TRANSFAC analysis using the GATHER website indicated that E2F1 was the most highly represented transcription factor binding site in the 150 promoters analyzed (p value <0.0001) (Table 3). These results suggest that E2F1-UHRF2 complexes may regulate expression of a higher frequency of apoptotic target genes. This is in agreement with our finding that UHRF2 knockdown impairs apoptosis induction.

We manually tested the effect of UHRF2 loss on E2F1 induction of 14 of these target genes by qPCR. Control and shUHRF2 U2OS cell lines were infected with empty or E2F1-expressing adenovirus and qPCR was used to analyze some of the genes
TABLE 2
Identification of E2F1 target genes requiring UHRF2 for expression

The E2F-induced genes that display the greatest failure in induction upon UHRF2 depletion are shown. F.I. refers to “fold-induction” by E2F1 compared with CMV infection in control U2OS cells. shUHRF2 is the percent remaining expression of the gene in shUHRF2 U2OS cells compared with E2F1 induction of the gene in control cells.

| Gene                | F.I. | shUHRF2 | Definition                                                                 |
|---------------------|------|---------|---------------------------------------------------------------------------|
| SLC32A1             | 9.6  | 30%     | Solute carrier family 32 (GABA vesicular transporter), member 1 (SLC32A1), mRNA. |
| FHDC1               | 3.3  | 31%     | FH2 domain containing 1 (FHDC1), mRNA.                                    |
| CKB                 | 3.8  | 35%     | Creatine kinase, brain (CKB), mRNA.                                       |
| LY6H                | 3.2  | 36%     | Lymphocyte antigen 6 complex, locus H (LY6H), mRNA.                       |
| AKT1                | 4.0  | 36%     | v-akt murine thymoma viral oncogene homolog 1 (AKT1), transcript variant 3, mRNA. |
| ZNF672              | 3.3  | 39%     | Zinc finger protein 672 (ZNF672), mRNA.                                   |
| LHX3                | 8.3  | 39%     | LIM homeobox 3 (LHX3), transcript variant 1, mRNA.                        |
| CRIP2               | 5.8  | 41%     | Cysteine-rich protein 2 (CRIP2), mRNA.                                    |
| ADIPOR2             | 2.5  | 41%     | Adiponectin receptor 2 (ADIPOR2), mRNA.                                   |
| PHF21B              | 4.1  | 41%     | Phosphoglycerate kinase 2 (PHF21B), mRNA.                                 |
| NFkBIB              | 8.3  | 45%     | Nuclear factor of light polypeptide gene enhancer in B-cells inhibitor, β |
| TRAF3               | 2.8  | 46%     | TNF receptor-associated factor 3 (TRAF3), transcript variant 3, mRNA.      |
| JAG2                | 10.1 | 48%     | Jagged 2 (JAG2), transcript variant 1, mRNA.                              |
| PPP1R13B            | 1.2  | 49%     | Protein phosphatase 1, regulatory (inhibitor) subunit 13B (PPP1R13B), mRNA. |
| SIVA                | 3.6  | 52%     | CD27-binding (Siva) protein (SIVA), transcript variant 1, mRNA.           |
| CBX7                | 4.2  | 53%     | Chromobox homolog 7 (CBX7), mRNA.                                         |
| CCNE1               | 7.1  | 56%     | Cyclin E1 (CCNE1), transcript variant 2, mRNA.                            |
| CSE1L               | 3.6  | 56%     | CSE1 chromosome segregation 1-like (yeast) (CSE1L), transcript variant 2, mRNA. |
| MOAP1               | 2.5  | 57%     | Modulator of apoptosis 1 (MOAP1), mRNA.                                   |
| PDCD5               | 3.5  | 58%     | Programmed cell death 5 (PDCD5), mRNA.                                    |
| BCL2L11             | 32.0 | 65%     | BCL2-like 11 (apoptosis facilitator) (BCL2L11), transcript variant 6, mRNA. |
| HRK                 | 15.1 | 70%     | Harakiri, BCL2-interacting protein (contains only BH3 domain) (HRK), mRNA. |
| FOX3                | 4.4  | 74%     | Forkhead box 3 (FOX3), transcript variant 2, mRNA.                        |
| FOX1                | 9.8  | 77%     | Forkhead box 1 (FOX1), mRNA.                                              |
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TABLE 3
GO and TRANSFAC analysis of genes co-regulated by E2F1 and UHRF2

The top 150 E2F1-induced genes displaying the greatest reduction in expression in shUHRF2 cells were analyzed for over-represented GO categories or putative transcription factor-binding sites using the GATHER website. Apoptosis and programmed cell death were the most prevalent relevant TRANSFAC site. E2F target genes were least induced in shUHRF2 cells.

| No. | GO annotation | p value | BF  |
|-----|---------------|---------|-----|
| 1   | GO:0006915 : apoptosis | 0.01    | 1   |
| 2   | GO:0012501 : programmed cell death | 0.01    | 1   |
| 3   | GO:0009896 : positive regulation of catabolism | 0.01    | 1   |
| 4   | GO:0016572 : histone phosphorylation | 0.01    | 1   |
| 5   | GO:00040023 : nuclear positioning | 0.01    | 1   |
| 6   | GO:0045732 : positive regulation of protein catabolism | 0.01    | 1   |
| 7   | GO:0007097 : nuclear migration | 0.01    | 1   |
| 8   | GO:0012501 : programmed cell death | 0.01    | 1   |
| 9   | GO:0045732 : positive regulation of protein catabolism | 0.01    | 1   |
| 10  | GO:0008219 : cell death | 0.01    | 1   |
| 11  | GO:0012625 : death | 0.01    | 1   |

| TRANSFAC | Gene | p value | BF  |
|----------|------|---------|-----|
| 1        | V$E2F1_Q3_01 | <0.0001 | 15  |
| 2        | V$KROX_Q6 | <0.0001 | 15  |
| 3        | V$MYCMAX_B : c-Myc:Max binding sites | 0.0001 | 5   |
| 4        | V$CEBPDELTA_Q6 | 0.0001 | 5   |
| 5        | V$E2F1DP2_01 : E2F-1:DP-2 heterodimer | 0.0002 | 5   |
| 6        | V$SOD2Q2 | 0.0003 | 4   |
| 7        | V$E2F1DP1_01 : E2F-1:DP-1 heterodimer | 0.0005 | 4   |
| 8        | V$S2F_Q3_01 | 0.0005 | 4   |

identified by the microarray approach. We determined by what percentage these E2F1-induced targets were reduced following loss of UHRF2, by comparing their E2F1-dependent expression in shUHRF2 with expression levels in control cells. The results are displayed with the genes showing the greatest dependence for their induction by E2F1 on the left of Fig. 8A. For example, TRAF3, which is induced by E2F1 around 16-fold, displays a 70% drop in E2F1-dependent expression in shUHRF2 cells. Other proapoptotic E2F1 targets include SIVA, PDCD5, and HRK, which also showed a significantly reduced induction by E2F1 following UHRF2 knockdown. In total, 9 of 14 genes we tested showed a significant drop in E2F1-dependent expression in shUHRF2 cells compared with induction in control cells. E2F1 mediated fold-induction results presented in Fig. 8B are in the order based on their positions in Fig. 8A. Target genes were induced ranging from 4- (PDCD5) to 40-fold (JAG2).

We used chromatin immunoprecipitation (ChIP) to test if UHRF2 and E2F1 both bound to the promoters of co-regulated target genes identified by microarray and confirmed by qPCR. U2OS cells were treated with serum-deprived media to more closely resemble cellular situations with increased E2F1 levels but lacking growth factor-induced proliferation stimuli. Chromatin from wild-type U2OS cells was prepared and immunoprecipitated with control IgG, anti-E2F1, or anti-UHRF2 antisera. Our ChIP analysis indicates that E2F1 binds to the BIM, cyclin E1 (CCNE1), SIVA, TRAF3, and dihydrofolate reductase promoters, but not to the β-actin control (Fig. 9A). Units represent fold-enrichment of PCR product generated from chromatin precipitated with anti-E2F1 and control IgG. In Fig. 9B, anti-UHRF2 ChIP analysis reveals that BIM, CCNE1, SIVA, and TRAF3 are bound by UHRF2, however, neither dihydrofolate reductase nor β-actin is bound by UHRF2. These results indicate that four genes identified by microarray and qPCR as reliant on E2F1 and UHRF2 for expression are bound at their promoters by both E2F1 and UHRF2. Dihydrofolate reductase was not identified in the microarray analysis as reliant on UHRF2 for expression, and it is not bound by UHRF2 by ChIP.

DISCUSSION

The Rb/E2F pathway is deregulated in a wide variety of human solid tumors. Rampant E2F activity caused by loss of Rb promotes unrestrained activation of proliferation target gene expression, critical to tumor cell growth. However, E2F activity also promotes distinct cell fate outcomes such as cell death, senescence, differentiation, and others, but the key regulatory mechanisms underlying these decisions are not clearly understood. We therefore utilized an unbiased shRNA screening pro-
procedure to identify regulators of E2F1-induced apoptotic cell death. Overall, the genes we identified as putative mediators of E2F1-induced apoptosis can be classified into three broad functional categories. These categories include cell signaling (MIA2, WDFY3, CCNA2, and AZIN1), protein translation (eIF5A1), and DNA transcription/chromatin state modification (UHRF2 and KBTBD7).

MIA2 (melanoma inhibitory activity 2) is an SH3 containing protein that is down-regulated in hepatocellular carcinomas and its overexpression causes growth arrest in hepatocytes (26). WDFY3 (WD repeat and FYVE domain containing 3) can bind to phosphatidylinositol 3-phosphate (PI3K) and regulate aggregated protein clearance by autophagy (27). CCNA2 (cyclin A2) is a member of the conserved cyclin family of proteins that binds to and controls cyclin-dependent kinases and function as regulators of G1/S and G2/M cell cycle transitions. Interestingly, CCNA2-CDK2 complexes bind to and phosphorylate the amino terminus of E2F1, inhibiting its DNA binding affinity (28, 29). AZIN1 (antizyme inhibitor 1) indirectly regulate polyamine biosynthesis through the stabilization of ornithine decarboxylase, a mediator of the ornithine to putrescine conversion (30). eIF5A2 (eukaryotic translation initiation factor 5A2) is amplified and appears oncogenic in a variety of human tumors (31). eIF5A2, and the family member eIF5A1, are the only eukaryotic proteins that utilize the unique, polyamine-derived amino acid hypusine (32). KBTB7 (kelch repeat and BTB (POZ) domain containing 7) is a member of a large family of kelch motif containing proteins that are generally involved in protein/protein interactions. KBTB7, when overexpressed, was shown to activate the transcriptional activities of activator protein-1 and serum response element (SRE) factors (33). UHRF2 (ubiquitin-like with PHD and ring finger domains 2, E3 ubiquitin protein ligase) encodes a complex, multidomain nuclear protein with DNA and histone binding capacity and ubiquitin-ligase activity and is involved in cell cycle regulation and potentially tumorigenesis (17, 34).

UHRF2 was originally called NIRF and was cloned through a two-hybrid interaction with PEST-containing nuclear protein and described as highly expressed in proliferating cells but not in the G0/G1 cell cycle phase (34). Subsequent work demon-
UHRF2 Mediates E2F1 Apoptosis Induction

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