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Cell Cycle, Cell Death, and Senescence

Molecular Cancer Research

RBM38 Is a Direct Transcriptional Target of E2F1 that Limits E2F1-Induced Proliferation

Orit Feldstein, Rotem Ben-Hamo, Dana Bashari, Sol Efroni, and Doron Ginsberg

Abstract

The E2F family of transcription factors plays a pivotal role in the regulation of cell proliferation in higher eukaryotes and is a critical downstream target of the tumor suppressor pRB. The pRB/E2F pathway is defective in most human tumors, resulting in deregulated E2F activity that induces uncontrolled cell proliferation, a hallmark of tumor cells.

The RNA-binding protein RBM38, also named RNPC1, induces cell-cycle arrest in G_1, at least in part, via binding to and stabilizing the mRNA of the cyclin-dependent kinase inhibitor p21. RBM38 levels are altered in human cancer. Generally, RBM38 is overexpressed in various tumors; however, RBM38 mRNA levels are reduced in some breast tumors due to increased methylation of its promoter region.

We show here that expression of RBM38 is regulated by E2F1. Specifically, RBM38 mRNA and protein levels are elevated upon activation of either exogenous E2F1 or endogenous E2Fs. Moreover, endogenous E2F1 binds the human RBM38 promoter and E2F1 knockdown reduces RBM38 levels. Our data raise the possibility that E2F1 together with E2F1-regulated RBM38 constitute a negative feedback loop that modulates E2F1 activity. In support of this, inhibition of RBM38 expression increases E2F1-mediated cell-cycle progression. Moreover, in human ovarian cancer, high correlation between expression of E2F1 and RBM38 is associated with increased survival.

Overall, our data identify RBM38 as novel transcriptional target of E2F1 that restricts E2F1-induced proliferation. Furthermore, this negative feedback loop seems to restrict tumor aggressiveness, thereby promoting survival of patients with cancer. *Mol Cancer Res; 10(9); 1169–77. ©2012 AACR.*

Introduction

E2Fs are transcription factors best known for their involvement in the timely regulation of gene expression required for cell-cycle progression (1). In particular, E2Fs regulate the expression of genes required for entry into and progression through S-phase and, accordingly, ectopic expression of E2F1 in quiescent cells induces S-phase entry (1, 2). Members of the E2F family are downstream effectors of the tumor suppressor pRB. The critical role of the RB/E2F pathway in normal cellular proliferation is highlighted by the common incidence among human tumors of pathway mutations that result in deregulated E2F activity (3). This deregulated E2F activity leads to uncontrolled cell proliferation, a hallmark of tumor cells.

In addition to being fundamental regulators of proliferation, E2Fs modulate diverse cellular functions, such as DNA repair, differentiation, and development (4, 5). At least one member of the E2F family, namely E2F1, can also mediate apoptosis (1, 6) and autophagy (7–9). In line with E2F1 influencing both proliferation and cell death, it functions *in vivo* in a context-dependent manner as an oncogene or a tumor suppressor (10).

The pivotal role of E2F1 in transcriptional regulation of genes and the ensuing effect on RNA production are well documented. More recently, studies have shown that E2F1 affects additional aspects of RNA dynamics. First, E2F1 controls pre-mRNA processing of specific genes by regulating the expression of the splicing factor, SC35 (11–13). Second, E2F1 regulates RNA stability and availability for translation, activities attributed mainly to direct regulation of microRNA expression. Indeed, a number of E2F-regulated miRNAs have been identified, including miR-449a, miR-449b (14) and the miR-17-92, miR-106a-363, miR-106b-25, and miR-15-16 clusters (14–21). Another little explored possibility for E2F-mediated regulation of RNA dynamics is an effect on RNA-binding proteins (RBP), which influence not

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only RNA stability but also RNA metabolism, localization, and splicing (22, 23), as well as miR activity (24).

RBM38, also called RNPC1, is an RBP that contains one RRM domain and it is a transcriptional target of the p53 family of transcription factors (25). Rbm38 can be expressed as 2 isoforms and isoform a, but not isoform b, binds and stabilizes the mRNA of the CDK inhibitor, p21 thereby inducing cell-cycle arrest in G_1 (25, 26). RBM38 also binds and stabilizes the mRNA of another RBP HuR, which in turn facilitates RBM38-mediated growth arrest (27). Additional mRNAs bound by RBM38 include p63 and p53, however, in these instances RBM38 binding mediates a decrease in mRNA levels and attenuation of translation, respectively (28, 29). Recently, RBM38 was shown to regulate cell-cycle arrest also via limiting miRNA accessibility to specific mRNAs (26). Expression of RBM38 is abnormal in human cancer. Generally, RBM38 is overexpressed in colon carcinoma (30), lymphomas (29) and esophageal cancer, in the latter case its upregulation is associated with a poor response to radiation combined with chemotherapy (31). However, a recent study found that RBM38 is silenced by promoter hypermethylation in breast cancer (26).

Here, we show that RBM38 is a novel direct transcriptional target of E2F1. Specifically, we evidence that endogenous E2F1 binds the RBM38 promoter and inhibition of endogenous E2F1 leads to reduced RBM38 levels, whereas E2F1 activation results in increased RBM38 levels. Importantly, we show that RBM38 knockdown enhances E2F1-induced G_1 exit, indicating that RBM38 is part of a negative feedback loop, which restricts E2F1 activity by limiting cell-cycle progression at the G_1–S boundary. In agreement with this model, analysis of human tumors reveals that expression of E2F1 together with RBM38 is highly correlated with increased survival.

Materials and Methods

Cell culture

U2OS and SAOS-2 human osteosarcoma cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal calf serum or without serum. H1299 human lung adenocarcinoma cells were grown in RPMI-1640 medium supplemented with 5% fetal calf serum. Early-passage WI38 human embryonic lung fibroblasts were grown in minimal essential medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate and nonessential amino acids. Cells were maintained at 37°C in a humidified atmosphere containing 8% CO_2. To induce activation of ER-E2F1, cells were treated with 100 nmol/L 4-hydroxytamoxifen (OHT, Sigma) for the times indicated. Where indicated, cycloheximide (Sigma) was added at 10 μg/mL to the incubation for 8 hours.

Real-time reverse transcriptase PCR (quantitative PCR)

Total RNA was extracted from the cells using the Tri Reagent method. Real-time reverse transcriptase PCR (RT-PCR) was done using SYBR Green PCR Master Mix (Applied Biosystems) and the following primer pairs:

RBM38: 5′-CTGCCGTACCACACTACCG and 5′-GTCTTTGCAAGCCCTCTCAG
GAPDH: 5′-CATGTTCCAATATGATTCCACC and 5′-GATGGGATTTCCATTGATGAC
β-Actin: 5′-TCCCTGGAGAAGAGCTACGA and 5′-AGGAAGGAAGGCTGGAAGAG
Cyclin E: 5′-CAGATTGCAGAGCTGTTGGA and 5′-TCAAGGCAGTCAACATCCAG
p21: 5′-TGGAGACTCTCAGGGTCGAAA and 5′-GGCGTTTGGAGTGGTAGAAATC.
p53: 5′-CCCAAGCAATGGATGATTTGA and 5′-GGCATTCTGGGAGCTTCATCT.

Primers for RBM38 recognize both RBM38 isoforms. All real-time RT-PCR reactions were carried out using the Applied BiosystemsStepOnePlus Real-Time PCR Systems Machine. Results are presented as mean and SD for duplicate runs.

Western blotting

Cells were lysed in lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40] in the presence of protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails I and II (Sigma). Equal amounts of protein, as determined by the Bradford assay, were resolved by electrophoresis in a 12.5% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated with one of the following primary antibodies: anti-RBM38 (ARP40863_P050, AVIVA Systems Biology), of note, this antibody recognizes only isoform a; anti-E2F1 (sc-251, Santa Cruz Biotechnology); anti-P21 (sc-397, Santa Cruz Biotechnology); anti-Actin (sc-1616r, Santa Cruz Biotechnology); or anti-Cyclin E (sc-247, Santa Cruz Biotechnology). Binding of the primary antibody was detected using an enhanced chemiluminescence kit (ECL; Amersham).

Plasmids

The plasmids pBabe-neo-HA-ER-E2F1, pBABE-puro-16E7, pBABE-puro-E7-dl21-35, and pRETROSUPER-shp53 have been described previously (32).

Transfection/infection procedures

To generate retroviruses, cells (2×10^6) of the packaging cell line 293T were cotransfected with ecotropic packaging plasmid pSV-EMLV (10 μg), which provides packaging helper function, and the relevant plasmid (10 μg) using the calcium phosphate method in the presence of chloroquin (Sigma). After 8 hours, the transfection medium was replaced with fresh Dulbecco's Modified Eagle's Medium supplemented with 5% fetal calf serum. Subsequently, cell supernatants containing retroviruses were collected.

When conducting infection, cells were incubated for 5 hours at 37°C in 4.5 mL of retroviral supernatant

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supplemented with polybrene (8 μg/mL, Sigma H9268). Then, 5.5 mL of medium was added, and after a further 24 hours, the medium was replaced with fresh medium containing puromycin (2 μg/mL, Sigma P7130).

When transfecting U20S, SAOS-2, or H1299 cells with siRNA, interferin transfection reagent (PolyPlus-transfection) was used according to the manufacturer's instructions. The siRNAs against RBM38 (siRBM38#1: GGAAGUACUUCGAGGGCUUCGGCGA, siRBM38#3: GGCCAACGUGAACCUGGCAUAUCUG) and a scrambled sequence serving as a negative control were synthesized by Invitrogen. siRNA against E2F1 was synthesized by Sigma-Aldrich. Experiments were carried out 72 hours after transfection with RBM38 siRNA and 48 hours after transfection with E2F1 siRNA.

Fluorescence-activated cell-sorting analysis

Cells were trypsinized and then fixed by incubating in 70% ethanol at 4°C overnight. After fixation, cells were centrifuged for 4 minutes at 1,500 rpm before being incubated for 30 minutes at 4°C in 1 mL of PBS. Then, the cells were centrifuged again and resuspended in PBS containing 5 mg/mL propidium iodide and 50 μg/mL RNase A. After incubation for 20 minutes at room temperature, fluorescence intensity was analyzed using a Becton Dickinson flow cytometer.

Chromatin immunoprecipitation

DNA–protein complexes were immunoprecipitated from U2OS cells using the ChIP (chromatin immunoprecipitation) assay kit (Upstate Biotechnology) according to the manufacturer's protocol with the following antibodies: anti-E2F1 (sc-193; Santa Cruz Biotechnology), anti-HA (sc-805; Santa Cruz), and anti IgG (111-035-144, Jackson Immunoresearch). Anti-HA and anti-IgG served as a control for nonspecific DNA binding. The precipitated DNA was subjected to RT-PCR analysis using specific primers corresponding to the human RBM38 promoter (5′-AAGCTCACAGAGCCCCACTA and 5′-TTGCAGGCACTAAGCACAAC) as well as primers for β-actin that served as a negative control (5′-ACGCCAAAACTCTCCCTCCTCCTC and 5′-CATAAAAGGCAACTTTCGGAACGGC).

Computational analysis

Data were obtained from The Cancer Genome Atlas dataset (TCGA) http://cancergenome.nih.gov/ (ovarian cancer) and the Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/geo/ databases [glioblastoma—accession number GSE13041 (33); breast cancer—accession number GSE20685 (34)]. The data consist of gene expression microarray data and clinical information from 506 patients with ovarian cancer from, 191 patients with glioblastoma, and 255 patients with breast cancer.

Feature selection algorithms were applied to E2F1 and RBM38 expression levels to classify a subgroup with significant strong correlation, distinct from the remaining set in which there was insignificant correlation. Kaplan–Meier survival analysis was conducted on the 2 groups to determine the stratification power.

Results

E2F directly regulates expression of Rbm38 in a p53-independent manner

To explore whether E2F1 regulates RBPs, we conducted a meta-analysis of published microarray data collected from studies aimed at identifying E2F-regulated genes. We found that the mRNA levels of a number of RBPs increase upon E2F activation (in at least 2 independent studies). From the group of RBPs putatively regulated by E2F, we chose to focus on RBM38 because the E2F-associated upregulation of this RBP is observed in dissimilar biologic systems (35, 36). We took advantage of a conditionally active E2F1 (ER-E2F1), which is activated by the addition of 4-hydroxytamoxifen (37), to validate that Rbm38 expression is indeed E2F-regulated. We found that activation of this conditional E2F1 in U2OS human osteosarcoma cells was associated with a significant increase in Rbm38 mRNA levels (Fig. 1A). As expected, addition of 4-hydroxytamoxifen to cells lacking the inducible E2F1 did not affect Rbm38 mRNA levels, validating the role of E2F1 in the upregulation (Fig. 1A). Next, Western blot analysis was used to discover whether expression changes seen at the mRNA level are also apparent at the protein level. Indeed, the protein levels of RBM38 were upregulated significantly following activation of conditional E2F1 (Fig. 1B).

Rbm38 expression is known to be regulated by the tumor suppressor p53 (25) and E2F1 can activate p53 via a number of pathways (38). To test whether E2F1-induced upregulation of Rbm38 occurs indirectly via p53, we examined the effect on Rbm38 expression of activating conditional E2F1 in SAOS-2 human osteosarcoma cells, which lack wild-type p53. Activation of ER-E2F1 in these cells resulted in a significant increase in RBM38 expression at both mRNA and protein level (Fig. 1C and D). Similar results were obtained using H1299 lung adenocarcinoma cells that are also p53-deficient (Supplementary Fig. S1A and S1B). Furthermore, we found that activation of conditional E2F1 induced RBM38 expression levels in the p53-proficient U2OS cell line even in the presence of short hairpin RNA directed against p53 (Supplementary Fig. S2). Taken together, these results indicate that E2F1-induced upregulation of Rbm38 is p53-independent.

Next, to test whether Rbm38 is regulated not only by exogenous E2F1 but also by endogenous E2F, we examined Rbm38 expression in nontransformed WI38 cells before and after deregulation of endogenous E2F activity. Endogenous E2F activity was deregulated by infecting the cells with a retrovirus encoding human papilloma virus 16 (HPV16) E7 protein, which disrupts RB/E2F complexes and thus, activates E2F. As a control, in parallel, cells were infected with a mutated E7 protein, E7△21–35, which does not bind RB family members and consequently does not deregulate E2F. As expected for a gene regulated by E2F, infection with E7, but not E7△21–35, resulted in

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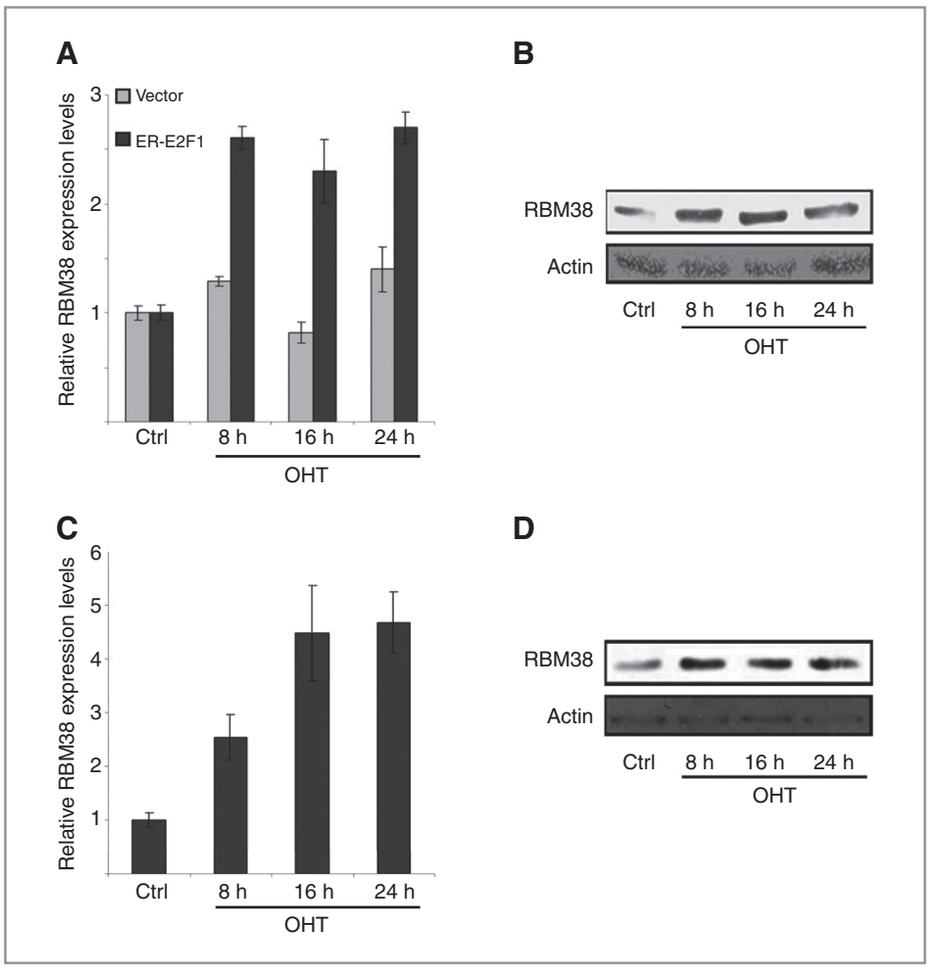

Figure 1. Activation of ectopic E2F1 upregulates RBM38 expression. U2OS cells infected with a control retrovirus (vector) or retrovirus expressing ER wild-type E2F1 (ER-E2F1) were incubated with 4-hydroxytamoxifen (OHT; 100 nmol/L) for the times indicated or left untreated (Ctrl). A, RNA was extracted and Rbm38 mRNA levels determined by real-time RT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. B, proteins were extracted from the cells and Western blot analysis conducted using antibodies directed against RBM38 or actin. SAOS-2 cells infected with retrovirus expressing ER wild-type E2F1 (ER-E2F1) were incubated with OHT (100 nmol/L) for the times indicated or left untreated (Ctrl). C, RNA was extracted and Rbm38 mRNA levels determined by real-time RT-PCR and normalized to GAPDH levels. D, proteins were extracted from the cells and Western blot analysis conducted using antibodies directed against RBM38 or ACTIN. All real-time RT-PCR experiments were carried out in duplicate and the average of 2 independent experiments is presented.

increased Rbm38 mRNA levels (Fig. 2A), implicating endogenous E2Fs in the regulation of Rbm38 expression. Another approach was taken to confirm the involvement of endogenous E2F1 in controlling Rbm38 expression, whereby the effect of E2F1 ablation on Rbm38 protein levels was studied. In both U2OS and SAOS-2 cells, introduction of an siRNA directed against E2F1 resulted in significantly reduced endogenous RBM38 protein levels (Fig. 2B). Similar results were obtained when E2F1 levels were reduced in H1299 cells (Supplementary Fig. S1C). In summary, we notice evidence for the first time another layer of RNA regulation by E2F, E2F regulates expression of an RBP, RBM38.

To examine whether E2F regulates Rbm38 directly or indirectly, exogenous E2F1 was activated in the presence of the protein synthesis inhibitor cycloheximide. In support of direct regulation, E2F activation in the presence of cycloheximide still resulted in increased Rbm38 mRNA levels (Fig. 2C). Further support for this premise was sought using ChIP analyses. First, chromatin was isolated from proliferating U2OS cells and incubated with an antibody directed against E2F1 and as predicted, endogenous E2F1 was detectably associated with the Rbm38 promoter (Fig. 2D). Next, chromatin was isolated from U2OS cells containing the inducible ER-E2F1 before and after activation of this exogenous E2F1. As predicted, binding of E2F1 to the RBM38 promoter was significantly enhanced upon ER-E2F1 activation (Fig. 2E).

E2f1 and Rbm38 form a negative feedback loop that impacts tumorigenesis

Having established that expression of Rbm38 is regulated directly by E2F1, we next tested whether Rbm38 plays a role in E2F1-mediated cell-cycle progression and/or apoptosis. RBM38 has been reported to affect the levels of the cyclin-dependent kinase inhibitor p21. Specifically, RBM38 binds the 3′-untranslated region of the p21 transcript and thus stabilizes p21 mRNA, leading to an increase in p21 expression at both the mRNA and protein level (25). Of note, p21 was suggested to be an E2F1-regulated gene as E2F1 binds to and activates the p21 promoter (39, 40). To test directly whether RBM38 plays a role in E2F1-induced cell-cycle progression, we reduced endogenous RBM38 expression and examined the effect(s) on E2F1-induced G_1 exit. RBM38 expression was inhibited in cycling U2OS cells expressing conditionally active E2F1 by introducing

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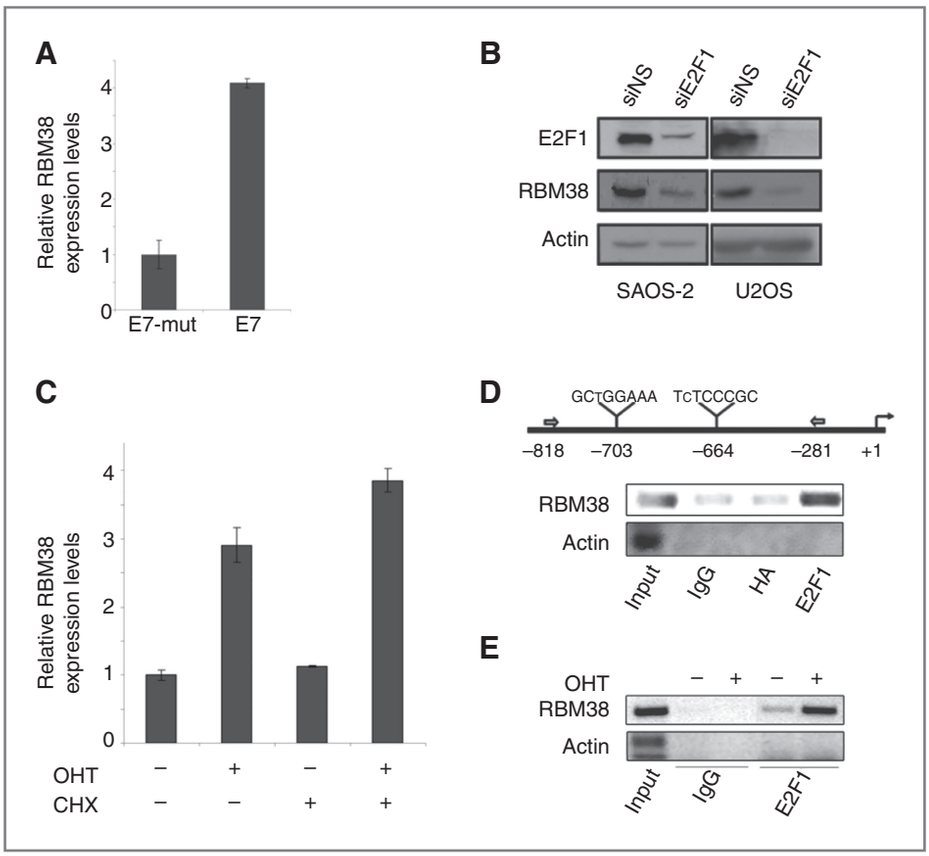

Figure 2. Endogenous E2F regulates RBM38 levels. A, WI38 cells were infected with a retrovirus expressing either wild-type E7 (E7) or an RB-binding–deficient mutant of E7 (E7-mut). Total RNA was extracted from the cells and expression levels of RBM38 determined by real-time RT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. B, U2OS or SAOS-2 cells were transfected with either a nonspecific siRNA (siNS) or an siRNA directed against E2F1 (siE2F1), proteins were extracted from the cells and Western blot analysis conducted using antibodies directed against E2F1, RBM38, or actin. C, U2OS cells infected with retrovirus expressing ER wild-type E2F1 were left untreated or incubated with 4-hydroxytamoxifen (OHT; 100 nmol/L) for 8 hours and then cells were treated or not with 10 μg/mL cycloheximide (CHX) for 8 hours. RNA was extracted and Rbm38 mRNA levels determined by real-time RT-PCR and normalized to GAPDH levels. D, top, a schematic representation of the human RBM38 promoter. The E2F-binding sites are represented as 8-mer nucleotide sequences. The transcription start site (+1) is indicated by an arrow. The DNA fragment amplified by RT-PCR is represented by arrows (−818/−281). –Bottom, ChIP analysis was conducted using U2OS cells. Cross-linked chromatin was precipitated using antibodies specific to E2F1, IgG, or HA. Then, RBM38 and actin promoter fragments were amplified by RT-PCR. Input DNA represents 0.5% of total chromatin. E, U2OS cells infected with a retrovirus expressing ER wild type-E2F1 (ER-E2F1) were incubated with OHT (100 nmol/L) for 6 hours (+) or left untreated (−). Then, ChIP analysis was conducted. Cross-linked chromatin was precipitated using antibodies specific to E2F1 or IgG. RBM38 and actin promoter fragments were amplified by RT-PCR. Input DNA represents 0.5% of total chromatin. All real-time RT-PCR experiments were carried out in duplicate and the average of 2 independent experiments is presented.

siRNA directed against Rbm38, siRBM38#1. As expected, the siRNA significantly reduced Rbm38mRNA levels (Fig. 3A) whereas introduction of nonspecific siRNA had no effect on RBM38 levels. In line with previous reports, activation of ectopic E2F1 resulted in a mild increase in both p21 mRNA and protein levels (Fig. 3A). The siRNA directed against Rbm38 reduced significantly both basal and E2F1-induced p21 mRNA and protein levels (Fig. 3A). Similar results were obtained using a different siRNA directed against Rbm38, siRBM38#3, (Fig. 3A), indicating that the effects observed after introduction of the 2 anti-RBM38 siRNAs are unlikely to be due to nonspecific siRNA targets. Analysis of cell-cycle distribution indicated that, as previously reported, activation of ectopic E2F1 resulted in G_1 exit. Knockdown of Rbm38 reproducibly promoted G_1 exit when using siRBM38#1, but not when using siRBM38#3, the siRNA that leads to a milder reduction in Rbm38 levels. Importantly, simultaneous activation of E2F1 and silencing of Rbm38 synergistically promoted G_1 exit (Fig. 3B and C). Similar results were obtained using serum-starved U2OS cells (Supplementary Fig. S3) as well as cycling SAOS-2 cells (data not shown). Silencing of Rbm38 did not have a significant effect on E2F1-induced apoptosis (data not shown). Taken together, these data support that Rbm38 attenuates E2F1-mediated cell-cycle progression

Next, to evaluate the physiologic relevance of RBM38 regulation by E2F1 in human tumors, we checked whether the levels of E2F1 and RBM38 correlate in a cohort of 506 patients with ovarian cancer. In agreement with Rbm38 being an E2F1-regulated gene, we detected a weak, yet

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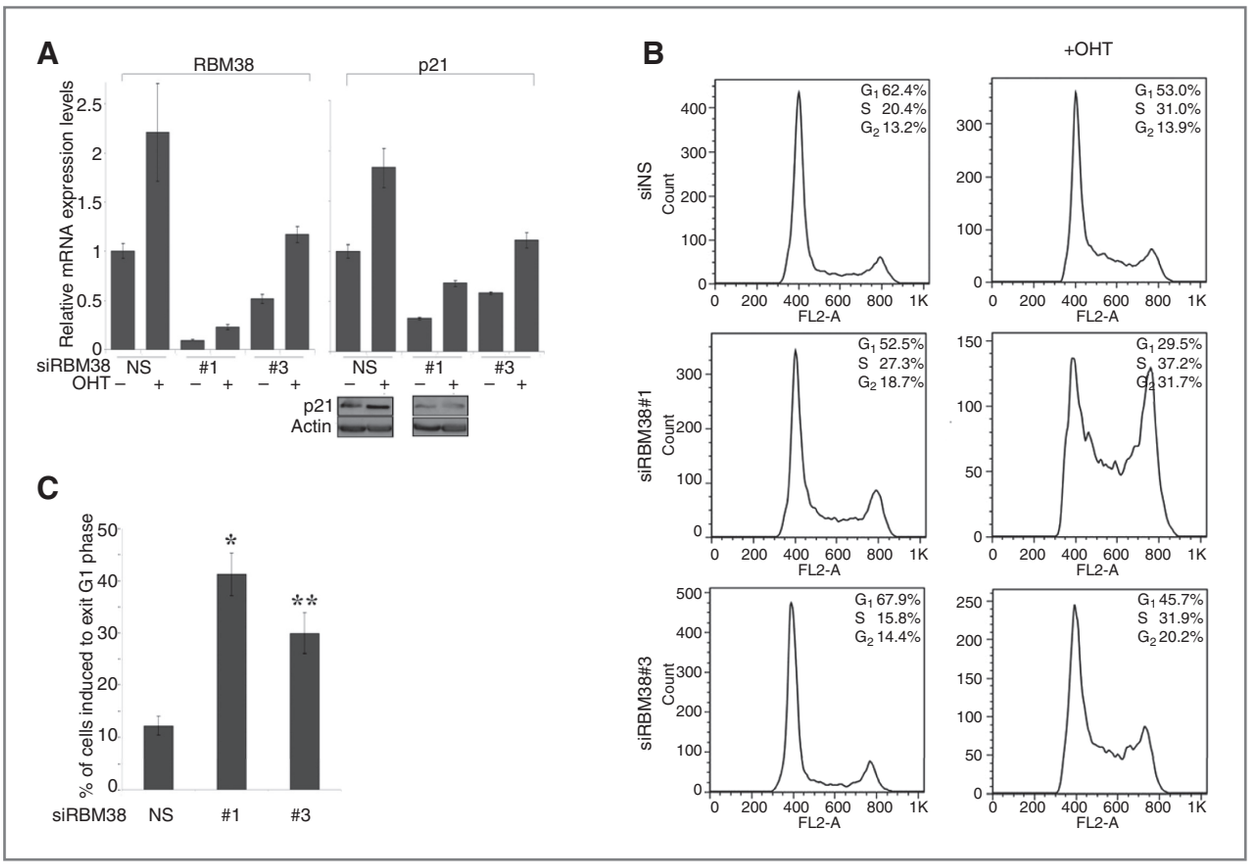

Figure 3. Rbm38 restricts E2F1-induced G_1 exit. A, U2OS cells stably expressing ER wild-type E2F1 were transfected with either a nonspecific siRNA (siNS) or an siRNA directed against RBM38 (siRBM38). Then, cells were left untreated or incubated with 4-hydroxytamoxifen (OHT; 100 nmol/L) for 20 hours. Top, RNA was extracted and Rbm38 and p21 mRNA levels determined by real-time RT-PCR and normalized to GAPDH levels. All real-time RT-PCR experiments were carried out in duplicate and the average of 2 independent experiments is presented. Bottom, proteins were extracted and Western blot analysis conducted using antibodies directed against p21 and actin. B, cells described in (A) were analyzed by fluorescence-activated cell sorting using propidium iodide (PI). One representative experiment is shown. The percentages of cells in G_1, S, and G_2–M cell-cycle phases are depicted in the top right side of each histogram. C, the percentages of cells induced to exit G1-phase are shown in bar graphs. The average of 3 independent experiments is presented. *, $P = 0.0081$; **, $P = 0.012$ (2-tailed Student t test, $n = 3$).

statistically significant, positive correlation between the expression levels of RBM38 and E2F1 in this group of patients (Fig. 4A). Notably, application of a feature selection algorithm that aims to identify the subgroup of patients with significant strong correlation, resulted in division of the cohort into 2 subgroups: one with high correlation between expression of RBM38 and E2F1 (Fig. 4B, 176 patients) and the other with no such correlation (Fig. 4C, 330 patients). Remarkably, it became apparent that the group with high correlation of expression exhibited significantly increased survival (Fig. 4D). Similar results were obtained when analyzing cohorts of 255 patients with breast cancer (Supplementary Fig. S4) and 191 patients with glioblastoma (Supplementary Fig. S5). These findings align with the model that RBM38 is an E2F1-regulated gene, which then functions as part of a negative feedback loop restricting E2F1-induced proliferation. We hypothesize that loss of correlation between E2F1 and RBM38, for example, due to methylation of the RBM38 promoter as reported to occur in breast cancer (26), unleashes the full proliferative potential of E2F1 and in this way contributes to the aggressiveness of the tumor.

Discussion

Rbm38 is a novel E2F1 transcriptional target

In this study, we identified that RBM38 expression is regulated by E2F1. Specifically, we show that activation of either ectopic E2F1 or endogenous E2Fs results in elevated levels of Rbm38 mRNA and protein. Moreover, we evidence that endogenous E2F1 binds the Rbm38 promoter and Rbm38 expression is reduced in response to knockdown of endogenous E2F1.

As described in the introduction, E2F1 is known to influence, directly and indirectly, various aspects of RNA dynamics. In addition, it was reported recently that E2F1 regulates the expression of the RBP QKI, thereby indirectly affecting RNA levels of critical cell-cycle regulators, such as c-fos, cyclin D1, and p27 (41). We show here that another RBP, RBM38, is a novel E2F1 transcriptional target. Our meta-analysis of published microarray data from studies

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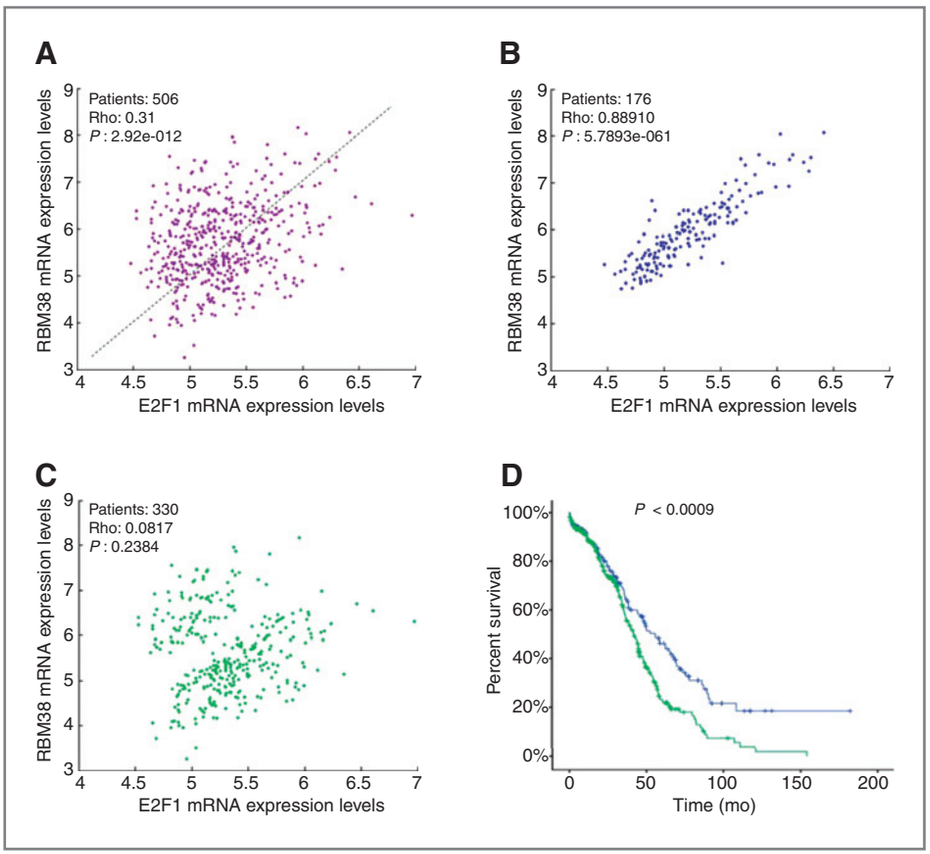

Figure 4. Correlated expression of E2F1 and RBM38 is associated with increased survival. A, the correlation between expression levels of E2f1 and Rbm38 in 506 patients with ovarian cancer is presented. The 506 patients with ovarian cancer shown in (A) were stratified into 2 groups according to E2F1 and RBM38 correlation status. B, group 1 contains 176 patients who clustered according to a significant strong positive correlation between the genes' expression levels. C, group 2 contains 330 patients who clustered on the basis of a nonsignificant correlation in gene expression levels. D, Kaplan–Meier survival curves of the 2 groups that emerged from this analysis. Group 1 (blue line) has a higher survival rate than group 2 (green line). Rho represents the correlation coefficient.

aimed at identifying E2F-regulated genes suggests that E2F regulates the expression of many additional RBPs. Validation of these predictions as well as elucidation of the role(s) such RBPs play on E2F1-mediated biologic functions awaits future research.

Rbm38 restricts E2F1-mediated cell-cycle progression at the G_1 exit

It is well documented that E2F1 promotes cell-cycle progression. In particular, E2F1 controls the expression of many genes required for S-phase entry and progression and accordingly, possesses an ability to induce G_1 exit and S-phase entry (42). Rbm38 was reported to exert the opposite effect, namely induce G_1 arrest, an activity attributed mainly to an ability to bind and regulate the stability of p21 mRNA (28, 43). We show here that Rbm38 knockdown synergizes with E2F1 activation to promote G_1–S transition and cell cycle-progression. Of note, RBM38 knockdown did not have any detectable effect on E2F1-induced apoptosis in the same experimental systems.

Our data reveal a new regulatory loop: E2F1 directly activates expression of the RBP RBM38, which in turn restricts the proproliferative function of E2F1. We suggest that this feedback loop serves to temper the full proliferative potential of E2F1. Loss of this loop, for example, via hypermethylation of the RBM38 promoter as reported to occur in breast cancer may contribute to the enhanced proliferation and increased tumor aggressiveness and observed reduced survival.

This is not the first example of E2F-dependent inhibition of E2F activity and in fact the RB/E2F pathway encompasses many negative feedback loops. For example, we have shown previously that E2F1 activates AKT via transcriptional regulation of the adaptor Gab2 and that this E2F1-dependent AKT activation serves to inhibit E2F1-mediated apoptosis (44). In addition, we found that E2F1 transcriptionally regulates the expression of miR-15 and miR-16, and these miRs in turn, repress E2F1-mediated cyclin E expression and G_1 exit (16). Furthermore, E2F1 was shown to upregulate expression of CDK inhibitors including p21 and p18 (45) and repressive E2Fs such as E2F7 and E2F8 (46–48), all of which counteract E2F1 activities. It remains to be investigated whether E2F1 expression correlates with the expression of any/all of these repressors and if the relationships are associated with cancer prognosis.

RBM38 is a transcriptional target of p53 and can induce cell-cycle arrest in G_1, at least in part, via binding to and stabilizing the p21 transcript (28). In addition, it has been suggested that E2F regulates expression of the p21 gene during cell-cycle progression (49) most probably via a p53-independent mechanism (40). Because we show here that Rbm38 is transcriptionally regulated by E2F1 and modulates E2F1-induced G_1 exit, we hypothesized that p21 could mediate the impact of Rbm38 on E2F1-dependent cell-cycle

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progression. Our data show that in U2OS cells, RBM38 knockdown leads to promotion of G_1 exit and a concomitant decrease in the mRNA and protein levels of p21. However, in SAOS-2 cells, a similar knockdown of RBM38 led to an enhanced G_1 exit that was not accompanied by a significant decrease in the mRNA and protein levels of p21 (data not shown). These data lead us to suggest that the effect of RBM38 on E2F1-induced cell-cycle progression is largely mediated by a yet undiscovered target(s) of RBM38 and probably only to a minor extent by p21. This model is in agreement with previous studies showing that RBM38 can suppress cell proliferation in a p21-independent manner (28). In addition to its effect on the p21 transcript, RBM38 was shown to bind the p53 transcript and repress its translation (29). However, we did not detect a significant and reproducible effect on p53 expression in our experimental system upon modulation of RBM38 levels (data not shown), suggesting that the p53–RBM38 regulatory loop likely operates in distinct biologic settings.

An intricate network of E2F1-regulated genes affects G_1–S transition

When the findings presented here are taken together with the growing body of research on E2Fs, a model emerges in which E2F1 regulates the expression of multiple genes, the products of which interact to generate a network that controls cell-cycle progression. The players in the network include cyclin E, a pivotal player in cell-cycle progression and one of the best documented targets of E2F1 (50), which is negatively regulated by p21 (which as mentioned was also suggested to be an E2F target; ref. 49); miR-106b and miR-17, which are targets of E2F (17, 51) that in turn target p21 (52–54); and now, to add to this list, there is RBM38, shown here to be yet another E2F1-regulated gene, an RBP known to bind p21 mRNA and thereby counteract the inhibitory effect of miR-106b and miR-17 (26). We surmise that progression along the cell cycle, across the G_1–S boundary depends on the outcome of crosstalk between these E2F targets.

To conclude, this work evidences a novel regulatory loop, consisting of a transcription factor, E2F1, and an RBP, RBM38. This loop is shown here to influence the G_1–S transition and thus, control cell proliferation. In addition, analysis of patients with ovarian cancer suggests that this regulatory loop impacts cancer biology, in particular survival. Given the key role of the E2F network in cancer biology, better understanding of the crosstalk between the players, such as between RBM38 and E2F, should ultimately advance our ability to develop effective therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: O. Feldstein, D. Ginsberg
Development of methodology: S. Efroni
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Feldstein, D. Bashari, S. Efroni, D. Ginsberg
Writing, review, and/or revision of the manuscript: O. Feldstein, D. Ginsberg
Study supervision: S. Efroni, D. Ginsberg
Computational analysis of the data in terms of survival analysis, sub-group regulation of microRNA and genes etc.: R. Ben-Hamo

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