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A Nucleolar Specificity Factor for E2F1 Induced Cell Death

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A NUCLEOLAR SPECIFICITY FACTOR FOR E2F1-INDUCED CELL DEATH

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ABSTRACT

The E2F family of transcription factors are important regulators of cell proliferation, and are often dysregulated in cancers. One member of the E2F family, E2F1, also has the ability to induce apoptosis; therefore, uncovering how E2F1-induced apoptosis is controlled is of interest in understanding tumorigenesis. To this end, we identified RRP1B as a novel target specifically induced by E2F1. RRP1B expression is specifically upregulated by E2F1 overexpression, but not other E2F family members. RRP1B expression is correlated with E2F1 expression during the cell cycle, and is significantly induced after DNA damage. The minimal RRP1B promoter region responsive to E2F1 was identified. Finally, E2F1, but not other E2F family members, was shown to bind endogenous RRP1B promoters through chromatin immunoprecipitation assays.

To determine the function of RRP1B in regulation of E2F1-induced apoptosis, we then constructed cell lines stably transfected with siRNAs against RRP1B. Knockdown of RRP1B inhibited apoptosis induced by genotoxic stimuli. Knockdown of RRP1B was able to inhibit the expression of selective E2F1 targets, including caspase-3 and -7 which are involved in apoptosis. We also determined that RRP1B and E2F1 interact both in vitro and in vivo, and also showed that RRP1B specifically bound to the promoters of E2F1 targets that were selectively affected by RRP1B knockdown. Together, this data suggests that E2F1-induced apoptosis is mediated in part by induction of RRP1B,

interaction between E2F1 and RRP1B, and binding to the promoters of selective proapoptotic E2F1 targets.

Keywords: E2F1, RRP1B, apoptosis, nucleolus

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DISCUSSION

INTRODUCTION

Cancer is fundamentally a disease of improper cellular growth (Hanahan and Weinberg, 2000). One of the critical regulators of cellular growth is the E2F family of proteins.

Rb, E2F, and control of proliferation

The E2F protein family is a group of transcription factors which upregulate genes important for synthesis of DNA and other regulators important for proper cellular division. Control of E2F activity revolves around a cell cycle checkpoint between the G1 and S phases of the cell cycle. A major controller of this checkpoint is the Retinoblastoma susceptibility protein or pRb (Burkhart and Sage, 2008; Nevins, 2001). During quiescence, pRb is hypophosphorylated and is able to bind to the E2Fs to repress their activity to induce S phase genes. When signals to begin cellular division arise, cyclin dependent kinases phosphorylate pRb, releasing the E2Fs to dimerize to their cofactors DP1/2 to activate S phase genes. The importance of pRb in human disease can be shown in cancers, where inactivation of pRb by mutation, germline deletion as in the disease retinoblastoma, or by mutations in upstream regulators of pRb such as p16Ink4a can contribute to cancer susceptibility and are a common occurrence. Indeed, most, if not all, human cancers have abnormally high E2F expression.

The portrait of the E2Fs presented above is complicated by the diverse functions of E2F family members, which now number eight (Polager and Ginsberg, 2009; van den Heuvel and Dyson, 2008). The E2Fs can be roughly divided into two parts – activators, which include E2F1-3a, and repressors which include E2F3b-E2F8. E2F1-3a "activate" E2F target genes and are bound and inactivated by pRb (Flemington et al., 1993; Helin et al., 1992; Lees et al., 1993; Shan et al., 1992), while E2F4/5 can be bound by p107 and p130 pocket proteins to repress E2F activity (Beijersbergen et al., 1994; Buck et al., 1995; Ginsberg et al., 1994; Hijmans et al., 1995). E2F6 does not bind pocket proteins but does require dimerization with DP1/2 (Cartwright et al., 1998; Gaubatz et al., 1998; Morkel et al., 1997; Trimarchi et al., 1998), and participates in repression via Polycomb silencing (Trimarchi et al., 2001). E2F7-E2F8 have less defined roles (Christensen et al., 2005; de Bruin et al., 2003; Di Stefano et al., 2003; Logan et al., 2004; Logan et al., 2005; Maiti et al., 2005) but have recently been shown to together bind and repress the E2F1 promoter (Li et al., 2008). Since E2F consensus sequence sites can theoretically be bound by all E2Fs, activators and repressors compete for the same sites. The composition of E2F responsive sites can be determined by the comparative levels of individual E2F family members and their repressor binding partners. At the G1/S checkpoint, expression of the activator E2Fs is at the highest as is their composition on responsive promoters, but during quiescence, E2F4/5 are the dominant E2Fs at E2F sites (Gaubatz et al., 2000; Moberg et al., 1996; Takahashi et al., 2000). Induction of genes important for cell division therefore can be attributed to E2F1, E2F2, and E2F3a, which each can induce genes important for cellular proliferation (Muller et al., 2001). There is also further complexity, as each of the E2Fs has distinct but overlapping sets of target genes that can

be dictated by DNA sequence, specific binding partners, and surrounding regulatory elements (DeGregori and Johnson, 2006).

E2F1 and apoptosis

The picture is further complicated by the paradoxical ability of E2F1 to induce apoptosis (Iaquinta and Lees, 2007; Polager and Ginsberg, 2009; Stanelle and Putzer, 2006). Ectopic E2F1 *ex vivo* can cause S-phase entry, but at the same time induce apoptosis (Kowalik et al., 1995; Qin et al., 1994; Shan and Lee, 1994). Induction of apoptosis can occur independent of the status of p53, though some specific mechanisms by which E2F1 induces apoptosis depend on p53 (Hsieh et al., 1997; Kowalik et al., 1995). Overexpression *in vivo* in squamous epithelium induced apoptosis and suppressed ras-dependent papilloma formation (Pierce et al., 1998). A physiological role for E2F1 in apoptosis can be shown in gene disruption mouse models where E2F1 is knocked out; thymocytes fail to undergo apoptosis during negative selection, leading to a hyperproliferative thymus. Furthermore, a wide variety of tumors form in E2F1 -/- mice, indicating E2F1 has tumor suppressive activities (Field et al., 1996; Yamasaki et al., 1996; Zhu et al., 1999). Apoptosis in the lens and nervous system of Rb -/- mice also requires E2F1 (Tsai et al., 1998). While some studies have shown that E2F2 and E2F3 can also induce apoptosis when overexpressed (DeGregori et al., 1997; Moroni et al., 2001), it appears that these must act through E2F1; each activating E2F is itself an E2F target; therefore E2F2 and E2F3 may induce apoptosis through activation of E2F1 (Lazzerini Denchi and Helin, 2005).

Furthermore, E2F1, and not the other activator E2Fs, appears to be induced by DNA damage, providing a physiological context for E2F1-induced apoptosis. (Blattner et al., 1999; Huang et al., 1997; Meng et al., 1999). Induction following DNA damage involves phosphorylation and subsequent stabilization of E2F1 by the ATM and Chk kinases (Lin et al., 2001; Stevens et al., 2003). Acetylation of E2F1 by p300 or PCAF is also induced following DNA damage, leading to stabilization and transactivation (Galbiati et al., 2005; Ianari et al., 2004; Martinez-Balbas et al., 2000; Pediconi et al., 2003).

The induction of apoptosis by E2F1 occurs primarily through increased expression of a large number of proapoptotic targets (**Figure 1**). Initial studies focused on the link between E2F1 and p14ARF, which sequesters MDM2 and thereby activates p53 (Bates et al., 1998), and p73, a p53 family member that can also induce apoptosis (Irwin et al., 2000; Lissy et al., 2000; Stiewe and Putzer, 2000). Unbiased microarray studies followed, using cells in which ectopically expressed or stably overexpressed E2F1 were used as a template. These studies identified a cadre of targets which were known to regulate apoptosis (Galbiati et al., 2005; Ianari et al., 2004; Martinez-Balbas et al., 2000; Pediconi et al., 2003). Validated E2F1 targets known to participate in apoptosis or DNA damage response are listed in **Table 1**, in which induction was biochemically recapitulated, promoter sites identified, and E2F1 promoter binding was shown. E2F1 can induce the expression of p73, Apaf-1 (Moroni et al., 2001), the caspases (Nahle et al., 2002), and BH3-only proteins which inactivate the anti-apoptotic Bcl-2 family (Hershko and Ginsberg, 2004).

Interestingly, many of the studies listed neglected to distinguish induction of target genes by E2F1 versus other E2Fs – of the ones that did, many were still induced by

E2F2 and E2F3. It is not surprising that only a small number of validated genes are induced only by E2F1 expression, and not the other activator E2Fs (**Table 2**). This is not an unexpected result considering that the consensus binding site for E2F1 is the same for E2F2 and E2F3 (Slansky and Farnham, 1996). Only p73, Chk2, and SirT1, a negative regulator of E2F1, have been identified as specifically upregulated by E2F1 expression and not the other activator E2Fs. β-catenin was also shown to be specifically repressed by E2F1 expression, and not the other activator E2Fs, for control of apoptosis (Morris et al., 2008). A number of additional E2F1 specific targets were identified but not biochemically validated in two microarray studies performed by the Nevins group. These studies attempted to identify genes that differentiate E2F1 and E2F3 expression (Black et al., 2005; Kong et al., 2007). We endeavor in this proposal to expand on this small number of genes to identify additional novel E2F1 specific targets, validate them biochemically, and characterize their function in apoptosis.

Alternative hypotheses

E2F1 apoptosis may be by default "turned on", and repression of apoptosis may require some external factor that may or may not involve E2F target regulation. Earlier evidence showed that apoptosis could be induced in states of serum starvation (DeGregori et al., 1997). Hallstrom et al. then showed that activation of the PI3K pathway was required for repression of E2F1-induced apoptosis (Hallstrom and Nevins, 2003). Interestingly, our lab showed that TopBP1, an E2F target which binds and specifically represses E2F1 activity, was activated by the PI3K pathway through Akt phosphorylation for repression (Liu et al., 2006). Furthermore, in a more recent

microarray study, Hallstrom et al. showed a subset of E2F1 targets which are repressed by PI3K activation. These E2F responsive genes presumably act to induce apoptosis when not supported by active PI3K (Hallstrom et al., 2008). These results provide a basis by which E2F1 apoptosis can be negatively regulated by PI3K, but they do not exclude a role for other E2F1 target genes that induce E2F1-induced apoptosis. In addition to the above mechanisms, E2F7 and E2F8 have been shown to bind and repress E2F1 promoter; loss of E2F7/8 lead to massive apoptosis which be rescued by concurrent loss of E2F1 or p53 (Li et al., 2008); these newly discovered E2Fs may also play an important role in specification of E2F1 apoptosis function.

Alternatively, levels of proteins not regulated by E2F1 may also play a role in establishing a cellular context in which E2F1 may be able to induce apoptosis. For example, Api5 was recently described in a genetic screen in Drosophila as being a repressor of E2F1 apoptosis, despite not affecting E2F1 transcriptional activity (Morris et al., 2006).

Finally, another level of regulation of E2F1 may be through binding of regulatory proteins specifically to E2F1. As of yet, only a few proteins are known to bind to E2F1 specifically for control of apoptosis (**Table 3**); most of the proteins listed act to repress E2F1 activity. The first specificity factor described was Jab1, which appears to play a role in assisting E2F1-induced apoptosis, and even then the mechanism of activation of apoptosis remains largely undescribed. MCPH1/Brit1 has also been described as a protein which specifically regulates E2F-driven transcriptional activity of p73 and Chk1, two important targets in DNA damage response; however, MCPH1 could also interact weakly with E2F2 (Yang et al., 2008). Taken together, the data presented above reveal the likely

multilayer regulation of E2F1-induced apoptosis, of which activation is likely context dependent, such as priming of a cell with proapoptotic proteins, fraction of E2F1 bound to pro- or anti-apoptotic cofactors, and inputs from growth factor signaling such as PI3K. Identification of gene which is specifically induced by E2F1, activates apoptosis, *and* binds E2F1, would be a significant advance in elucidating the mechanism of differential regulation of the activator E2Fs.

We therefore determined to identify genes either specifically regulated by E2F1 that potentially regulate E2F1-induced apoptosis or proteins which bind to E2F1 to regulate E2F1-induced apoptosis; preferably both. To this end, we performed a reanalysis of the microarray data presented by the Helin group in which expression profiles were compared between cells that overexpressed E2F1, E2F2, and E2F3 (Muller et al., 2001), and sought to identify those genes that were upregulated only by E2F1 and not the other E2Fs. Through this screen RRP1B was identified and validated in biochemical assays as a specific target of E2F1 transcription, and characterized for function in E2F1-induced apoptosis. Identification of a novel specificity factor for E2F1-induced apoptosis could further explain differential regulation amongst the activator E2Fs in the balance between proliferation and apoptosis, and would present an interesting target for improving the effectiveness of chemotherapeutics and radiation in the treatment of cancer.

Figure 1: E2F molecular networks regulating proliferation and apoptosis

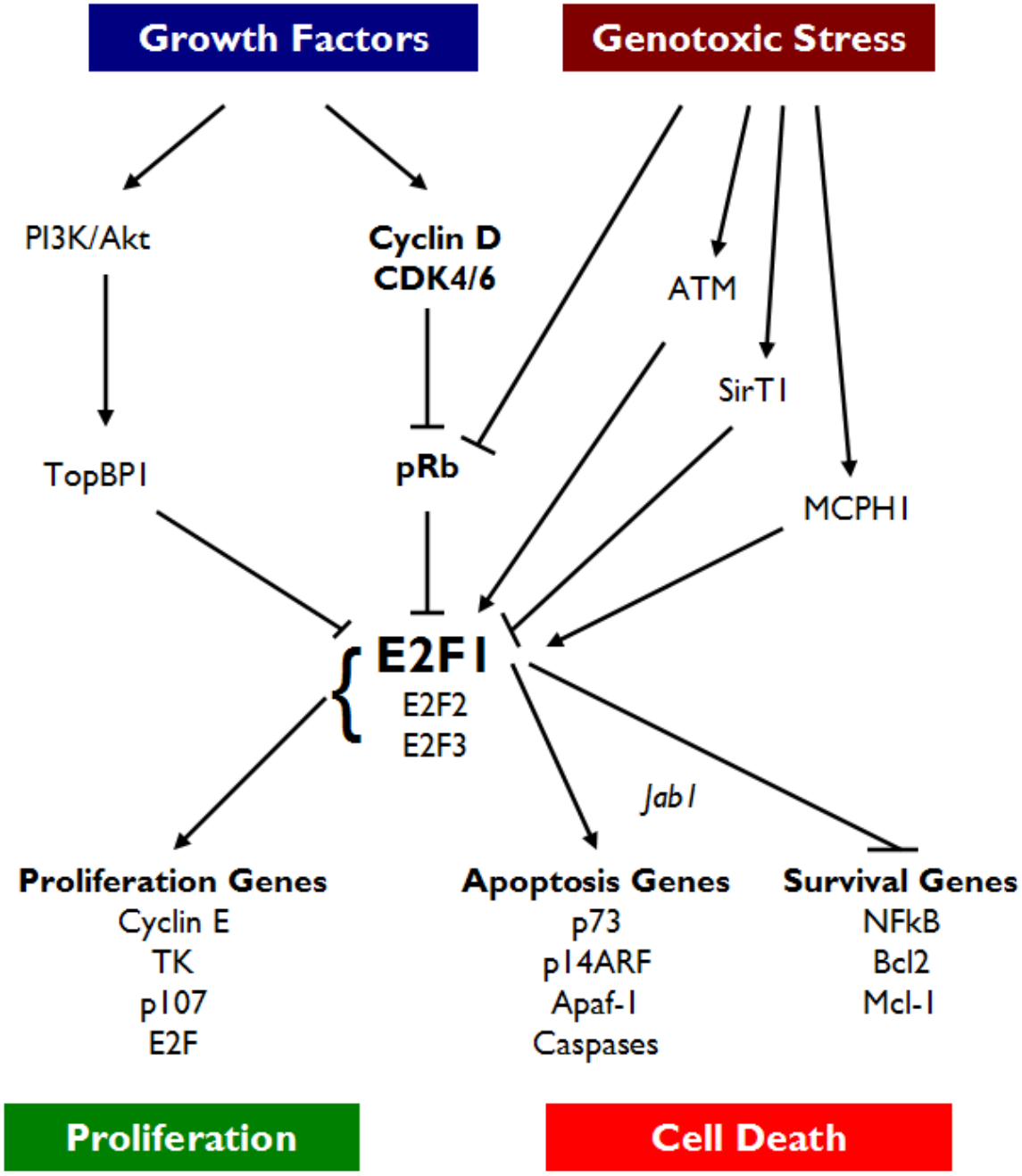

Table 1: Validated genes upregulated by E2F1 that participate in apoptosis or DNA damage response

Gene	Regulated specifically by E2F1?	Reference
p73	+/– (E2F2 slightly induces)	(Irwin et al., 2000; Pediconi et al., 2003)
Apaf-1	No	(Moroni et al., 2001)
Caspase-3/7/8/9	Unknown	(Nahle et al., 2002)
PUMA, NOXA	Unknown	(Hershko and Ginsberg, 2004)
Bim	Unknown	(Hershko and Ginsberg, 2004)
HRK/DP5	Unknown	(Hershko and Ginsberg, 2004)
ASPP-1/2	No	(Fogal et al., 2005; Hershko et al., 2005)
DIP	Unknown	(Stanelle et al., 2005)
Siva	No	(Fortin et al., 2004)
p14ARF	No (regulated only by E2F3 in unstressed cells)	(Aslanian et al., 2004; Bates et al., 1998)
PERP	No	(Attardi et al., 2000)
Chk2	Yes (only checked E2F1-2)	(Rogoff et al., 2004)
TopBP1	No	(Yoshida and Inoue, 2004)
Autophagy Genes	Unknown	(Polager et al., 2008)
DAPK2	Unknown	(Britschgi et al., 2008)
BIN1	No	(Cassimere et al., 2009)
N-Ras Isoprenylation Proteins	Variable (only checked E2F-1 and -3)	(Shamma et al., 2009)
EZH2	Unknown	(Wu et al., 2009)
HIC1	Unknown	(Jenal et al., 2009)
Bnip3	Unknown	(Yurkova et al., 2008)
SC35	Unknown	(Merdzhanova et al., 2008)
GRP78/BIP	Unknown	(Racek et al., 2008)
E1AF	+/- (E2F2/3 slightly induces)	(Wei et al., 2008)
Brip1/Bach1	No	(Eelen et al., 2008)
PAC1	Unknown	(Wu et al., 2007)
RhoBTB2/DBC2	Unknown	(Freeman et al., 2008)
β-catenin	Yes	(Morris et al., 2008)

Table 2: Studies of genes controlled specifically by E2F1, and not other E2Fs

Study	Comments	Reference
p73	Induction of p73 physiologically occurs after DNA damage, requiring E2F1 acetylation by PCAF.	(Pediconi et al., 2003)
SirT1	Negative feedback to repress specifically E2F1.	(Wang et al., 2006)
β-catenin	E2F1 *represses* transcription for control of Wnt signaling.	(Morris et al., 2008)
Black et al. Microarray study	A study comparing expression profiles of E2F1 and E2F3 overexpressing cells to identify differentially expressed genes.	(Black et al., 2005)
Kong et al. Microarray study	A study comparing expression profiles of E2F1/2/3 knockdown cells to identify differentially expressed genes.	(Kong et al., 2007)
Hallstrom et al. Microarray Study	PI3K repressed E2F1-target genes were identified that induce apoptosis.	(Hallstrom et al., 2008)

Table 3: Studies of proteins bound to E2F1 to control apoptosis

Protein	Comments	Reference
Jab1	Interacts predominantly with only E2F1 for apoptosis, no effects on proliferation. Unknown mechanism.	(Hallstrom and Nevins, 2006)
TopBP1	Akt induces TopBP1 binding to E2F1 to repress apoptosis by recruitment of Brg/Brm.	(Liu et al., 2003; Liu et al., 2004; Liu et al., 2006)
p53	p53 binds E2F1 via E2F's cyclin A domain for induction of apoptosis, but this domain exists in E2F1-3.	(Hsieh et al., 2002)
pRb	Specific domain within pRb binds only E2F1 to inhibit apoptosis.	(Dick and Dyson, 2003; Markham et al., 2006)
SirT1	Binds to E2F1 at promoters to block E2F1-induced apoptosis.	(Wang et al., 2006)
GABPγ1	ETS-related transcription factor that block apoptosis by inhibiting only caspase-3/7.	(Hauck et al., 2002)
MCPH1/Brit1	Binds E2F1 on proapoptotic gene promoters to activate apoptosis.	(Yang et al., 2008)
Kap1	Represses E2F1 induction of proapoptotic genes on promoters through HDAC recruitment.	(Wang et al., 2007)

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REGULATION OF E2F1-INDUCED APOPTOSIS BY THE NUCLEOLAR PROTEIN RRP1B

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ABSTRACT

Regulation of the E2F family of transcription factors is important in control of cellular proliferation; dysregulation of the E2Fs is a hallmark of many cancers. One member of the E2F family, E2F1, also has the paradoxical ability to induce apoptosis; however, the mechanisms underlying this selectivity are not fully understood. We now identify a nucleolar protein RRP1B as an E2F1-specific transcriptional target. We characterize the RRP1B promoter and demonstrate its selective response to E2F1. Consistent with the activation of E2F1 activity upon DNA damage, RRP1B is induced by several DNA damaging agents. Importantly, RRP1B is required for the expression of certain E2F1 pro-apoptotic target genes and the induction of apoptosis by DNA damaging agents. This activity is mediated in part by complex formation between RRP1B and E2F1 on selective E2F1 target gene promoters. Interaction between RRP1B and E2F1 can be found inside the nucleolus and diffuse nucleoplasmic punctates. Thus, E2F1 makes use of its transcriptional target RRP1B to activate other genes directly involved in apoptosis. Our data also suggest an under-appreciated role for nucleolar proteins in transcriptional regulation.

INTRODUCTION

E2F1 is a critical regulator of DNA damage response and apoptosis. As part of E2F family of transcription factors, E2F1 is also involved in regulation of a wide array of genes important for cell cycle progression and other functions (Dimova and Dyson,

2005). Paradoxically, E2F1 has the unique ability to induce apoptosis (Iaquinta and Lees, 2007). Overexpression of E2F1 *ex vivo* leads to apoptosis of breast cancer and other cells (Hsieh et al., 1997; Hunt et al., 1997; Phillips et al., 1997). Deletion of E2F1 *in vivo* shows a defect in thymocyte apoptosis and increased tumor incidence (Field et al., 1996; Yamasaki et al., 1996). An endogenous role for E2F1 apoptosis is illustrated by its activation and stabilization by genotoxic stimuli. Overexpression of E2F1 sensitizes cells to radiation and chemotherapy (Meng et al., 1999; Pruschy et al., 1999). DNA damage activates E2F1 expression and induces E2F1 stabilization through phosphorylation by DNA-damage responsive kinases ATM (Lin et al., 2001) and Chk2 (Stevens et al., 2003) and through acetylation (Ianari et al., 2004; Pediconi et al., 2003). E2F1 transactivates proapopotic genes such as p73 (Irwin et al., 2000; Lissy et al., 2000), Apaf-1 (Moroni et al., 2001), and caspases (Moroni et al., 2001) independently of p53, and cooperates with p53 in transactivation of p19ARF (Bates et al., 1998). Investigation of how E2F1 specifically regulates apoptosis through selective transcriptional regulation vis-à-vis other E2F family members may reveal targets for future study that might improve the sensitivity of cancer to radiotherapy and chemotherapy.

We therefore attempted to identify genes specifically regulated by E2F1 that potentially mediate E2F1-induced apoptosis. Previously, the Helin group published a microarray data set in which expression profiles were compared between cells that overexpressed E2F1, E2F2, and E2F3 (Muller et al., 2001). We screened their data set to include only those genes which were significantly induced by E2F1, but whose expression did not change more than one fold either positively or negatively upon E2F2 or E2F3 overexpression. The list of genes screened from this study is shown in Table 1**.**

Among them was the gene RRP1B (Ribosomal RNA Processing 1 homolog B), also known as KIAA0179 or NNP-1B (Novel Nucleolar Protein 1). RRP1B is related to RRP1 (Ribosomal RNA Processing 1), a protein involved in ribosomal biogenesis localized to the nucleolus (Fabian and Hopper, 1987; Horsey et al., 2004; Savino et al., 1999; Savino et al., 2001). Recent data have shown RRP1B is involved in suppression of metastasis and gene expression profile after overexpression predicted survival in breast cancers (Crawford et al., 2007). However, the mechanism of how RRP1B reduces tumor burden remains unclear.

We now provide evidence that RRP1B is specifically regulated by E2F1, and not other E2F family members. RRP1B is important for regulation of apoptosis induced by both DNA damage and E2F1 overexpression. Consistent with its pro-apoptotic function, RRP1B selectively regulates the expression of several pro-apoptotic E2F1 target genes through chromatin binding. We also demonstrate a direct interaction between RRP1B and E2F1 *in vitro* and *in vivo* in nucleoli and in punctate nucleoplasmic foci. Together, these data suggest that RRP1B is a novel E2F1 target and coactivator at the same time and may prime the cells for E2F1-dependent apoptosis.

EXPERIMENTAL PROCEDURES

Cell culture and transfection

HEK293, HEK293T, T98G, NIH3T3, H1299, HFF (human foreskin fibroblasts) and Ref52 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% fetal bovine serum (FBS), penicillin (50 IU/ml) and streptomycin (50 μg/ml). HCT116 and U2OS cells were grown in McCoy's 5A medium supplemented with 10% FBS, penicillin, and streptomycin. All cells were grown in a humidified incubator at 37°C with 5% CO_2 and 95% air. A standard calcium phosphate method was used for transfection of HEK293, HEK293T, and H1299 cells. NIH3T3 and Ref52 cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer instruction. After transfection, cells were incubated for 48hr before analysis.

Plasmid construction

The RRP1B promoter was cloned using PCR of genomic DNA, constituting genomic DNA from -2354 to +259 surrounding the cDNA start site. PCR primers contain a XhoI site 5' to the forward cloning primer and a HindIII site 5' to the reverse cloning primer. The primers used were: forward promoter: 5'-CGCCTCGAGCAGGGTTGGAG GCTGCA-3'; reverse promoter, 5'-CGCAAGCTTACTGAGAATGTCAGTGATGGGG GA-3'. PCR product was digested with XhoI and HindIII, then ligated together with pGL3-Basic digested with XhoI and HindIII.

A mutation at the putative E2F binding site at +150 was generated in pGL3-RRP1B promoter by changing two nucleotides (5'-GCGGTCAGCCGCTACACATGGC GGGC-3') using Quikchange Site-Directed Mutagenesis Kit (Stratagene). To construct pGL3-RRP1B with a mutation at -505 and -400, four nucleotides were changed in two consecutive cycles of a standard megaprimer mutagenesis protocol (Burke and Barik, 2003). For pGL3-RRP1B -505, the mutagenic primers used were: 5'-AGTGGGGCGTG

ATGATGCGCGCCTGTAGTC-3’ and GACTACAGGCGCGCATCATCACGCCCCAC T-3’, then 5’-GGGGCGTGATGATGCATGCCTGTAGTCTCAGC-3’ and 5’-GCTGAG ACTACAGGCATGCATCATCACGCCCC-3’. For pGL3-RRP1B -400, the mutagenic primers used were: 5’-AGCCAGGATCACCGCCAAGATATCGCCACTGCAT-3’ and 5’-ATGCAGTGGCGATATCTTGGCGGTGATCCTGGCT-3’, then 5’-TCACCGCCAAGATATCGATACTGCATTCCAGCCTGG-3’, and 5’-CCAGGCTGGAATGCAGTATCGATATCTTGGCGGTGA-3’.

To construct a tagged mammalian expression vector for RRP1B, RRP1B cDNA was obtained from ATCC in pBluescript II SK(+) (pBsII SK+). A FLAG-tag was inserted 5’ to the transcriptional start site using a PCR primer; a KpnI site, Kozak sequence, and methionine are 5’ to the FLAG tag, and a BglII site was inserted in between the FLAG tag and RRP1B cDNA. The following primers were used: forward: 5’-GCGGGTACCGCCACCATGGATTACAAGGATGACGACGATAAGAGATCTAT GGCCCCCGCCATGCAGCCGG-3’, reverse, 5’-AGCTTCGAAGACACCCCGAGCTA T-3’. Amplified PCR product was digested with KpnI and BstBI and cloned into pBsII SK+-RRP1B digested with KpnI and BstBI. pBsII SK+-FLAG-RRP1B was then digested with KpnI and NotI and the cDNA insert was ligated with pcDNA3 digested with KpnI and NotI.

pcDNA3-FLAG-RRP1B (1-473), FLAG-RRP1B (474-589), or FLAG-RRP1B (590-758) was cloned from full length RRP1B with the addition of a BglII site at the 5’ end of the forward primer, and a NotI site at the 5’ end of the reverse primer flanking the 3’ end of the coding sequence. PCR products were then digested with BglII and NotI and ligated with the vector sequence from modified pcDNA3-FLAG-RRP1B digested with

BglII and NotI. The BglII site in the backbone of pcDNA3 vector was first destroyed by Klenow enzyme. The following primer sets were used: FLAG-RRP1B (a.a. 1-473), forward sequence same as full length forward sequence, reverse, 5'-CGCGCGGCCGCTCATTTCCTTTTATTGTGCATGGG-3'; FLAG-RRP1B (a.a. 474-589), forward, 5'-GCGAGATCTCGGCCACGGAAGAAGAGCCCG-3', reverse, 5'-CGCGCGGCCGCTCATGTTTTCTGGCTGGGCAGGCC-3'; FLAG-RRP1B (a.a. 590-758), forward, 5'-GCGGGTACCGCCACCATGGATTACAAGGATGACGACGATAA GAGATCTGCAAGTTTGAAAAAGAGGAAG-3', reverse, 5'-CGCGCGGCCGCTCA GAAGAAATCCATAGC-3'.

E2F1 domain mutants were constructed into the pGEX-6P1 system (GE). To construct pGEX-6P1-E2F1 (aa 1-109), pAS2-1-E2F1 (1-109) (Liu et al., 2003) was digested with EcoRI and SalI, and the insert was ligated with pGEX-6P1 vector which was digested with EcoRI and SalI. pGEX-6P1-E2F1 (110-284), pGEX-6P1-E2F1 (285-358), and pGEX-6P1-E2F1 (359-437) were cloned by PCR, using full length E2F1 cDNA as a template, with addition of a 5' BamHI site and a 3' EcoRI site flanking primer sequences. PCR products were then digested with BamHI and EcoRI and ligated with pGEX-6P1 digested with BamHI and EcoRI. pGEX-6P1-E2F1 (110-282) was cloned using 5'- GCGGGATCCGGCAGAGGCCGCCATCCA-3' and 5'-AGCGAATTCTCAAAAGTTCTCCAAG AGTC-3'; pGEX-6P1-E2F1 (283-358) was cloned using 5'-GCGGGATCCCAGATCTCCCTTAAG AGC-3' and 5'-AGCGAATTCTCACAACAGCGGTTCTGCTC-3'; pGEX-6P1-E2F1 (359-437) was cloned using 5'-GCGGGATCCTCCCGGAT GGGCAGCCTG-3' and 5'-AGCGAATTCTCAG AAATCCAGGGGGGT-3'.

For bimolecular complementation assays, RRP1B was first shuttled from pcDNA3-FLAG-RRP1B by digestion with BglII and BamHI and ligated with pEGFP-C1 digested with BamHI; orientation was checked by digestion with BglII and BamHI. RRP1B was then excised from pEGFP-C1-RRP1B by BspEI and NheI and inserted into pcDNA3.1 yellow fluorescent protein 1 (YFP1, containing eYFP aa 1-158) (Remy et al., 2004) digested by BspEI and NheI.

Immunoprecipitation and Western blot analysis

Cells prepared for endogenous immunoprecipitation were washed and scraped in phosphate buffered saline (PBS); nuclei were then extracted twice by incubation on ice for 10 min with nuclear extraction buffer (10 mM Tris, 85mM KCl, 5 mM EGTA, 0.5% NP-40) supplemented with protease inhibitors (1 mM dithiothreitol, 1mM NaF, 1 mM sodium orthovanadate, 20 nM microcystin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml antipain, and 1 μg/ml chymostatin). Nuclei were then lysed in TNN buffer (50 mM Tris, 0.25 M NaCl, 5 mM EDTA, 0.5% NP-40) with protease inhibitors, sonicated, precleared by nutation at 4°C for 1hr with protein G agarose beads (Pierce), then nutated at 4°C overnight with 2 μg of E2F1 antibody (KH95, Santa Cruz) or nonspecific mouse IgG (Pierce). Protein G beads were then added and the sample nutated at 4°C for 2 hr, then washed 5 times with ice cold TNN buffer. Beads were eluted with SDS sample buffer, subjected to sodium docecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE), and electrotransferred to Immobilon-P membrane (Millipore).

Cells prepared for immunoprecipitation of overexpressed proteins were washed and directly lysed in TNN with protease inhibitors and nutated at 4°C overnight with anti-FLAG agarose beads (M2, Sigma). An aliquot of lysate was saved for protein input control. Beads were washed 5 times with ice cold TNN buffer, eluted, electrophoresed, and blotted as above.

Cells prepared for direct protein analysis were lysed in SDS lysis buffer (1% SDS, 60 mM Tris). Equal protein amounts were electrophoresed and blotted as above. Equal loading was confirmed by Ponceau S staining. DNA damage was induced by addition of 1 μM doxorubicin (doxo), 20 μM cisplatin (CDDP), or 0.05 μg/ml, 0.3 μg/ml or 1.0 μg/ml neocarzinostatin (NCS). Densitometric analysis was performed using ImageJ (NIH); measurement of RRP1B protein level was normalized against corresponding GAPDH protein level. For all experiments, specific proteins were detected with the appropriate antibodies. An RRP1B antibody was raised in rabbits against a peptide (ATHPPGPAVQLNKTPSSSKK) by Open Biosystems. Crude rabbit sera were affinity purified using peptide-conjugated NHS-activated Sepharose (GE). Antibodies against E2F1 (KH95 and C20), E2F2 (C20), E2F3 (C18), E2F4 (WUF11), E2F5 (MH5), HA (Y11), and glyceraldehyde-3-phosphate dehydrogenase (0411) were purchased from Santa Cruz. FLAG antibody (F7425) was purchased from Sigma.

Lentivirus production and transduction

Knockdown of RRP1B was achieved by infection of cells with lentiviruses expressing RRP1B small interfering RNA (siRNA). pLKO.1 plasmids expressing siRNA sequences (Moffat et al., 2006) were obtained from the RNAi Consortium (Open

Biosystems) and screened for knockdown of RRP1B by transient transfection of HEK293T cells, followed by Western blotting. A control nonspecific siScramble pLKO.1 plasmid (Sarbassov et al., 2005) and pMDG and pCMV ΔR8.2 packaging vectors were obtained from Addgene. Two plasmids containing the following siRNA sequences achieved high knockdown; A, 5'-GATGACCAAATCCTCAGTCAA-3'; B, 5'-GCACATTTGTTCTGCAGACTA-3'. Plasmids achieving high knockdown were used for lentivirus production by cotransfection of pLKO.1 containing siRNA sequences, pMDG, and pCMV ΔR8.2 in HEK293T cells; supernatants containing virus were collected every 24 hr, filtered using a .30 μm filter, added to target cells, incubated for 48 hr, then selected for stable transduction by addition of puromycin for 96 hr. Knockdown was confirmed by Western blotting.

Luciferase assays

The expression constructs (5 μg for pcDNA3-E2F1, pcDNA3-E2F2, or pcDNA3-E2F3 or empty vector), the promoter plasmids (1 μg for pGL3-RRP1B and point mutants, pGL3-rRNA promoter and proximal mutant (Ayrault et al., 2006b), caspase-7 promoter (Nahle et al., 2002), E2F1 promoter (Johnson et al., 1994) and thymidine kinase (TK) promoter (Li et al., 1994)) and 1 μg of pCMV-β-galactosidase plasmids were cotransfected in HEK293T or stably transduced siScramble or siRRP1B H1299 cells. Cells were harvested 48 hr later in PBS; an aliquot was lysed in SDS lysis buffer for Western blotting, while the rest of the sample was lysed in reporter lysis buffer (Promega). Luciferase activity and β-galactosidase activity were measured according to

manufacturer instruction. Luciferase activity was normalized against β-galactosidase activity. All transient expression assays were performed in triplicate.

Apoptosis assays

DNA damage induced apoptosis was assayed in stably transduced siScramble or siRRP1B U2OS cells which were untreated or treated with 20 μM cisplatin for 30 hr before harvest. Cells were then stained with annexin V-APC or annexin V-PE (BD Biosciences) and 7-amino-actinomycin (BD Biosciences). At least 10000 cells were profiled for surface annexin-V/7-AAD positivity by flow cytometry. Annexin V^+/7-AAD^- and Annexin V^+/7-AAD^+ cells were scored as apoptotic. Alternatively, stably transduced siScramble or siRRP1B U2OS cells were untreated or treated with 1 μM doxorubicin for 8 hr, harvested, then assayed for caspase-3/7 cleavage according to manufacturer instruction (Caspase-Glo 3/7, Promega).

E2F1 induced apoptosis was assayed in stably transduced siScramble or siRRP1B U2OS cells infected by adenoviruses expressing E2F1. Adenoviruses were produced in the AdEasy system as previously described (DeGregori et al., 1997). Cells were starved in 0.25% fetal bovine serum for 48 hr, followed by adenovirus infection (MOI 100) for 28 hr. Cells were then harvested and analyzed for surface annexin-V/7-AAD positivity by flow cytometry as above. All apoptosis assays were performed in triplicate.

Cellular proliferation assay

$1x10^5$ stably transduced siScramble and siRRP1B U2OS cells were each plated in six replicates in 3.5cm diameter 6 well plates, grown for 72 hr prior to confluence,

trypsinized and collected. Two aliquots from each plate were counted using a hemacytometer. One quarter of the remaining cells were replated. Assay was repeated on day 6 and day 9. Cells were harvested at day 9 in SDS sample buffer for Western blotting.

Real time and semiquantitative PCR

For analysis of RRP1B dependency on E2F family member expression, T98G cells were starved in DMEM containing 0.25% FBS for 48 hr, then infected with adenoviruses expressing E2F1, E2F2, E2F3, E2F4, E2F5, or empty vector for 24 hr. RNA was then extracted using TRIzol (Invitrogen); 1µg of RNA was used to produce cDNA using MMLV reverse transcriptase (Promega), then expression of specific targets was assayed by PCR. For analysis of RRP1B expression after E2F family knockdown, U2OS cells were stably transfected with pSuperior.puro containing siGFP, siE2F1, or siE2F3, and puromycin selected. Parallel aliquots of cells were prepared for RTPCR and SDS-PAGE as above. Construction and sequences were previously described (Liu et al., 2004).

For analysis of cell cycle dependent RRP1B RNA levels, HFF cells were starved in DMEM containing 0.25% FBS for 48 hr, then stimulated with 20% FBS at various timepoints. Harvesting of RNA and semiquantitative PCR was then performed as above. A parallel set of cells was treated identically, harvested, and analyzed for DNA content by propodium iodine flow cytometry as previously described (DeGregori et al., 1997).

For analysis of RRP1B knockdown and E2F1 target expression, stably transduced siScramble and siRRP1B U2OS cells were harvested in TRIzol and RNA extracted and

semiquantitatively analyzed as above. Quantitative PCR was performed in triplicate on an MX3005p thermal cycler (Stratagene) using SYBR Green dye to measure amplification and ROX as a reference dye (Brilliant II SYBR Green QPCR Master Mix, Stratagene). Transcript levels were normalized with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels, which were assayed in parallel with test genes. Results were analyzed with MxPro 4.1 QPCR software (Stratagene).

For all experiments, PCR was performed using the following primer sets: RRP1B, 5'-CCCGTCCCTGGAACAGAAC-3', 5'-CTCGGGCCACTCTGAGACA-3', size 249bp; p73, 5'-CATGGTCTCGGGGTCCCACT-3' and 5'-CGTGAACTCCTCCTTGATGG-3', size 471bp; Apaf-1. 5'-AATGGACACCTTCTTGGACG-3', 5'-GCACTTCATCCTCATGAGCC-3', size 331bp; Caspase-3, 5'-TCGGTCTGGTACAGATGTCG-3', 5'-CATACAAGAAGTCG GCCTCC-3', size 398bp; Caspase-7, 5'-CAAAGCCACTGACTGAGATG-3', 5'-CAACCCAATGAATAAATGAT-3', size 259bp; p107, 5'-TGGTGTCGCAAATGATGCTGG-3', 5'-AGGAGCTGATCCAAATGCCTG-3', size 362bp; Cyclin E 5'-CTCCAGGAAGAGGAAGGCAA-3', 5'-GTAAAAGGTCTCCCTGTGAAG-3', size 421bp; TK, 5'-ATGAGCTGCATTAACCTGCCCACT-3', 5'-ATGTGTGCAGAAGCTGCTGC-3', size 204bp; GAPDH, 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3', 5'-AAATGAGCCCCAGCCTTCTCCA TG-3', size 325bp. We ensured linear amplification in all cases.

Chromatin immunopreciptation (ChIP) assay

U2OS cells were grown in 15cm diameter dishes, crosslinked with 1% formaldehyde, washed and scraped with PBS, and nuclei extracted on ice twice with nuclear extraction buffer with protease inhibitors. Cells were then resuspended in chromatin extraction buffer (1% SDS, 10 mM EDTA, 20 mM Tris) with protease inhibitors and sonicated to an average fragment size of 1000bp; 0.5% of supernatants were used for control input PCR. All other chromatin was diluted in dilution buffer (0.01% SDS, 1% Triton X100, 2 mM EDTA, 20 mM Tris, 150 mM NaCl) and precleared with salmon sperm DNA/bovine serum albumin blocked protein G plus protein A agarose beads (Pierce) for 3hr, then immunoprecipitated with 4ug of each antibody (E2F1, C20, Santa Cruz; E2F2 (C18), E2F3 (C20), E2F4 (C20); RRP1B, rabbit IgG, Pierce) by nutation at 4°C overnight. Blocked protein G+A agarose beads were added for 2hr, then beads were washed and nutated for 5min at 4°C consecutively with ice cold low salt buffer (0.1% SDS, 1% Triton X100, 2 mM EDTA, 20 mM Tris, 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X100, 2 mM EDTA, 20 mM Tris, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris), and twice with TE (10 mM Tris, 1 mM EDTA). Chromatin was eluted in fresh elution buffer (0.1 M $NaHCO_3$, 1% SDS); crosslinks were then reversed by incubating samples in high salt conditions for > 4hr at 65°C, followed by digestion of RNA by RNase A and protein by proteinase K. DNA was then purified by dilution in buffer PB (Qiagen) then purification using a silica column (Qiaquick gel extraction kit, Qiagen).

For reChIP assays, cells and chromatin were treated as before; chromatin was immunoprecipitated using 4 μg of antibodies (E2F1, KH95, Santa Cruz, mouse IgG,

Pierce); prior to chromatin elution, antibody/chromatin complexes were eluted in 10mM DTT and incubated at 37°C for 30 min. Supernatants were then diluted 20:1 in reChIP buffer (1% Triton X100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris) and nutated at 4°C overnight with 4 μg of antibodies (RRP1B, rabbit IgG, Pierce). Blocked protein G+A agarose beads were added for 2 hr, then beads were washed, eluted, and DNA purified as above.

For all experiments, PCR was performed using the primer sets which flank putative E2F-binding sites within the promoters of the following genes: E2F1, 5'-AGGAACCGCCGCCGTTGTTCCCGT-3', 5'-CTGCCTGCAAAGTCCCGGCCACTT-3', size 124bp; p73, 5'-CTCTGCCGAAGATCGCGGTCGG-3', 5'-GGCCGCGTCCAAGTCGGGGTCC-3', size 170bp; β-actin, 5'-ACGCCAAAACTCTCCCTCCTCCTC-3', 5'-CATAAAAGGCAACTTTCGGAACGGC-3', size 166bp; caspase-7, 5'-TTTGGGCACTTGGAGCGCG-3', 5'-AAGAGCCCAAAGCGACCCGT-3', size 220bp; GAPDH, 5'-AAAAGCGGGGAGAAAGTAGG-3', 5'-CTAGCCTCCCGGGTTTCTCT-3', size 270bp; p107, 5'-TCTTTCAGAATCTGAGGTAC-3', 5'-CCGACTTCTTTCTCCCTCC-3', size 198bp; rRNA, 5'-GTTTTTGGGGACAGGTGT-3', 5'-CCAGAGGACAGCGTGTCAGCA-3', size 146bp; TK, 5'-TCCCGGATTCCTCCCACGAG-3, 5'-TGCGCCTCCGGGAAGTTCAC-3', size 200bp; RRP1B, 5'-CGGTGAAGAGCTGCGCCAGT-3', 5'-CGCAAGCTTACTGAGAATGTCA GTGATGGGGGA-3', size 180bp. We ensured linear amplification in all cases.

GST pulldown assay

Escherichia coli strain BL21 transformed with pGEX or pGEX-E2F1 were cultured in LB medium containing ampicillin at 37°C to an A600 value of 0.5. GST fusion proteins were induced by 0.02 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 25°C for 3 hr; cells were then lysed by sonication in PBS containing protease inhibitors, and then purified using Glutathione Sepharose 4B (GE) (Lin et al., 2001). ^{35}S-tagged RRP1B was produced from rabbit reticulocyte lysates according to manufacturer instruction (TnT Quick Coupled Transcription/Translation System, Promega). 1 µg of GST or GST-E2F1 on sepharose beads was combined with ^{35}S-tagged RRP1B in NETN-A buffer (50 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, 0.5% NP-40) with protease inhibitors and nutated overnight at 4°C. Sepharose beads were washed 4 times with NETN-B buffer (100 mM NaCl, 1 mM EDTA), eluted in SDS sample buffer, then subjected to SDS-PAGE, fixed, enhanced for autoradiography (Enlightening, Dupont), dried, and exposed to film for 1 hr at -80°C. Equal loading of GST proteins was assessed in parallel by SDS-PAGE followed by Coomassie staining.

Alternatively, GST-fusion proteins were induced, lysed, and purified by the above method. 2 µg of GST-NHERF-PDZ2 (Shuyu et al., 2009) (as a control irrelevant protein), E2F1, or E2F1 mutants on sepharose beads were nutated overnight at 4°C with cellular lysates prepared from HEK293T cells which had been transfected with pcDNA3 or pcDNA3-FLAG-RRP1B, FLAG-RRP1B (1-473), FLAG-RRP1B (474-589), or FLAG-RRP1B (590-758), incubated for 48 hr, and lysed with NETN-A buffer with protease inhibitors. Sepharose beads were washed 5 times with NETN-B buffer, eluted in SDS

sample buffer, and subjected to SDS-PAGE and immunoblotting. Equal loading of GST proteins was assessed in parallel by SDS-PAGE followed by Coomassie staining.

Immunofluorescence studies

HEK293, NIH3T3, U2OS, or Ref52 cells were plated on collagen-coated coverslips in six-well plates, and then transfected with pcDNA3-FLAG-RRP1B using the appropriate transfection protocol and incubated for 48 hr. Cells were then fixed in 3% paraformaldehyde for 20 min, followed by permeabilization in 0.5% Triton-X-100 in PBS for 10 min. Cells were then blocked in 50% horse serum/50% PBS at room temperature for 30 min, then incubated with primary antibody in blocking solution for 60 min, washed, blocked again, then incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) or Texas-Red-X goat anti-mouse IgG (Molecular Probes, 1:400 dilution) for 1 hr. Cells were then washed, nuclei were stained using Hoescht 33258, then mounted. For immunostaining, FLAG antibody (F7425, Sigma, 1:250 dilution), and nucleolin antibody (MS-3, Santa Cruz, 1:100 dilution) were used. Images were captured on a Zeiss fluorescent microscope (Axioplan 2 imaging system).

For bimolecular fluorescence complementation assays (Kerppola, 2006), YFP1-RRP1B, YFP2-E2F1 (Yang et al., 2008), or nonspecific YFP1-zipper and YFP2-zipper (Remy et al., 2004) were transfected in HEK293 or NIH3T3 cells by appropriate transfection protocol, incubated for 48 hr, fixed, nuclei-stained, and mounted as above.

RESULTS

Expression of RRP1B is specifically controlled by E2F1

We first investigated the potential role and specificity of E2F1 on RRP1B expression. We overexpressed E2F1 through E2F5 using adenoviruses encoding E2F1-5 cDNAs or no cDNA (pCMV) in serum-starved T98G cells, and then checked for expression of RRP1B by semiquantitative RT-PCR. RRP1B expression was induced upon overexpression of E2F1, but not the other E2F family members E2F2-5 (Fig. 1*A*). We also tested the expression of RRP1B upon knockdown of E2F1, E2F3, or a nonspecific GFP using U2OS cells in which siRNAs against each target were stably transfected. RRP1B transcripts were decreased after knockdown of E2F1 by quantitative RT-PCR, but not upon knockdown of E2F3 or nonspecific GFP (Fig. 1*B*).

To support a role for E2F1 in the control of expression of RRP1B, we investigated the expression of RRP1B during cellular states where E2F1 expression is endogenously induced. E2F1 transcriptional activity is induced following DNA damage (Pediconi et al., 2003); if RRP1B is an E2F1 target, RRP1B expression will be increased following DNA damage. Using U2OS cells in which DNA damage was induced by neocarzinostatin (NCS) or cisplatin (CDDP) for varying times and dosages, we observed that RRP1B expression was induced by genotoxic agents as soon as 15 minutes following administration, peaking 60 minutes after administration (Fig. 1*C*), with decrease afterwards. We also observed similar induction in HCT116 cells after doxorubicin treatment (Fig. 1*C*). To determine whether RRP1B transcripts were induced following DNA damage, we performed quantitative RT-PCR on U2OS cells that were treated with

doxorubicin on a time course. RRP1B transcripts were significantly induced after 15 minutes of doxorubicin treatment (Fig. 1*D*).

E2F1 expression is controlled during the cell cycle, where expression peaks at the G1/S transition. We therefore investigated whether RRP1B expression also peaks at the G1/S transition, consistent with E2F1 expression, using semiquantative RT-PCR and protein blotting for RRP1B expression in primary foreskin fibroblasts that had been serum-starved to quiescence for cell cycle phase synchronization, then stimulated with serum to reinduce cycling. RRP1B transcripts were observed to be induced, peaking at 18 hours after cell cycle induction (Fig. 1*E*), with levels falling afterward, suggesting that RRP1B expression peaks at the G1/S transition (Fig. 1*F*). This is further supported by observation of RRP1B protein levels which peaked at 20 hours after cell cycle induction, with levels falling afterward (Fig. 1*E*).

To further test the role of E2F1 in control of RRP1B expression, we cloned the endogenous RRP1B promoter into a reporter luciferase plasmid, and assayed the ability of E2F1 to induce RRP1B promoter-driven luciferase activity. A schema of the endogenous RRP1B promoter with putative E2F sites as determined by computer screening (Kel-Margoulis et al., 2000) is shown in Fig. 2*A*. We also tested the ability of E2F1 to induce RRP1B promoter reporter activity where putative E2F sites were inactivated by point mutation. E2F1 induced luciferase activity of the wild type promoter, but mutation of the putative E2F site at position +150 from the RRP1B ATG completely abolished induction by E2F1 (Fig. 2*B*). Two other E2F sites at position -505 and -400 were also mutated, but the ability of E2F1 to induce luciferase activity was unaffected when compared to the wild type, indicating these two sites are not relevant to E2F1

induction of RRP1B (Fig 2*C*). We finally tested the specific ability of E2F1 to induce RRP1B promoter driven luciferase activity. Consistent with Fig. 1*A*, overexpression of E2F1, but not E2F2 or E2F3, was able to significantly induce luciferase activity (Fig. 2*D*)

Finally, we determined whether E2F1 protein binds to the RRP1B promoter in an endogenous chromatin immunoprecipitation (ChIP) assay. Using a primer set which encompasses the RRP1B promoter from position +79 to +259, containing the E2F site at +150, we observed binding of E2F1 to the RRP1B promoter (Fig. 2*E*). Specificity of E2F binding was further shown by immunoprecipitation with E2F2-4; while binding of all E2Fs was seen when the p107 promoter was assayed, little binding was seen between E2F2-4 on the RRP1B promoter, indicating that E2F1 specifically binds to the RRP1B promoter.

Knockdown of RRP1B decreases apoptosis induced by genotoxic agents and E2F1

Since the data above suggest proapoptotic E2F1, and not the other E2Fs, specifically regulates the expression of RRP1B, we investigated what effect RRP1B would have on apoptosis induced by both DNA damaging genotoxic agents and by overexpression of E2F1 during serum starvation. The effect of RRP1B was tested in U2OS cells which were stably transduced with siRNAs against RRP1B or control scramble siRNA by means of a lentiviral system. Two independent siRNAs against RRP1B were used. We first determined the effect of RRP1B knockdown on apoptosis as induced by cisplatin (CDDP). RRP1B knockdown significantly decreased the ability of cisplatin to induce surface annexin V positivity, an early marker for apoptosis, as compared to control cells expressing a nonspecific siRNA (siScr), as analyzed by flow

cytometry (Fig. 3*A*). Knockdown of RRP1B protein levels reached nearly 100% in this assay (Fig. 3*A*, inset). We further tested the role of RRP1B in apoptosis in a caspase cleavage assay in the same cell lines. After treatment with the genotoxic agent doxorubicin (doxo), RRP1B knockdown cells had significantly reduced activated caspase activity as compared to control siScr cells (Fig. 3*B*). Finally, we tested the ability of E2F1 to induce apoptosis in serum-starved U2OS cells expressing siRNAs against RRP1B. Knockdown of RRP1B significantly reduced the ability of E2F1 to induce surface annexin V positivity as compared to control siScr cells (Fig. 3*C*).

RRP1B does not affect cellular proliferation

Since E2F1 also regulates genes important for cellular proliferation, and RRP1B belongs to the Nop52 family, which is known to regulate ribosomal RNA production, a limiting factor for cellular growth, we assayed the role of RRP1B in cellular proliferation. U2OS cells stably transduced with siRNAs against RRP1B or control nonspecific siScr siRNAs were plated equally, grown, trypsinized and harvested, and counted using a hemacytometer. Knockdown of RRP1B did not appear to change the rate of proliferation of U2OS cells (Fig. 3*D*). This result suggests that RRP1B is not required for cellular proliferation. However, it is possible that Nop52, a homolog of RRP1B, compensate for loss of RRP1B in rRNA production. Furthermore, proliferation was only measured in cell lines or in serum-supplemented media; proliferation in primary cells or in cells undergoing stress or grown in low-serum conditions was not assayed.

RRP1B selectively regulates transcription of E2F1 target genes

Based on results above showing decrease of the ability of E2F1 to induce apoptosis after knockdown of RRP1B, we investigated whether knockdown of RRP1B could affect the transcription of E2F1 target genes by examining expression in stably tranduced U2OS cells expressing siRNAs against RRP1B. E2F1 target genes related to apoptosis, such as p73, Apaf-1, caspase-3, and caspase-7, as well as target genes related to the cell cycle, such as cyclin E and thymidine kinase (TK) were examined. Transcripts of specific genes were analyzed by quantitative (Fig. 4*A*) and semiquantitative (Fig. 4*B*) RT-PCR assays. The effectiveness of RRP1B siRNAs was confirmed, where a 75-80% knockdown of transcripts was observed in both siRNAs tested. Knockdown of RRP1B expression appeared to reduce the expression of caspase-3 and caspase-7 (Fig. 4*A*), consistent with the caspase cleavage assay above (Fig. 3*B*), and also reduced the expression of proapoptotic Apaf-1. Interestingly, p73, an E2F1 target gene known to be important for apoptosis, and other target genes involved in proliferation such as TK and Cyclin E were not significantly affected by RRP1B knockdown (Fig. 4*A*). These results suggest a selective role for RRP1B in regulation of E2F1 target genes.

Recently, several nucleolar proteins have been shown to regulate transcription through binding to chromatin (Gonzalez et al., 2009; Li et al., 2008). We therefore examined a role for RRP1B in E2F1 regulation by assaying the presence of RRP1B on E2F1 target gene promoters through ChIP assays. E2F1 was seen on the promoters of all E2F1 target genes assayed. E2F1 was also seen on the rRNA promoter (Ayrault et al., 2006b) and the RRP1B promoter (Fig. 2*E*). Interestingly, RRP1B antibodies precipitated chromatin from the caspase-7 promoter, the rRNA promoter, and the RRP1B promoter,

but not from promoters of other E2F1 target genes assayed, including p73, TK, and E2F1 (Fig. 4*C*). These data show that RRP1B binds only to the promoters of E2F1 target genes which were affected by RRP1B knockdown, but not to the promoters of E2F1 target genes not affected by RRP1B knockdown. Collecting these results suggests that RRP1B binding to specific promoters is important for regulation of E2F1 target genes. We then investigated whether RRP1B and E2F1 were bound together on E2F1 target gene promoters in a ChIP-reChIP assay, where two consecutive immunopreciptitations using E2F1 and RRP1B antibodies were performed. RRP1B and E2F1 were shown to interact together on the caspase-7, rRNA, and RRP1B promoters, but not on the p73 promoter, suggesting that RRP1B regulation of E2F1 target genes occurs through interaction with E2F1 (Fig. 4*D*).

We further tested the ability of RRP1B to regulate E2F1 target genes in promoter reporter luciferase assays. We used H1299 cells that were stably transduced with lentiviruses encoding siRNAs against RRP1B. First, we tested the ability of E2F1 to induce the caspase-7, TK, and E2F1 promoters. Consistent with Fig. 4*A*, RRP1B knockdown inhibited the ability of E2F1 to induce luciferase activity of the caspase-7 promoter reporter (Fig. 5*A*), but not the E2F1 (Fig. 5*B*) and TK (Fig. 5*C*) promoter reporters, further supporting specificity in RRP1B regulation of E2F1 target genes.

Since E2F1 has been reported to bind rRNA promoter and up-regulate its promoter activity (Ayrault et al., 2006b), we assayed the ability of E2F1 to induce the rRNA promoter in H1299 cells or stably transduced siRRP1B cells. RRP1B knockdown significantly reduced both endogenous and E2F1-induced reporter luciferase activity (Fig. 5*D*). Similar results were seen in stably transduced U2OS cells expressing RRP1B

siRNAs (data not shown). Since the previous assay does not rule out a nonspecific RRP1B effect on transcription, we tested the effect of RRP1B knockdown on reporter luciferase activity of an rRNA promoter containing a mutation through which induction by E2F1 is lost. Consistent with figure 5*D*, RRP1B knockdown significantly reduced the endogenous reporter activity of the wild type promoter (Fig. 5*E*). However, RRP1B knockdown was not observed to decrease promoter reporter activity in cells transfected with the mutant rRNA promoter, suggesting that an intact E2F site is required for knockdown of RRP1B to regulate transcriptional activity (Fig. 5*B*).

E2F1 interacts directly with RRP1B

Based on the results above showing coimmunoprecipitation of E2F1 and RRP1B on the chromatin of E2F target gene promoters (Fig. 4*D*), we tested whether there was a physical interaction between E2F1 and RRP1B in biochemical assays. We examined *in vitro* binding between purified GST-E2F1 and RRP1B produced in an *in vitro* transcription/translation system. When ^{35}S-labelled RRP1B was incubated with either GST or GST-E2F1 and pulled down by glutathione sepharose, GST-E2F1, but not GST, pulled down RRP1B, demonstrating a direct interaction between E2F1 and RRP1B (Fig. 6*A*).

Next, we examined whether RRP1B could interact with E2F1 *in vivo*. We detected an endogenous interaction between E2F1 and RRP1B in nuclear extracts from both U2OS and HCT116 cells (Fig. 6*B*). DNA damage increased the interaction between RRP1B and E2F1, but this was due to induction of both E2F1 and RRP1B (data not shown).

We further investigated the ability of RRP1B to interact with E2F1 by dissecting the domains of interaction between RRP1B and E2F1. E2F1 was coexpressed with FLAG-tagged RRP1B or RRP1B N-terminal domain (aa 1-473), Middle Domain (aa 474-589), or C-terminus domain (aa 590-758); when cells were lysed and immunoprecipitated with FLAG, E2F1 was pulled down with full length RRP1B and RRP1B (1-473) and RRP1B (590-758), indicating two separate domains of interaction (Fig. 6*C*). We also dissected the domains of interaction between RRP1B and E2F1. Purified GST-tagged full length E2F1, or GST-tagged E2F1 domain peptides corresponding to the N-terminus (aa 1-109), DNA binding domain (aa 110-284), marked box domain (aa 285-358), or Rb/Dimerization domain (aa 359-437) were incubated with lysates from HEK293T cells in which FLAG-tagged RRP1B was overexpressed. Only GST-E2F1 and GST-E2F1 (110-284) were able to pull down FLAG-tagged RRP1B (Fig. 6*D*). Unlike TopBP1 (Liu et al., 2003) and 14-3-3τ (Wang et al., 2004), interaction between RRP1B and E2F1 was not perturbed by mutation of E2F1 serine 31 (data not shown), as expected because RRP1B does not interact with the N-terminus of E2F1.

RRP1B and E2F1 interact in the nucleolus and punctate nucleoplasmic foci

To further investigate the role of RRP1B in E2F1 regulation, we assayed the localization of RRP1B and E2F1. We overexpressed FLAG-tagged RRP1B in HEK293 cells and probed for intracellular localization using antibodies against FLAG. RRP1B was localized to areas within the nucleus corresponding to nucleolin staining, a marker for the nucleolus. In addition, punctate nucleoplasmic foci were also observed, which did

not correspond to nucleolin staining (Fig. 7*A*). We observed similar patterning in other cell lines (Fig. 7*A*).

We next investigated the localization of interaction between RRP1B and E2F1. We assayed the localization of interaction using a bifluorescence complementation assay (Kerppola, 2006). No fluorescence was seen when either RRP1B or E2F1 was coexpressed with a nonspecific leucine zipper control, but when both YFP-tagged RRP1B and E2F1 were coexpressed, fluorescence was seen within intracellular locations similar to those seen in Figure 7*A* (Fig. 7*B*). These results suggest that the RRP1B and E2F1 interaction is located within nucleoli and punctate nucleoplasmic foci.

DISCUSSION

With a role for E2F1 in apoptosis during either DNA damage response or thymocyte development, the molecular details that dictate the pro-apoptotic activity of E2F1 have drawn much attention. For example, association of Jab1 (Hallstrom and Nevins, 2006) and MCPH1/BRIT1 (Yang et al., 2008) has been identified to contribute to this activity (Hallstrom and Nevins, 2006), although how these interactions specifically leads to activation of E2F1-dependent apoptosis remains unclear. In this report, we identify the nucleolar protein RRP1B as an E2F1-specific target (Fig. 1&2), which in turn selectively up-regulates certain E2F1 target genes such as caspase 3 and 7 and Apaf-1 (Fig. 4&5), and is required for E2F1-induced apoptosis (Fig. 3*C*). These data unravel a

novel function for RRP1B and identify it as one of the factors that activate the pro-apoptotic activity of E2F1.

The nucleolar localization of RRP1B is also worth noting (Fig. 7*A&B*). While the role of the nucleolus in ribosome production is well known, a role for the nucleolus in cancer, including in regulation of cellular proliferation and apoptosis, has only recently been established (Maggi and Weber, 2005; Ruggero and Pandolfi, 2003). We now show RRP1B as an example of a multifunctional nucleolar protein that regulates apoptosis through E2F1-medicated transcription. A role for nucleolar and ribosomal proteins in transcriptional regulation has also only been recently recognized (Boisvert et al., 2007; Lindstrom, 2009; Warner and McIntosh, 2009). Two nucleolar proteins have been extensively investigated in transcriptional regulation. Nucleophosmin was the first histone chaperone identified (Laskey et al., 1978), and has been shown to bind to histone acetyltransferases (Shandilya et al., 2009; Swaminathan et al., 2005) and regulate transcriptional activity through GCN5 (Zou et al., 2008), AP2α (Liu et al., 2007), c-myc (Li et al., 2008), and the androgen receptor (Leotoing et al., 2008). Nucleolin is a histone chaperone with FACT-like activity (Angelov et al., 2006), and regulates transcriptional activity of pRb (Grinstein et al., 2006), KLF2 (Huddleson et al., 2006), AP-1 (Samuel et al., 2008), c-myc (Gonzalez et al., 2009), and IRF-2 (Masumi et al., 2006). Other nucleolar and ribosomal proteins involved in transcriptional regulation through binding of chromatin include RPS3 in NFκB dependent transcription (Wan et al., 2007), L11 in c-myc depdendent transcription (Dai et al., 2007), Nopp140 (Chiu et al., 2002), ApLLP (Kim et al., 2006), and Drosophila ribosomal proteins (Ni et al., 2006). To these examples, we now add RRP1B as a specific regulator of transcription by a nucleolar

protein in a manner similar to that seen in nucleolin or nucleophosmin regulated transcription.

Another nucleolar protein which is induced by E2F1 but also regulates E2F1 is ARF. ARF binds to MDM2 to activate the growth suppressive functions of p53, but can also exert its tumor suppressor activity independently of p53: for example, ARF has been shown to inhibit the transcriptional activity of E2F1 through regulation of both E2F and DP1 (Datta et al., 2002; Datta et al., 2005). More recently, ARF has been shown to inhibit ribosomal RNA processing, and to interact with the rRNA promoter (Ayrault et al., 2004) and inhibit rRNA transcription by blocking Upstream Binding Factor phosphorylation (Ayrault et al., 2006a). These inhibitory functions toward E2F1 by ARF are in contrast to the promoting function by RRP1B, at least in the aspect of certain E2F1 target gene expression and the rRNA promoter activity.

RRP1B binds together with E2F1 on the chromatin of specific E2F1 target genes (Fig. 4*C*&*D*): however the mechanism by which E2F1 transcriptional activity is controlled by RRP1B remains unclear. RRP1B does not contain any known DNA binding or transcriptional regulatory motifs; therefore its role may be in binding to chromatin or in recruitment of chromatin modifiers. Nucleophosmin and nucleolin have been shown to direct bind to histones and act as histone chaperones to regulate transcription (Angelov et al., 2006; Laskey et al., 1978). Consistent with these examples, a recent study has shown RRP1B to bind generally to chromatin, including to general chromatin components such as histone H1X (Crawford et al., 2009). However, because our data suggest selective and promoter-specific regulation of E2F1 target genes, it may be more likely that general binding of RRP1B to ubiquitous histones is uninvolved in regulation of E2F1 target

genes. Alternatively, RRP1B may recruit histone modifiers, such as histone acetyltransferases, to upregulate E2F1 target genes. This is similar to the mode of action seen for both nucleophosmin and nucleolin, which recruit GCN5 and P/CAF respectively, to specific promoters for transcriptional regulation (Masumi et al., 2006; Zou et al., 2008). Consistent with this hypothesis, RRP1B has been shown to bind acetylated lysine 5 of histone 4 and other nonubiquitous chromatin binding proteins (Crawford et al., 2009). Further investigation of the ability of RRP1B to recruit chromatin modifiers is warranted.

We also show that RRP1B is localized to the nucleolus and punctate nucleoplasmic foci in multiple cell lines (Fig. 7*A&B*). This observeration is consistent with other studies showing localization of the RRP1, a RRP1B homolog, to the nucleolus (Savino et al., 1999; Savino et al., 2001), and also with proteomic studies suggesting nucleolar localization (Andersen et al., 2005; Andersen et al., 2002; Scherl et al., 2002). However, our results are inconsistent with a recent study, suggesting localization of RRP1B to the nucleoplasm and nuclear lamina, to the exclusion of the nucleolus (Crawford et al., 2009); this disparity might be because of the different cell lines used.

One possible reason for the selective ability of RRP1B to regulate particular E2F1 target genes is the localization of gene promoters during interphase in proximity to the nucleolus. The rRNA promoter, an E2F1 and RRP1B regulated promoter (Fig. 4*C*), is situated within nucleolar organizing regions inside the nucleolus (Raska et al., 2006). Whether the promoters of caspase-3, caspase-7, or RRP1B are located within or near the nucleolus remains to be determined. RRP1B was also observed to be localized with E2F1 in punctate nucleoplasmic foci. While the type and nature of these foci are unknown,

regulation of E2F1 target genes unrelated to ribosome biogenesis, such as caspase-3 or caspase-7, may be localized to these foci. Finally, because the nucleolus is not membrane bound, proteins may freely enter and exit the nucleolus into the nucleoplasm; regulation of E2F1 target gene promoters may be situated within the nucleoplasm as a consequence.

Identification of RRP1B as a promoter of apoptosis may also suggest an explanation for the observation of higher survival in breast cancers with an expression profile driven by high RRP1B expression (Crawford et al., 2007; Crawford et al., 2009). RRP1B may be an important factor in apoptotic response to genotoxic agents and aberrant proliferation (Fig. 3*A*-*C*); therefore it is possible that increased survival seen in breast cancers with high RRP1B expression may be due to increased responsiveness to genotoxic therapy. It would be interesting to see whether expression profiles seen in RRP1B overexpression also show increases in E2F1-dependent target genes involved in apoptosis.

In summary, we have identified RRP1B as a novel specific target of E2F1 involved in the regulation of apoptosis. Loss of RRP1B expression inhibits the cellular apoptotic response to genotoxic agents as well as E2F1 overexpression. RRP1B selectively regulates E2F1 target gene expression through binding with E2F1 on target gene promoters. These data suggest that RRP1B is a new specificity factor for E2F1-mediated apoptosis (Fig. 8). Furthermore, we have identified a novel nucleolar protein in regulation of apoptosis through binding of chromatin.

FOOTNOTES

We greatly appreciate the gift of rRNA promoter plasmids from Dr. Paule Séité (University of Poitiers), caspase-7 promoter plasmid from Dr. Zaher Nahle (Washington University) and GST-NHERF-PDZ2 from Fannie Lin (UAB). We also thank Marion Spell at the UAB CFAR Flow Cytometry Core Facility and Enid Keyser at the UAB Arthritis and Musculoskeletal Center Flow Cytometry Core Facility for assistance with flow cytometry analysis. We also thank Dr. Susan Nozell (UAB) for technical assistance with ChIP assays. This work was supported by National Institutes of Health Grants T32 GM008361 (to J.C.P.) and CA100857 (to W.-C.L.), and Department of Defense Breast Cancer Research Program Grants W81XWH-09-1-0338 (to W.-C.L.) and W81XWH-06-1-0708 (to J.C.P.). W.-C.L. is a Leukemia and Lymphoma Society Scholar. This work is submitted in partial fulfillment of the requirements for the UAB Cell Biology Graduate Program (J.C.P.).

The abbreviations used are: ATM, ataxia telangiectasia mutated; Chk2, checkpoint kinase 2; RRP1B, Ribosomal RNA Processing 1 homolog B; NNP-1B, Novel Nucleolar Protein 1 homolog B; YFP, yellow fluorescent protein; doxo, doxorubicin; CDDP, cisplatin; NCS, neocarzinostatin; siRNA, small interfering RNA; 7-AAD, 7-amino-actinomycin; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; ChIP, chromatin immunoprecipitation; GST, glutathione S-transferase; rRNA, ribosomal ribonucleic acid; aa: amino acid

Table 1: Post-hoc analysis of Muller et al. (Muller et al., 2001) for genes specifically upregulated by E2F1, but not other E2Fs. Number values indicate fold induction or repression.

Gene	E2F1	E2F2	E2F3	Description
EPAS1	4.1	-0.6	-0.8	endothelial PAS domain protein 1
	3.8	-0.7	-0.7	Homo sapiens mRNA; cDNA DKFZp434E1515
	3.4	0.5	-0.5	Homo sapiens mRNA; cDNA DKFZp564E1363
ARHH	2.1	0.8	0.2	ras homolog gene family, member H
CHML	4.8	0.2	0.3	choroideremia-like (Rab escort protein 2)
NFRKB	2.4	0.5	0.4	nuclear factor related to kappa B binding protein
KIAA0179	**2.5**	**0.7**	**0.5**	**KIAA0179 protein (RRP1B)**
ABCB2	8.5	-0.3	0.6	ATP-binding cassette, sub-family B (MDR/TAP)
CAMKK2	3.1	0.6	0.6	calcium/calmodulin-dependent protein kinase kinase
NCOA1	2.9	-0.7	0.6	nuclear receptor coactivator 1
C3	2.5	-0.3	0.6	complement component 3
MAOA	2.7	0.5	0.7	monoamine oxidase A
OSTF1	3.5	-0.6	0.8	osteoclast stimulating factor 1
FBLN5	3.2	-0.5	0.8	fibulin 5

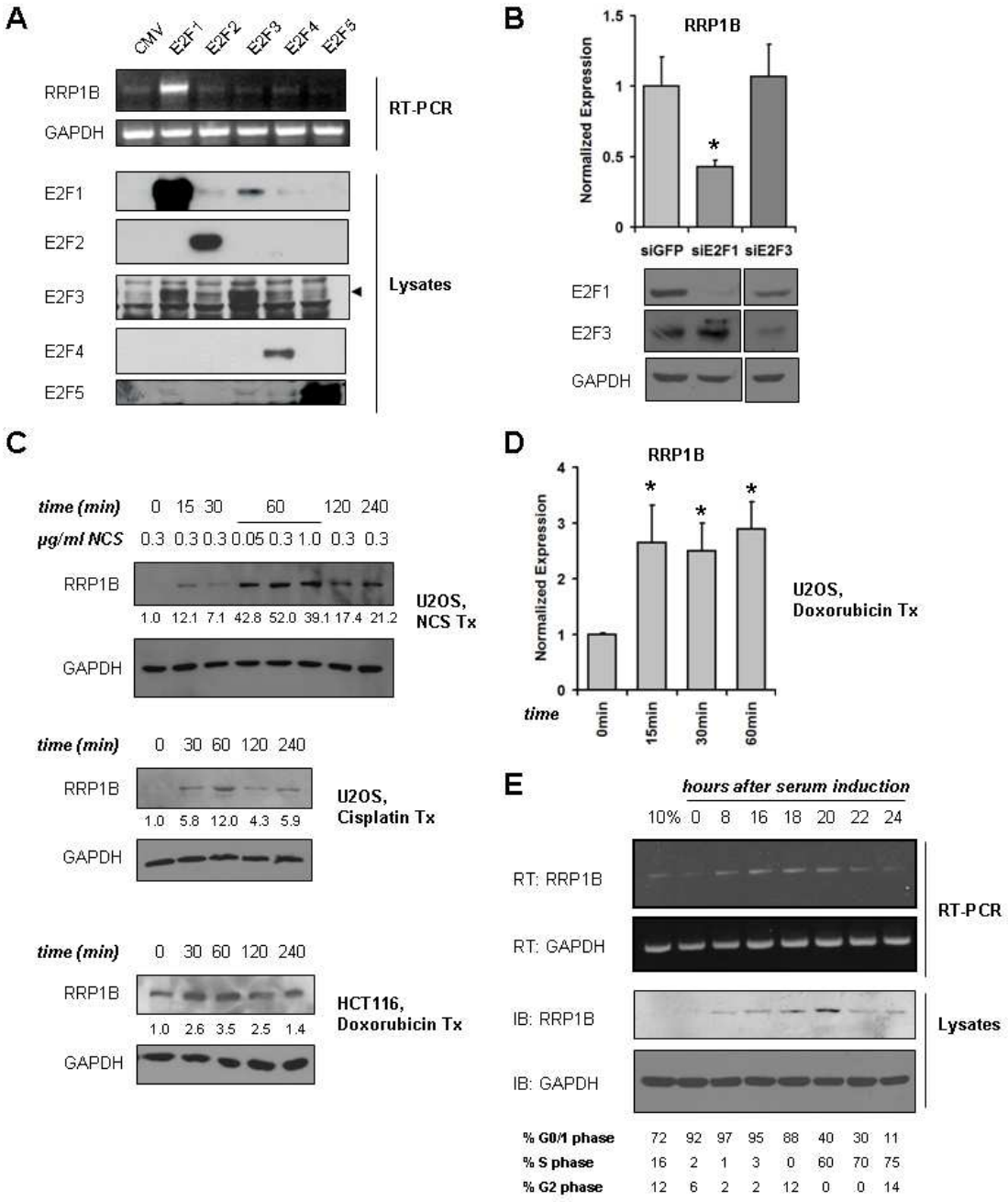
A
CMV
E2F1
E2F2
E2F3
E2F4
E2F5
RRP1B
GAPDH
RT-PCR
E2F1
E2F2
E2F3
E2F4
E2F5
Lysates
B
RRP1B
Normalized Expression
1.5
1
0.5
0
siGFP siE2F1 siE2F3
E2F1
E2F3
GAPDH
C
time (min) 0 15 30 60 120 240
μg/ml NCS 0.3 0.3 0.3 0.05 0.3 1.0 0.3 0.3
RRP1B
1.0 12.1 7.1 42.8 52.0 39.1 17.4 21.2
GAPDH
U2OS, NCS Tx
time (min) 0 30 60 120 240
RRP1B
1.0 5.8 12.0 4.3 5.9
GAPDH
U2OS, Cisplatin Tx
time (min) 0 30 60 120 240
RRP1B
1.0 2.6 3.5 2.5 1.4
GAPDH
HCT116, Doxorubicin Tx
D
RRP1B
Normalized Expression
4
3
2
1
0
time
0min
15min
30min
60min
U2OS, Doxorubicin Tx
E
hours after serum induction
10% 0 8 16 18 20 22 24
RT: RRP1B
RT: GAPDH
RT-PCR
IB: RRP1B
IB: GAPDH
Lysates
% G0/1 phase 72 92 97 95 88 40 30 11
% S phase 16 2 1 3 0 60 70 75
% G2 phase 12 6 2 2 12 0 0 14

Figure 1

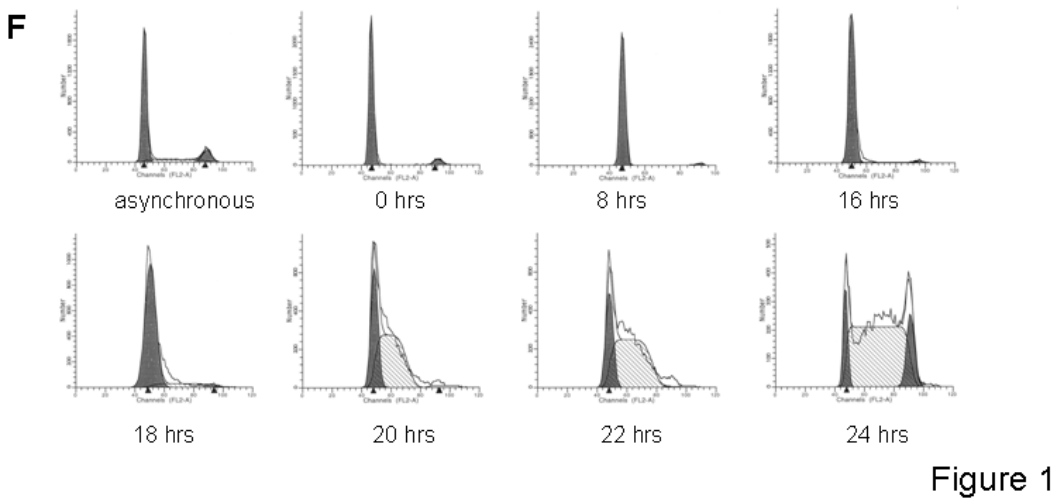

Figure 1: Regulation of RRP1B expression by E2F1.

A. Serum-starved T98G cells were infected with adenoviruses containing either E2F1, E2F2, E2F3, E2F4, E2F5, or the CMV promoter alone. RNA was extracted and subjected to semiquantitative RT-PCR for RRP1B and GAPDH. Cell lysates were also collected for each infection and probed with the indicated antibodies.

B. RNA was extracted from U2OS cells that were stably transfected with pSuperior encoding siRNAs against GFP, E2F1, or E2F3 and subjected to quantitative PCR for RRP1B, levels of which were normalized against GAPDH. Cell lysates for independent experiments were collected for siGFP, siE2F1, and siE2F3 cell lines and probed with the indicated antibodies. * $P < 0.05$ compared to both siGFP and siE2F3.

C. U2OS or HCT116 cells were treated with 10 μM doxorubicin, neocarzinostatin (NCS), or 20 μM cisplatin for the indicated times and dosages, lysed, electrophoresed, and immunoblotted with the indicated antibodies. Numbers below each lane indicate densitometry of RRP1B levels normalized to GAPDH levels.

D. RNA was extracted from U2OS cells treated with 1 μM doxorubicin for the indicated time points and subjected to quantitative PCR for RRP1B, levels of which were normalized against GAPDH. * $P < 0.01$ for all treated time points compared to untreated.

E. Human foreskin fibroblasts were brought to quiescence by serum starvation (0.25% FBS) for 48hr, and then reinduced with 20% serum at the indicated timepoints. Cells were lysed, RNA and protein extracted, subjected to semiquantitative RT-PCR or blotting with the indicated primer sets or antibodies. Numbers below each lane indicate percentage of cells in G0/G1, S, and G2 phases of the cell cycle as assayed by propidium iodide DNA histogram analysis. *F.* Representative DNA histogram analysis by propidium iodide flow cytometry.

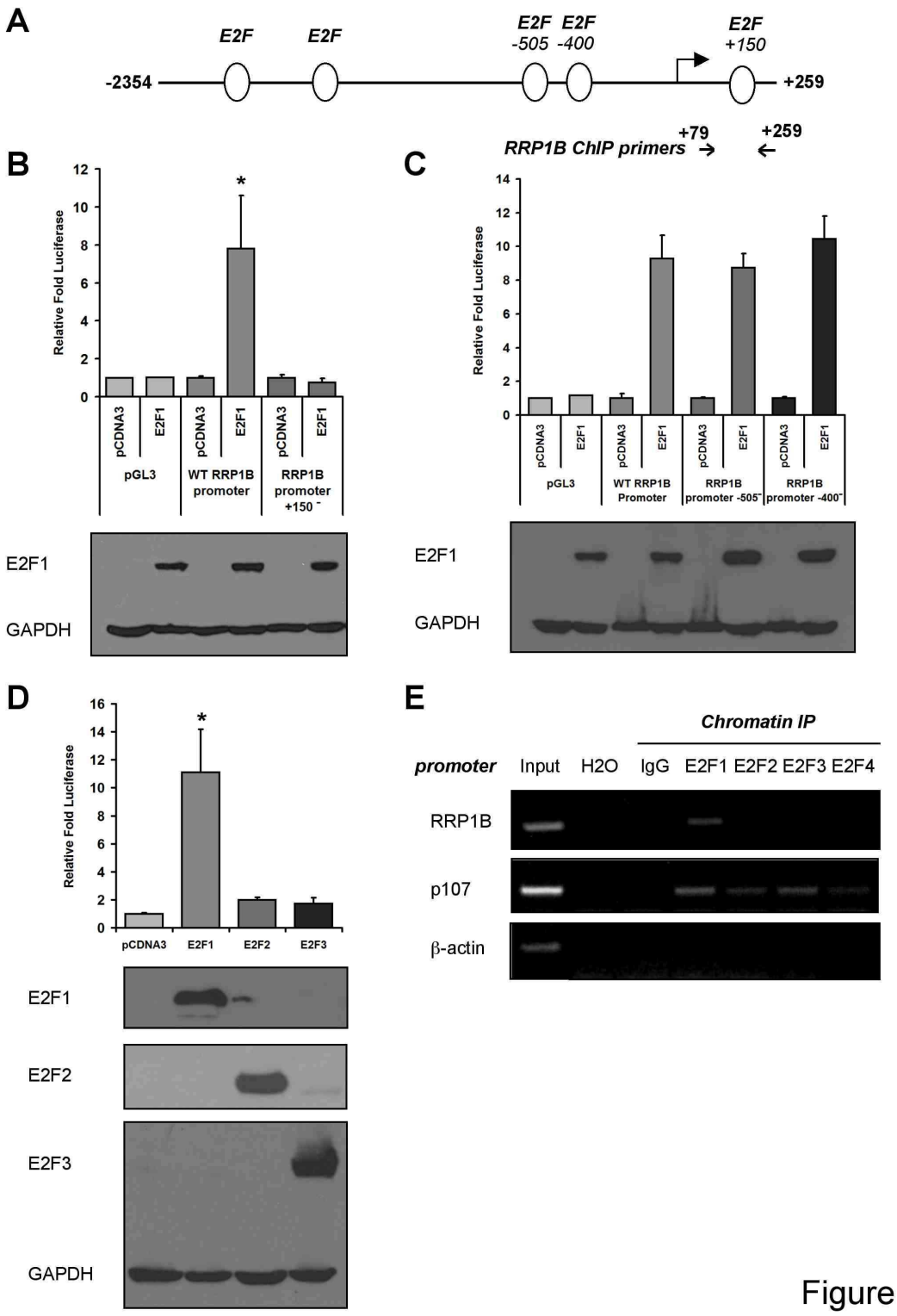
A
E2F
E2F
E2F -505
E2F -400
E2F +150
-2354
+259
B
Relative Fold Luciferase
pCDNA3
E2F1
pGL3
WT RRP1B promoter
RRP1B promoter +150
E2F1
GAPDH
C
RRP1B ChIP primers
+79
+259
Relative Fold Luciferase
pGL3
WT RRP1B Promoter
RRP1B promoter -505
RRP1B promoter -400
D
Relative Fold Luciferase
pCDNA3
E2F1
E2F2
E2F3
GAPDH
E
Chromatin IP
promoter
Input
H2O
IgG
E2F1
E2F2
E2F3
E2F4
RRP1B
p107
β-actin

Figure 2

Figure 2: E2F1 specifically drives RRP1B expression and binds to the RRP1B promoter.

A. Schema of the wild type RRP1B promoter.

B. HEK293T cells were transfected with either empty vector, wild type RRP1B promoter reporter luciferase vector, or with RRP1B promoter vector in which a single E2F site is mutated at +150, with either E2F1 or empty vector and β-galactosidase. 48 hr later, cells were lysed for determination of luciferase activity. β-gal activity was used as a control for transfection efficiency. A protein aliquot from each experimental arm was blotted and probed with the indicated antibodies. * $P < 0.02$ between E2F1 transfected arms.

C. HEK293T cells were transfected as before but with empty vector, wild type RRP1B promoter, or with RRP1B promoter in which a single E2F site is mutated at -505 or -400, and either E2F1 or empty vector. Luciferase analysis and protein blotting was done was before.

D. HEK293T cells were transfected with a RRP1B promoter reporter and either E2F1, E2F2, or E2F3 or empty vector. Luciferase analysis and protein blotting was done was before. * $P < 0.01$ between E2F1 arm and all other arms.

E. U2OS cells were crosslinked, nuclei extracted, sonicated, and incubated with the indicated antibodies, followed by washes and decrosslinking. Chromatin was then used for PCR amplification using the indicated primer sets.

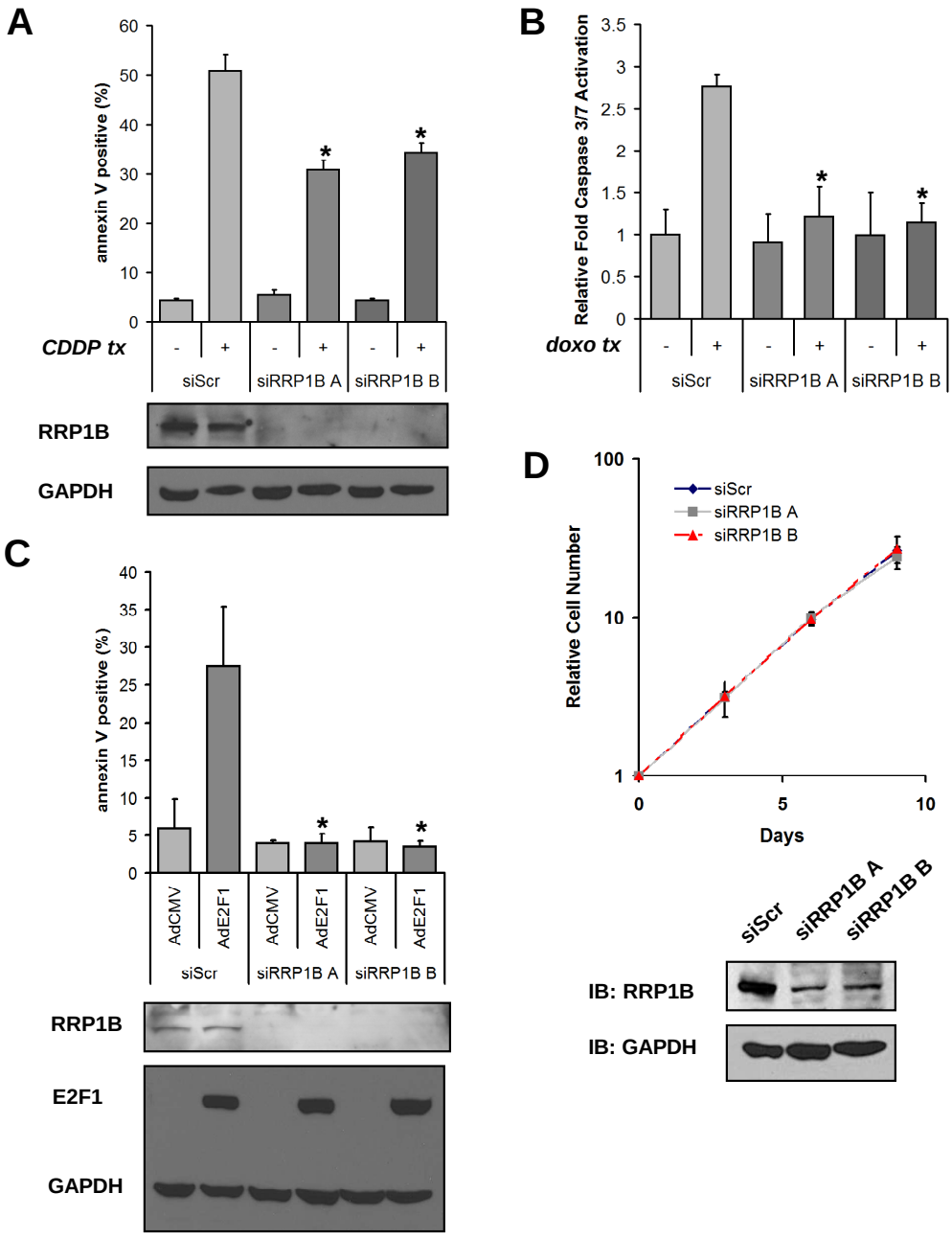
A
annexin V positive (%)
60
50
40
30
20
10
0
CDDP tx
-
+
-
+
-
+
siScr
siRRP1B A
siRRP1B B
RRP1B
GAPDH
B
Relative Fold Caspase 3/7 Activation
3.5
3
2.5
2
1.5
1
0.5
0
doxo tx
siScr
siRRP1B A
siRRP1B B
C
annexin V positive (%)
40
35
30
25
20
15
10
5
0
AdCMV
AdE2F1
siScr
siRRP1B A
siRRP1B B
RRP1B
E2F1
GAPDH
D
Relative Cell Number
100
10
1
siScr
siRRP1B A
siRRP1B B
0
5
10
Days
siScr
siRRP1B A
siRRP1B B
IB: RRP1B
IB: GAPDH

Figure 3

Figure 3: Knockdown of RRP1B reduces DNA-damage and E2F1-induced apoptosis but does not affect cellular proliferation.

A. Stably transduced U2OS cells expressing either nonspecific siScramble (siScr) sequence or two siRNA against RRP1B (siRRP1B A and siRRP1B B) were seeded equally and induced for apoptosis with 20 µM cisplatin for 30 hr, then analyzed by FACS for surface Annexin V staining. Experiments were done in triplicate. An aliquot of protein from each experimental arm was blotted and probed with the indicated antibodies. * $P < 0.02$ between treated siScr and siRRP1B arms.

B. Stably transduced siScr or siRRP1B A or B U2OS cells were seeded equally and induced for caspase cleavage with 1 µM doxorubicin for 8 hr. * $P < 0.01$ between treated siScr and siRRP1B arms.

C. Stably transduced siScr or siRRP1B A or B U2OS cells were seeded equally, starved for 48 hr, then infected with 200 MOI of either CMV adenovirus (empty) or E2F1 adenovirus for 36 hr, then analyzed by FACS for surface Annexin V staining. Experiments were done in triplicate. An aliquot of protein from each experimental arm was electrophoresed, blotted, and probed with the indicated antibodies. * $P < 0.01$ between E2F1 induced siScr and siRRP1B arms.

D. Stably transduced siScr or siRRP1B A or B U2OS cells were seeded equally on six-well plated and counted by a hemacytometer at 3 d prior to confluence. Cells were diluted 1:4 and replated, and counted at 6 d and 9 d. Cells were lysed at the end of the experiment, electrophoresed, blotted, and probed with the indicated antibodies.

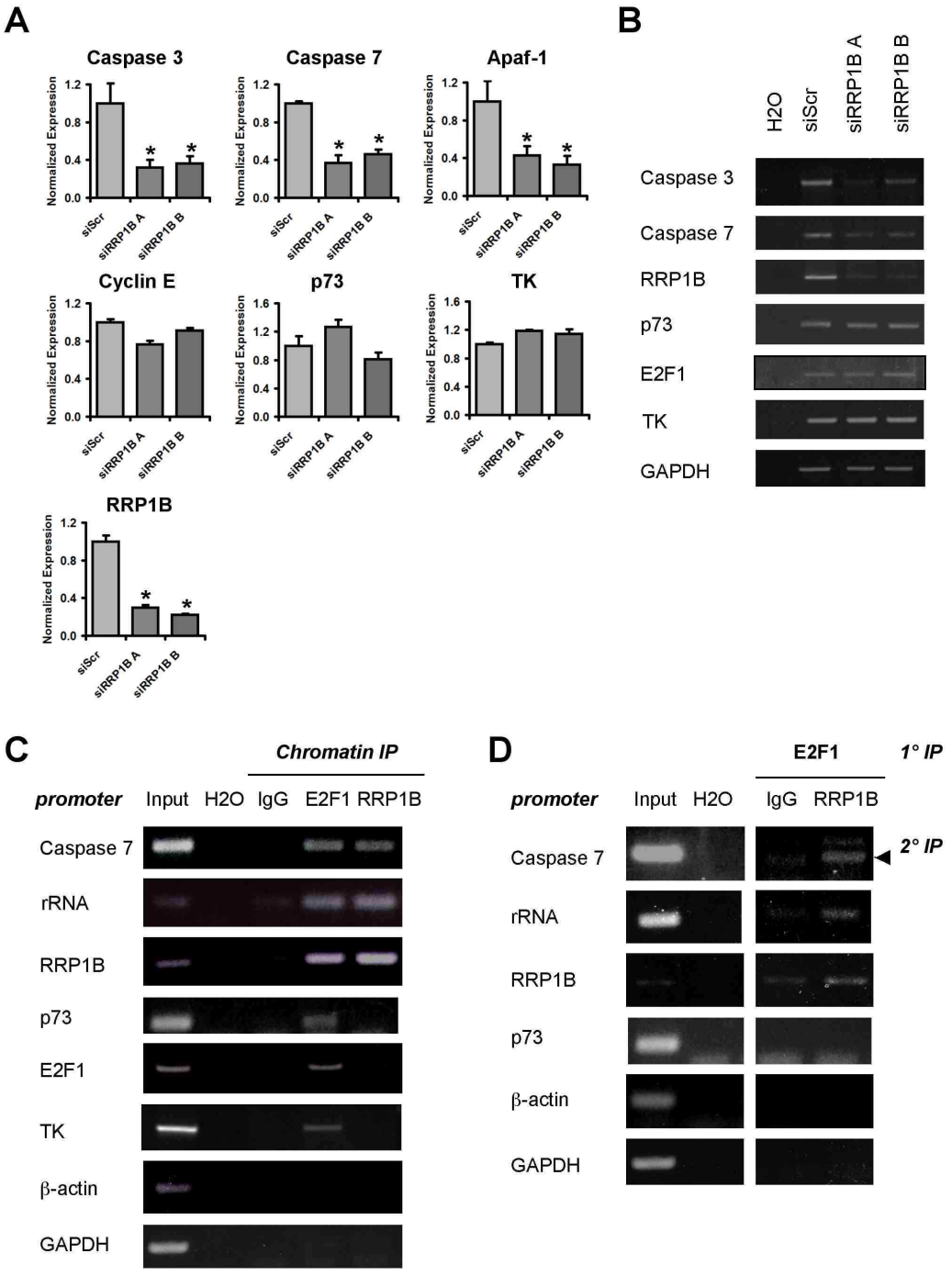
A
Caspase 3
Caspase 7
Apaf-1
Cyclin E
p73
TK
RRP1B
Normalized Expression
siScr
siRRP1B A
siRRP1B B
B
H2O
siScr
siRRP1B A
siRRP1B B
Caspase 3
Caspase 7
RRP1B
p73
E2F1
TK
GAPDH
C
Chromatin IP
promoter
Input
H2O
IgG
E2F1
RRP1B
Caspase 7
rRNA
RRP1B
p73
E2F1
TK
β-actin
GAPDH
D
E2F1
1° IP
promoter
Input
H2O
IgG
RRP1B
2° IP
Caspase 7
rRNA
RRP1B
p73
β-actin
GAPDH

Figure 4

Figure 4: Knockdown of RRP1B selectively affects E2F1 target levels by binding with E2F1 on E2F-responsive promoters.

A. RNA extracted from U2OS cells stably transduced with siScr or siRRP1B A or B were subjected to quantitative PCR for the indicated targets. Expression level was normalized to GAPDH. * $P < 0.02$ between siScr and siRRP1B arms.

B. In an independent experiment, semiquantative RTPCR was performed on RNA extracted from U2OS siScr or siRRP1B cells for the indicated targets. H2O indicates no template control.

C. U2OS cells were crosslinked, nuclear extracted, sonicated, precleared, and immunoprecipitated with 4 µg of the indicated antibodies overnight followed by incubation with protein A+G beads for 3 hr and stringent washes. Chromatin was eluted from beads, decrosslinked, incubated with RNase A and proteinase K, purified, and subjected to PCR for the indicated E2F responsive promoters. H2O indicates no template control.

D. U2OS cells were crosslinked, nuclear extracted, sonicated, precleared, and immunoprecipitated with 4 µg of the indicated antibodies overnight followed by incubation with protein A+G beads for 3hr and stringent washes. Chromatin-protein complexes were eluted with 1 mM DTT followed by a second immunoprecipitation with the indicated antibodies. Binding to beads, washes, elution, purification, and PCR were done as in Fig. 4D. Arrowheads indicate expected size of PCR products.

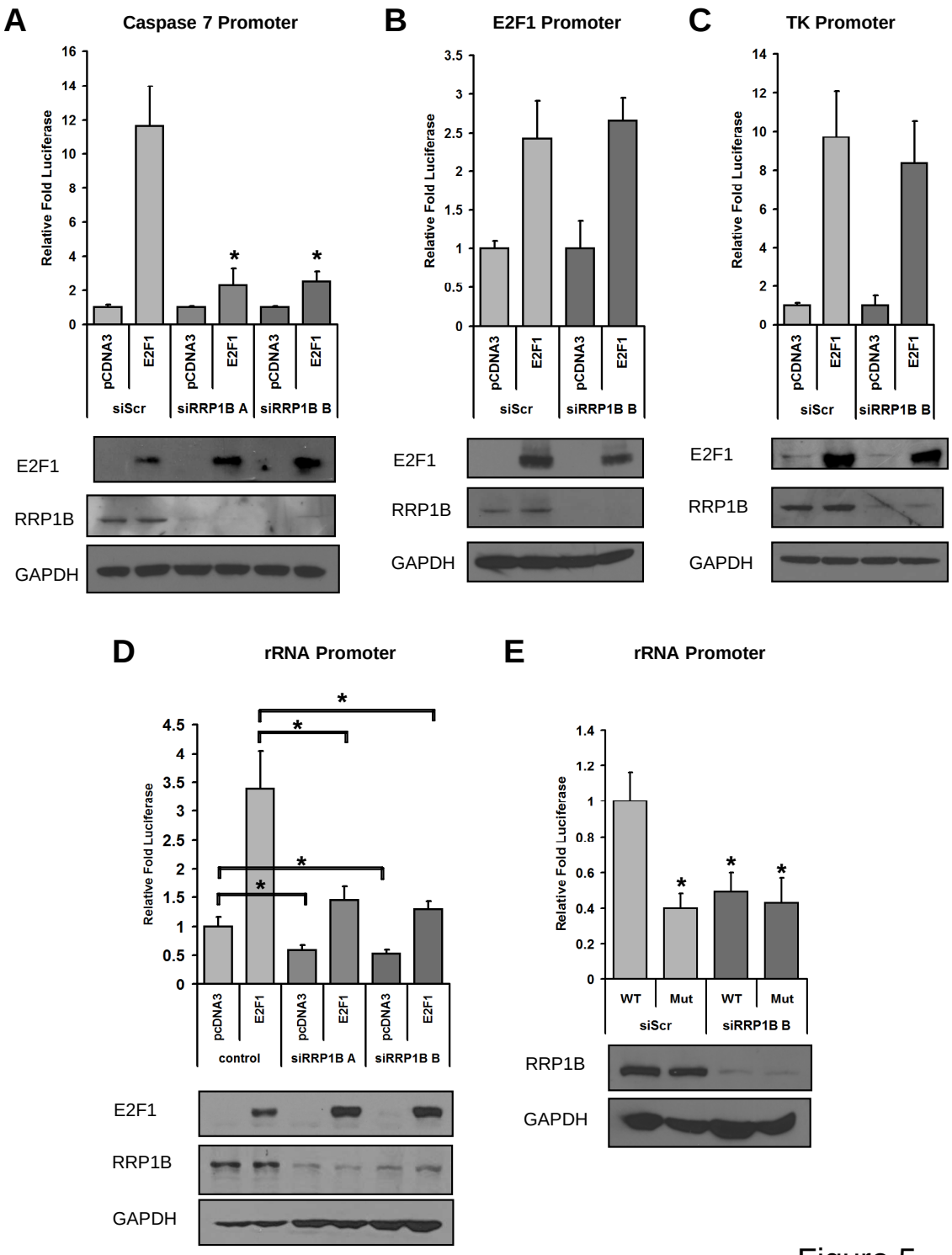
A
Caspase 7 Promoter
Relative Fold Luciferase
pCDNA3
E2F1
siScr
siRRP1B A
siRRP1B B
B
E2F1 Promoter
C
TK Promoter
E2F1
RRP1B
GAPDH
D
rRNA Promoter
control
E
WT
Mut

Figure 5

Figure 5: Knockdown of RRP1B selectively affects E2F induced promoter reporter luciferase activity.

A. H1299 siScr or siRRP1B A or B cells were transfected with caspase-7 promoter reporter, E2F1 or empty vector, and β-galactosidase, incubated for 48 hr and subjected to luciferase assay. β-gal activity was used as a control for transfection efficiency. A protein aliquot from each experimental arm was blotted with the indicated antibodies. * $P <$ 0.005 between E2F1 transfected siScr cells and both E2F1 transfected siRRP1B cells.

B. H1299 siScr or siRRP1B B cells were transfected with E2F1 promoter reporter, E2F1 or empty vector, and β-galactosidase. Luciferase analysis and protein blotting was done was before.

C. H1299 siScr or siRRP1B cells were transfected with TK promoter reporter, E2F1 or empty vector, and β-galactosidase. Luciferase analysis and protein blotting was done was before.

D. H1299 cells transduced with siRRP1B A or B or no virus were seeded equally and transfected with rRNA promoter reporter, E2F1 or empty vector, and β-galactosidase. Luciferase analysis and protein blotting was done was before. * $P < 0.01$ between pcDNA3 transfected arms and between E2F1 transfected arms.

E. H1299 cells stably transduced with siScr or siRRP1B B were seeded equally and transfected with an intact rRNA promoter reporter or rRNA promoter in which the E2F binding site for activation has been mutated, and β-galactosidase. Luciferase analysis and protein blotting was done was before. * $P < 0.05$.

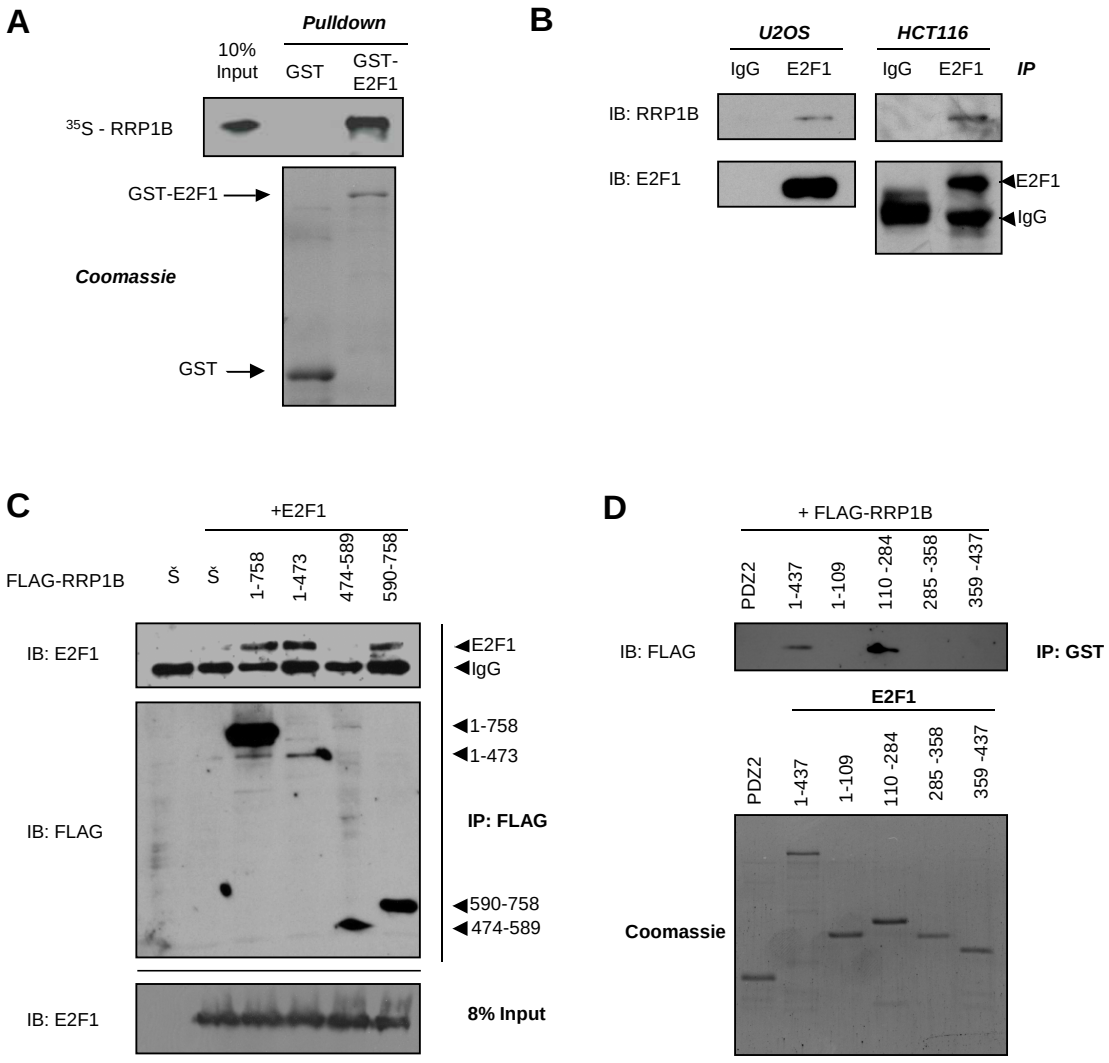
A
Pulldown
10% Input
GST
GST-E2F1
35S - RRP1B
GST-E2F1
Coomassie
GST
B
U2OS
HCT116
IgG
E2F1
IgG
E2F1
IP
IB: RRP1B
IB: E2F1
E2F1
IgG
C
+E2F1
FLAG-RRP1B
Ś
Ś
1-758
1-473
474-589
590-758
IB: E2F1
E2F1
IgG
1-758
1-473
IB: FLAG
IP: FLAG
590-758
474-589
IB: E2F1
8% Input
D
+ FLAG-RRP1B
PDZ2
1-437
1-109
110 -284
285 -358
359 -437
IB: FLAG
IP: GST
E2F1
PDZ2
1-437
1-109
110 -284
285 -358
359 -437
Coomassie

Figure 6

Figure 6: Physical interaction between RRP1B and E2F1.

A. FLAG-tagged RRP1B was produced by in vitro transcription/translation in the presence of ^{35}S-methionine and added to buffer containing either GST-E2F1 bound to glutathione agarose or GST alone, nutated, washed, separated by SDS-PAGE, fixed, enhanced, and exposed to film. Equal loading of GST proteins indicated by a parallel Coomassie stain.

B. Nuclei from U2OS and HCT116 cells were extracted, sonicated, lysed, and immunoprecipitated with E2F1 antibodies. Beads were washed, blotted, and probed with the indicated antibodies.

C. FLAG-tagged RRP1B or RRP1B domain mutants were coexpressed with E2F1 in HEK293T cells. Cells were lysed, immunoprecipitated with anti-FLAG agarose, washed, blotted, and probed with the indicated antibodies.

D. FLAG-tagged RRP1B was expressed in HEK293T cells, lysed, and incubated with a control irrelevant protein GST-PDZ2, GST-E2F1, or GST-E2F1 mutants bound to glutathione agarose overnight, washed, blotted, and probed with the indicated antibodies. Equal loading of GST proteins indicated by a parallel Coomassie stain.

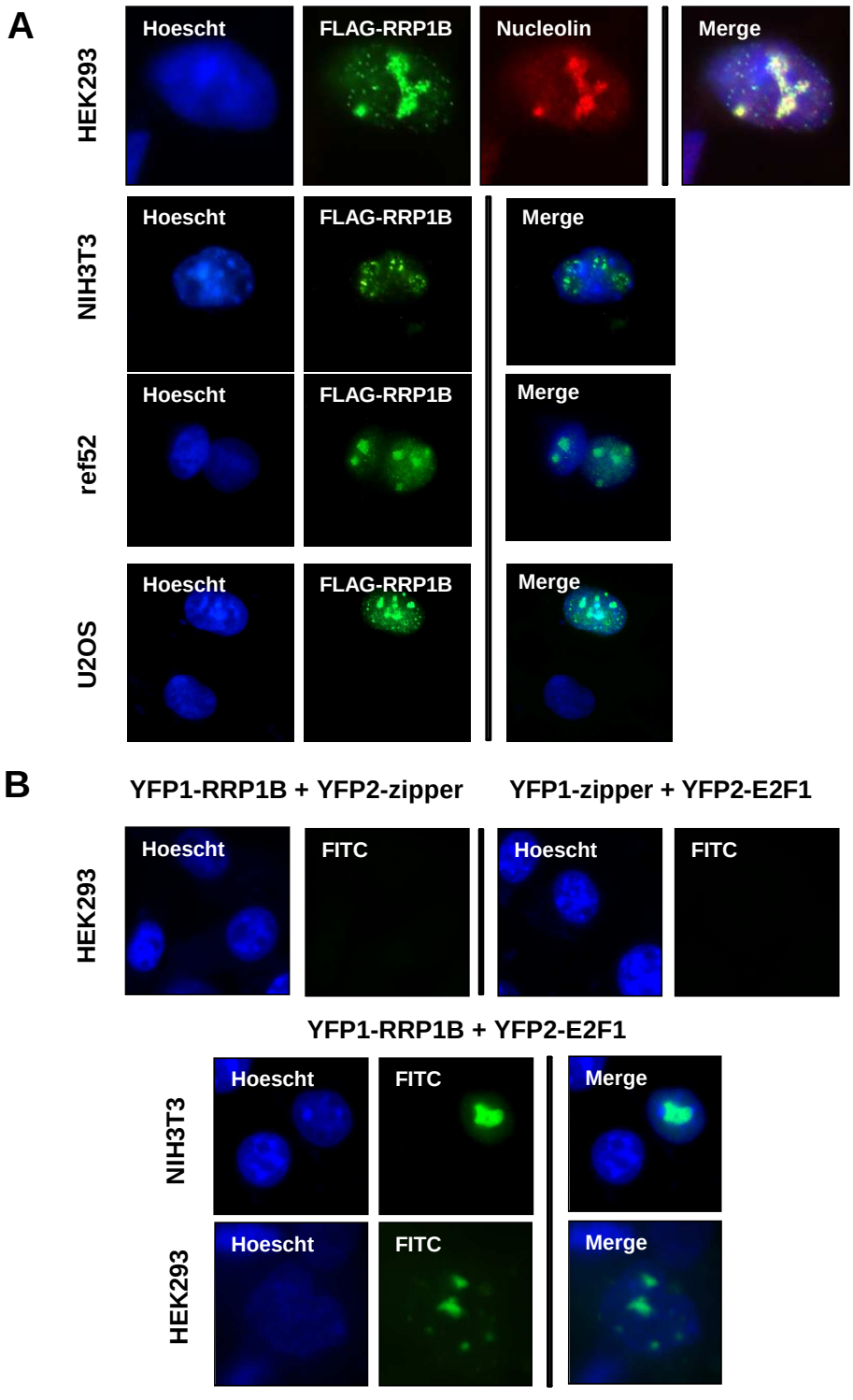
A
HEK293
Hoescht
FLAG-RRP1B
Nucleolin
Merge
NIH3T3
Hoescht
FLAG-RRP1B
Merge
ref52
Hoescht
FLAG-RRP1B
Merge
U2OS
Hoescht
FLAG-RRP1B
Merge
B
YFP1-RRP1B + YFP2-zipper
YFP1-zipper + YFP2-E2F1
HEK293
Hoescht
FITC
Hoescht
FITC
YFP1-RRP1B + YFP2-E2F1
NIH3T3
Hoescht
FITC
Merge
HEK293
Hoescht
FITC
Merge

Figure 7

Figure 7: RRP1B localizes and interacts with E2F1 in nucleoli and punctate nucleoplasmic foci.

A. Indicated cells were transfected with FLAG-RRP1B, fixed, probed with the indicated antibodies, nuclei stained with Hoescht 33258, and mounted for microscopy.

B. RRP1B and E2F1 were each cloned into vectors expressing one part each of YFP in a single continuous cDNA and transfected into the indicated cells. YFP subunits expressing a nonspecific leucine zipper were used as a negative control. Green fluorescence indicates colocalization of YFP subunits, and the subcellular location of interaction. Cells were fixed in paraformaldehyde, nuclei stained with Hoescht 33258, and mounted for immunofluorescence.

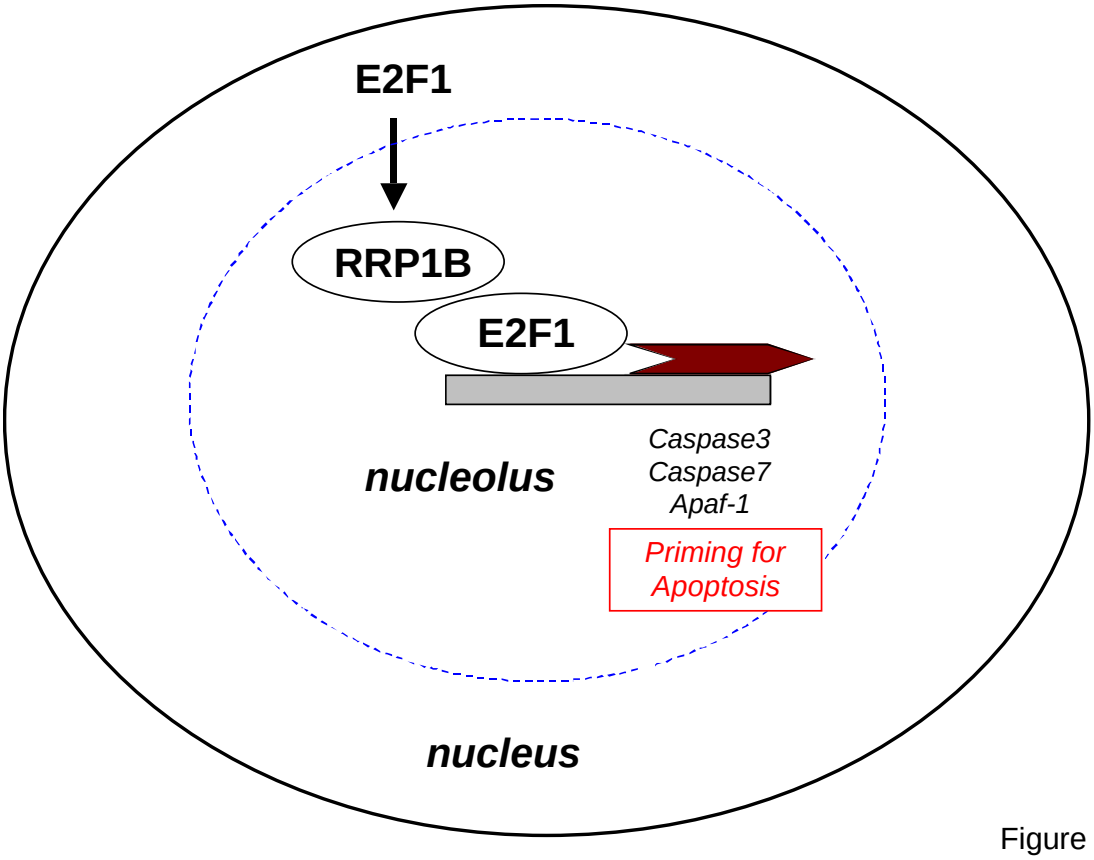

Figure 8

Figure 8: Proposed model of regulation of E2F1 and RRP1B.

RRP1B is specifically stimulated by E2F1 expression. RRP1B then binds E2F1 at specific E2F1 promoters, acting as a cofactor for expression of those specific E2F1 targets to upregulate E2F1-mediated apoptosis.

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DISCUSSION

A novel specificity factor for E2F1 induced apoptosis

An important question in cancer research is how cells regulate the switch between cellular proliferation and cell death. The transcription factor E2F1 has been hypothesized to play a key role in this switch by preventing runaway cellular proliferation through activation of a cell suicide mechanism. However, the role and regulation of specific E2F1 induced cell death has not been fully delineated. Multiple molecular mechanisms, including transcriptional regulation, regulation of protein half-life, interactions with physical binding partners, regulation of protein translation, and regulation of transcript splicing have all been hypothesized to participate in specific regulation of E2F1. Other mechanisms outside of a biochemical context, including differences in cell type, context within tissues and organs, and cell autonomy also are likely to play a role in regulation of E2F1 induced apoptosis. In this study, we have identified RRP1B as one of the regulators that allows E2F1 to act as a switch between cell death and cellular proliferation.

Through a screen of E2F regulated transcripts, we identified RRP1B as a specific transcriptional target of E2F1 through biochemical assays. We also show the ability of RRP1B to act as a positive regulator of both DNA damage induced and E2F1-induced apoptosis, and that this occurs through direct promoter binding with E2F1. This finding establishes another level of regulation of E2F1 in addition to the other known E2F1 apoptosis specificity factors Jab1 and MCPH1 (**Figure 1**). RRP1B can also be added to

the short list of genes specifically regulated by E2F1, which include AMPKα2, p73, Chk1, and β-catenin. The combination of specificity at a transcriptional and at a cofactor level suggests the importance of RRP1B in regulation of E2F1 induced apoptosis, and is unique amongst all known E2F1 induced transcripts and cofactors.

How does RRP1B fit into current models of specific E2F1 regulation?

RRP1B likely plays a role in concert with other cofactors in regulation of E2F1 induced apoptosis. Like almost all of the other regulators of apoptosis in general, ability to regulation is likely context dependent on both a biochemical and tissue level. Our results indicate that RRP1B occupies a unique place in proapoptotic cofactors of E2F1 in that it binds to the DNA binding domain of E2F1. In comparison, the two other positive regulators of E2F1 induced apoptosis, Jab1 (Hallstrom and Nevins, 2006) and MCPH1 (Yang et al., 2008), are bound to the marked box domain and N-terminus of E2F1 respectively. While suggesting again that there are multiple layers of control of E2F1 induced apoptosis, it also shows that the regulatory ability of RRP1B on E2F1 is not likely dependent on Jab1 or MCPH1 regulation. This hypothesis is further supported by the inability of RRP1B to regulate p73, an important E2F1-induced proapoptotic target which is regulated by MCPH1 (Yang et al., 2008). Furthermore, unlike TopBP1 and MCPH1, N-terminal phosphorylation of E2F1 in response to DNA damage (Liu et al., 2004) is not likely to play a role in RRP1B regulation of E2F1, since that region is not bound by RRP1B. Similarly, Akt/PI3K signaling, which is important to control E2F1 induced apoptosis in a negative feedback loop with TopBP1, is also not likely to play a role for RRP1B regulation of E2F1 (Liu et al., 2006).

The possibility exists for RRP1B to participate in regulation of E2F1 apoptosis by displacing cofactors that negatively regulate E2F1. While potential RRP1B displacement of TopBP1 might be ruled out due to differences in binding site, RRP1B may be involved in displacement of other negative regulators of E2F1 apoptosis. For example, the ETS-related transcription factor GABPγ1 has been shown to bind to the E2F1 DNA binding domain, and negatively regulate the ability of E2F1 to transduce caspase-3 and caspase-7 (Hauck et al., 2002). pRb also appears to have an independent binding E2F1 ability; while pRb does bind other E2Fs, a separate domain within pRb is capable of binding E2F1 at a site outside of the C-terminal Rb/dimerization domain. This site includes the E2F1 DNA binding domain (Dick and Dyson, 2003) to which RRP1B also binds. In contrast, the negative regulator KAP1 binds to the marked box domain of E2F1, and may not be regulated by competitive or cooperative binding with RRP1B to E2F1 (Wang et al., 2007). Another negative regulator, SirT1, also binds to the C-terminus of E2F1, suggesting a potential lack of participation of RRP1B on this interaction (Wang et al., 2006). Investigation into a potential role of RRP1B, GABPγ1, or pRb competitive interactions might be of interest.

Independent of other cofactors, the ability of RRP1B to bind to the DNA binding domain of E2F1 on promoters also warrants investigation. One potential mechanism by which RRP1B controls E2F1 transcriptional activity is by acting as a necessary cofactor for E2F1 promoter binding on specific promoters. A proposed experiment could be to assay the presence of E2F1 on E2F1 responsive proapoptotic promoters in an RRP1B depleted context through a chromatin immunoprecipitation assay. If RRP1B is important for E2F1 binding to promoters, E2F1 promoter binding should be absent upon RRP1B

knockdown. If true, it would represent an important advance, as it would open up new lines of investigation; since RRP1B appears to bind specific E2F1-induced promoters, further promoter studies are warranted to determine if RRP1B binding to E2F1 promoters requires E2F1, or if RRP1B can independently bind promoters at specific sites in a similar manner as nucleolin, nucleophosmin, or several other previously described nucleolar proteins participating in transcriptional regulation (Boisvert et al., 2007; Lindstrom, 2009; Warner and McIntosh, 2009). Direct promoter binding occurs despite the fact that these proteins do not contain canonical transcription factor DNA binding motifs. Other proteins which are known to bind to the E2F1 DNA binding domain include the required cofactors DP1 and DP2 (Hitchens and Robbins, 2003). RRP1B binding may improve DP1 and DP2 colocalization on E2F responsive promoters, providing a mechanism by which RRP1B can specify E2F presence on promoters; this can also be assayed using a similar chromatin immunoprecipitation assay.

Finally, regulation of E2F1 activity by RRP1B can occur via recruitment of chromatin modifying cofactors, such as histone modifying enzymes, in a site specific manner. Because E2F responsive sites in promoters can potentially bind to other E2Fs in a promiscuous manner, the composition of recruited cofactors and other chromatin binding proteins at the E2F site itself and surrounding promoter regions likely plays a large role in determining which E2F is specifically able to regulate transcription at a particular site. Differences between activator and repressor E2F binding specificities have been ascribed to variation in cofactor recruitment (Blais and Dynlacht, 2007). For example, activator E2Fs are known to recruit chromatin modifiers such as Tip60, Arid1B, and SWI/SNF complexes to acetylate Histone H1 in a repressor E2F antagonizing

manner (Blais and Dynlacht, 2007; Nagl et al., 2007; Takahashi et al., 2000; Taubert et al., 2004); activator E2Fs have also been shown to recruit HCF-1 and MLL/Set1 to methylate Histone H3 as well (Takeda et al., 2006; Tyagi et al., 2007). Nucleolar proteins have been shown to serve directly as chromatin modifiers or to recruit chromatin modifying proteins in other contexts as well (Shandilya et al., 2009; Swaminathan et al., 2005). While RRP1B does not contain the acidic domains in nucleophosmin and nucleolin previously described as mediating their intrinsic histone chaperone function, based on our data, it would be interesting to investigate a role for RRP1B in recruitment of histone modifying enzymes such as histone acetyltransferases or histone methyltransferases. A first experiment that may be performed is to analyze the status of important histone acetyl or methyl substrates by using monoclonal antibodies against acetyl-histone or methyl-histone in chromatin immunoprecipitation assays in cells where RRP1B is knocked down. If RRP1B is absent, and a change in acetylation or methylation is detected, further studies could be done to determine which chromatin modifying enzymes are present at those sites normally and are absent without RRP1B. RRP1B may also displace chromatin modifying enzymes which negatively affect E2F1 transcriptional activity, as well.

Once a mechanism for RRP1B in E2F1 proapoptotic activity has been described, investigation of a role for RRP1B in E2F1 regulation *in vivo* could be pursued. While we have provided evidence that RRP1B participates in regulation if E2F1 induced apoptosis in cell culture, effects seen in cancer cell lines may or may not translate well to preclinical or clinical *in* vivo models. One proposed experiment is to construct or acquire an RRP1B knockout murine model. While RRP1B does participate in ribosomal

biogenesis (Savino et al., 1999; Savino et al., 2001), it is possible that RRP1B knockout alone will not cause a severe metabolic or translational deficit, because RRP1, the other family member with RRP1B, could compensate in ribosomal biogenesis. Whether RRP1B knockout leads to a phenotypes similar to E2F1 knockout (Field et al., 1996; Yamasaki et al., 1996) remains to be seen. A viable RRP1B knockout model would also be useful to test the ability of E2F1 targets to be induced following chemotherapeutic treatment, and potentially the relative effectiveness of chemotherapy in the absence of RRP1B. A murine model in which RRP1B levels might be inducibly transduced could also provide valuable information; based on our biochemical studies, induction of RRP1B levels may potentially increase the ability of chemotherapeutics or radiation to affect tumor growth, an important step in suggesting RRP1B as a molecular target for improving the effectiveness of current anticancer treatment.

A noncanonical role for a nucleolar protein in transcription and apoptosis

The canonical role for the nucleolus is production of ribosomes, primarily through manufacture of ribosomal RNA and assembly of associated ribosomal proteins into ribosomal subunits, finally leading to a complete ribosomal supercomplex (Maggi and Weber, 2005). However, it has become increasingly clear that the nucleolus plays a surprising number of roles outside of ribosomal biogenesis, including regulation of proteins important in tumorigenesis. Individual components of the ribosomes and associated nucleolar proteins have been shown to regulate p53 stability; a large number of recent studies have shown multiple ribosomal proteins acting as mediators of MDM2 dependent p53 degradation (Zhang and Lu, 2009). Overproduction of ribosomes and

nucleolar proteins in response to oncogenic mitogen activity has also been hypothesized to play a role in tumorigenesis; myc and PTEN have both been shown to regulate numerous nucleolar proteins, and disruption of proper ribosome biogenesis has been implicated in dyskeratosis congenita and Diamond-Blackfan anemia (Ruggero and Pandolfi, 2003). Mutations and translocations of nucleophosmin, a nucleolar protein, have been shown to play an important role in leukemia (Falini et al., 2007). To this we add RRP1B as another example of a nucleolar protein which may be important in cancer. In contrast to regulation of localization of critical apoptosis regulators, we show that RRP1B acts as a proapoptotic cofactor for E2F1 directly on promoters. This is similar to RPS3 in NFκB regulation (Wan et al., 2007) and NPM in Miz1 regulation (Wanzel et al., 2008). Interestingly, as a nucleolar protein, RRP1B has been shown in microarray studies to be upregulated by c-myc (Schlosser et al., 2005; Schlosser et al., 2003), suggesting an intriguing possibility; that during oncogenesis, overactive myc may activate E2F1 proapoptotic activity through the upregulation of RRP1B and transactivation of proapoptotic E2F1 targets. A potential linkage between myc and E2F1 through RRP1B may warrant investigation.

RRP1B is not the first nucleolar protein known to bind to E2F1; E2F1 has previously been shown to interact with ARF, whose canonical function is to regulate MDM2 through sequestration from p53. Interestingly, ARF is a nucleolar protein with other functions including ribosomal biogenesis and negative regulation of activator E2Fs (Datta et al., 2002). While RRP1B has not been shown by us to regulate other activator E2Fs, its role in regulating E2F1 on the rRNA promoter is antagonistic to ARF functions

in rRNA promoter activity (Ayrault et al., 2006). An antagonistic relationship between RRP1B and ARF might also exist on other promoters, which may warrant investigation.

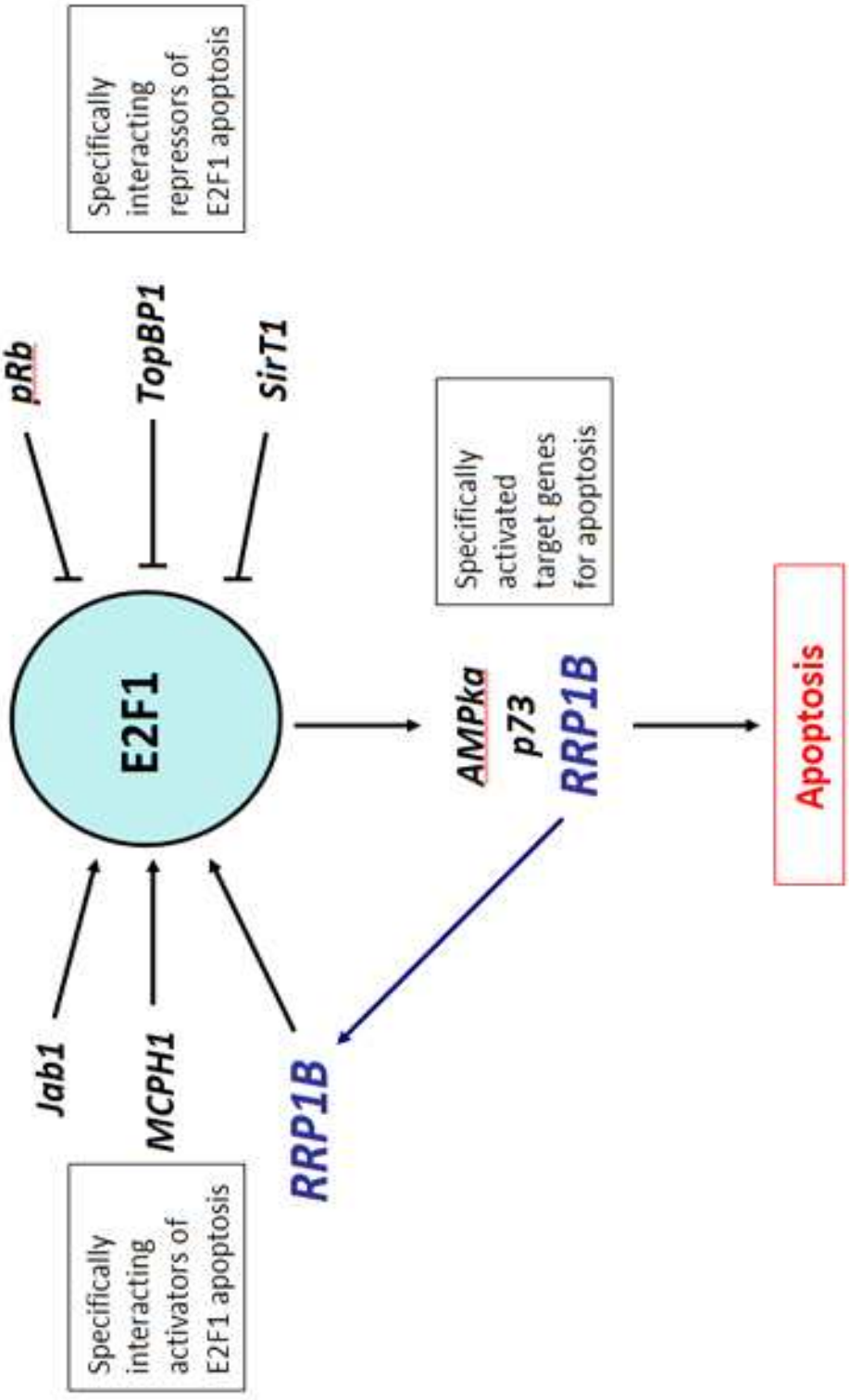
Also of interest is the potential function of RRP1/Nop52, the other member of the protein family which includes RRP1B. Interestingly, we have shown that the N-terminus of RRP1B is able to bind to E2F1, which corresponds to the entirety of Nop52. It is not known which of the two domains within RRP1B is important for regulation of E2F1 transcriptional activity; these two domains do not contain any homology to the other, suggesting two separate functions. One intriguing possibility is that RRP1/Nop52 may also bind to E2F1 and moreover may have a slightly different function as well.

Finally, while many proteins are known to localize in the nucleolus, it is not membrane bound; many proteins can diffuse in and out of the nucleolus in an energy-dependent or independent manner. Based on our localization data, RRP1B interacts with E2F1 inside nucleoli, but these complexes may or may not be functional; it is possible that the active complexes in transcriptional regulation lie within the nucleoplasm at punctuate sites, or diffusely within the nucleoplasm at levels below the detection limit of our assay. Nucleoli are organized around very active sites of transcription on particular chromosomes called nucleolar organizer regions; one hypothesis for how RRP1B only affects particular E2F targets may be due to proximity of particular proteins within or near to nucleoli (Maggi and Weber, 2005). To investigate this, FISH analysis using probes bound to particular E2F1 promoters showing localization at or near nucleoli may suggest that specificity derives primarily from localization, and not from variable promoter binding elements. Alternatively, a recent study showed a specific role for acetylated nucleophosmin in regulation of transcriptional activity within the nucleoplasm

(Shandilya et al., 2009). Other nucleolar proteins, including RRP1B, may also be subject to changes in localization after phosphorylation or acetylation, providing a different level of control of E2F1 apoptosis through RRP1B localization change. Determination of whether RRP1B is functionally regulated by posttranslational modification could also be of interest.

In summary, discovery of an E2F1/RRP1B genetic and physical interaction is significant advance towards understanding the specific regulation of E2F1 proapoptotic activity. A role for RRP1B in E2F1 regulation is also a novel example of a nucleolar protein which regulates cell death through transcriptional regulation.

Figure 1: Specific regulators of E2F1 apoptosis



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APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

DATE: 10/17/09

MEMORANDUM

TO: **Jason Paik**
Principal Investigator

Weei-Chin Lin
Co-Investiagor

Sheila Moore, CIP

FROM: Sheila Moore, CIP
Director, UAB OIRB

RE: Request for Determination—Human Subjects Research
IRB Protocol #N091006009– The Role of a Novel Nucleolar Protein in Regulation of E2F1 in Breast Cancers

An IRB Member has reviewed your application for Designation of Not Human Subjects Research for above referenced proposal.

The reviewer has determined that this proposal is **not** subject to FDA regulations and is **not** Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.

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