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The role of a novel nucleolar protein in regulation of E2F1 in breast cancer

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14. ABSTRACT

Breast cancer is the second most common cancer in man and accounts for significant morbidity and mortality both from disease and treatment. Identifying molecular markers for survival and sensitivity to therapy is therefore important both for prognosis as well as elucidating mechanisms associated with tumorigenesis. A recent study has identified a novel protein, RRP1B, as being associated with reduced proliferation and metastasis and improved survival in breast cancer; however, the mechanism remains unclear.

In this study, we identify RRP1B in a screen for specific targets of the DNA damage induced pro-apoptotic transcription factor E2F1. RRP1B is induced specifically by E2F1, but not the other non-apoptotic E2Fs, in a direct manner by promoter binding. Knockdown of RRP1B levels is associated with reduced sensitivity to DNA-damaging chemotherapeutic agents. RRP1B knockdown is associated with reduced transcription of selected other E2F1 targets, including the apoptosis effectors caspase-3 and -7. Finally, we show that RRP1B and E2F1 directly interact with each other *in vitro* and *in vivo*, and show that RRP1B and E2F1 bind selectively together on E2F1 transcriptional targets.

All together, we identify a mechanism by which RRP1B can be associated with improved survival by affecting sensitivity to chemotherapy. We identify E2F1 as an important inducer of RRP1B, and furthermore identify a mechanism by which RRP1B can affect apoptosis directly through protein-protein interactions with E2F1 to control transcription of proapoptotic targets.

15. SUBJECT TERMS
E2F, E2F1, apoptosis, breast cancer, cell cycle, RRP1B, NNP1B, nucleolus

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Introduction

We would like to update the grant program with a progress report for the second year of the PI's training grant in breast cancer research. Over the course of the past year, the PI in conjunction with his mentor, has made significant progress in investigating the role of a novel nucleolar protein in cancer.

We would like to summarize our previous research results in brief: through protein-protein interaction screening assays, we determined that a novel nucleolar protein, which we provisionally named EONR (E2F One Nucleolar Repressor) as an interacting protein with the important cell cycle and apoptosis transcription factor E2F1. This interaction was confirmed by though *in vitro* and *in vivo* immunoprecipitation (IP) assays in which E2F1 and EONR are overexpressed and in which E2F1 and EONR are expressed endogenously. We also determined by IP that EONR and E2F1, but not E2F2-4 could interact with E2F1. This is important since E2F1 specifically amongst the E2F family members has a role in the induction of apoptosis. We hypothesized that EONR regulates E2F1 to control apoptosis, which was tested in apoptosis assays where EONR was knocked down by lentiviruses expressing EONR specific siRNA. In preliminary studies, EONR knockdown was associated with decreased apoptosis as induced by DNA damaging agents, suggesting EONR positively regulates E2F1 apoptosis. This was further supported by measurement of E2F1-induced transcripts in RTPCR assays: EONR knockdown was associated with knockdown of specific E2F1 targets important for apoptosis and cell cycle regulation.

Body

We would like to first discuss progress in the training program. The PI has enrolled in a new cancer biology journal club offered by the sponsoring institution (Task 3b). He also continues to be enrolled and in attendance at various cancer biology associated seminars, many of which focus on breast cancer (Task 3a). The PI has also attended the 2008 Era of Hope Breast Cancer Research Conference, and has presented an abstract including results from studies described in this progress report (**Appendix).** The PI will not be attending the AACR annual meeting due to cost considerations and timeline to graduation (Task 3c). The PI will also not be initiating training in animal studies, but will initiate training in chromatin biology in the upcoming year (Task 1b & 1c).

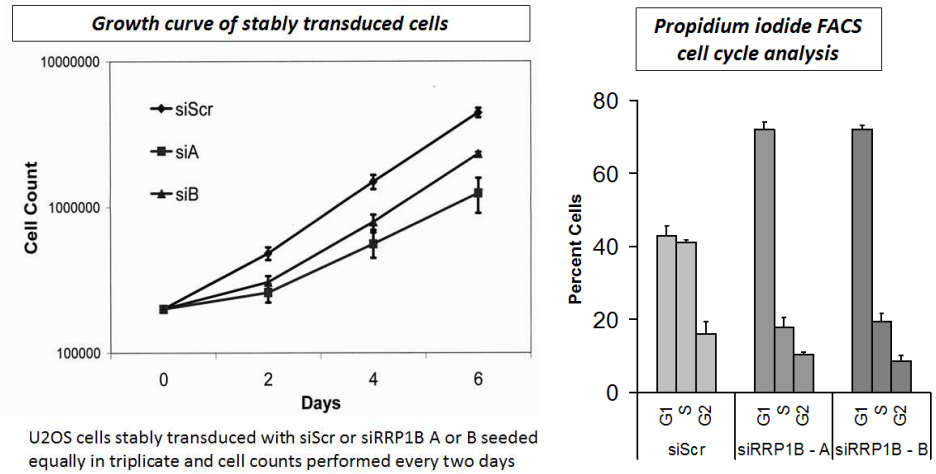
We would now like to discuss progress in the research program. To preface, in the past year, another group has discovered a role for our EONR in breast cancer metastasis [1]. The alternative name of this protein is **"RRP1B"** (ribosomal RNA processing 1 homolog) – all subsequent mentions of EONR will now be replaced by RRP1B in this report. In this recent report however, there was not any discussion relating RRP1B to apoptosis or E2F1, nor was there any mechanism described for how RRP1B controls metastasis or proliferation.

Task 1. Characterize the role and regulation of EONR on E2F1-mediated cell cycle progression, apoptosis, and sensitization to DNA damage in breast cancers. (Months 1-36)

d. Assay the effect of knockdown of EONR in MCF-7 cells on cell cycle progression to elucidate the physiological role of EONR in regulation of the cell cycle, as seen by BrdU incorporation and measurement of DNA content by propidium iodide staining. (Months 6-9)

We tested the effect of knockdown of RRP1B in U2OS cells on cell cycle progression, using lentivirally-transduced siRRP1B. We first tested for cellular proliferation; equal numbers of siScr control transduced or two different siRRP1B transduced cells were plated in triplicate, then incubated. Every two days, cells were trypsinized and cell counts were obtained using a hemocytometer. As can been seen in Figure 1-1, siRRP1B A and B cells both had decreased cellular proliferation vis-à-vis the control siScr cells. Given this data, we also determined the cell cycle profile status of siRRP1B A and B cells versus siScr control cells. As can be seen in Figure 1-1, both siRRP1B A and B cells had a increase in the fraction of cells in G1 versus the control siScr cells. This indicates that RRP1B loss leads to a cell cycle checkpoint at G1.

Figure 1-1:

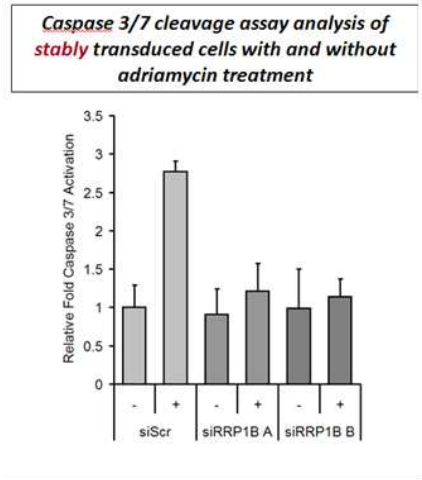


U2OS cells stably transduced with siScr or siRRP1B A or B seeded equally in triplicate and cell counts performed every two days

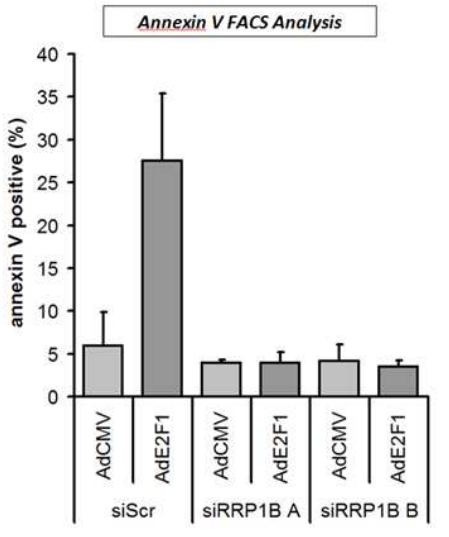
e, Assay the effect of knockdown of EONR on apoptosis in MCF-7 cells to elucidate the physiological role of EONR in regulation of apoptosis, as seen by surface annexin V and 7-AAD staining. Investigate the dependency of apoptosis induced by EONR knockdown on E2F1 by expression of siRNAs against E2F1. Also express siRNAs against E2F2 and E2F3 as a negative control. (Months 6-9)

Continuing from the previous annual report, we again used lentivirally transduced U2OS cells to determine the effect of DNA damaging agents on cells with either knockdown of RRP1B or control cells. Previous preliminary data suggested that RRP1B was able to reduce adriamycin induced apoptosis. In figure 1-2, we show that in a caspase cleavage assay (Promega, Madison, WI), a measure of caspase activation and apoptosis, RRP1B knockdown reduced the effect of the apoptosis inducing agent adriamycin to induce caspase activation. Because RRP1B is known to be involved in E2F1 activities through direct interaction and by regulation of E2F1 apoptotic targets, we tested the effect of RRP1B knockdown on apoptosis induced by E2F1 expression through adenoviruses following serum starvation, a classic test for E2F1-induced apoptosis [2]. Apoptosis was tested by analyzing annexin V surface markers followed by flow cytometry. As can be seen in figure 1-2, both siRRP1B viruses were able to repress the ability of E2F1 to induce annexin V surface positivity to almost nil. We also show knockdown of RRP1B by QPCR analysis of RRP1B transcripts. Taken together, these results indicate that RRP1B is a positive regulator of both DNA damage induced apoptosis and E2F1-induced apoptosis.

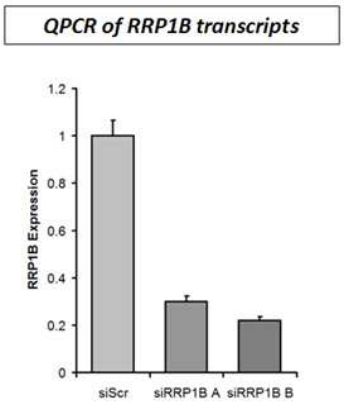
Figure 1-2



U2OS cells stably transduced with RRP1B siRNA, followed by adr tx 2uM, 8hr



Cells were starved for 48hr, then infected with 200 MOI of either CMV adenovirus (empty) or E2F1 adenovirus for 36hr, then analyzed by FACS for surface Annexin V staining by a standard protocol. Cells were washed, staining with FITC-conjugated anti-Annexin V antibodies, and then sorted by FACS for fluorescence levels. Experiments were done in triplicate.



Transduced cells were lysed, RNA was extracted, and QPCR was performed for RRP1B transcripts; expression is shown relative to GAPDH expression levels.

f. In collaboration with Donald Buchsbaum (UAB), assay the effect of knockdown of EONR on growth of MCF-7 xenografts in a nude mouse model. Assay the effectiveness of a chemotherapeutic DNA damaging agent after knockdown of EONR. (Months 18-36)

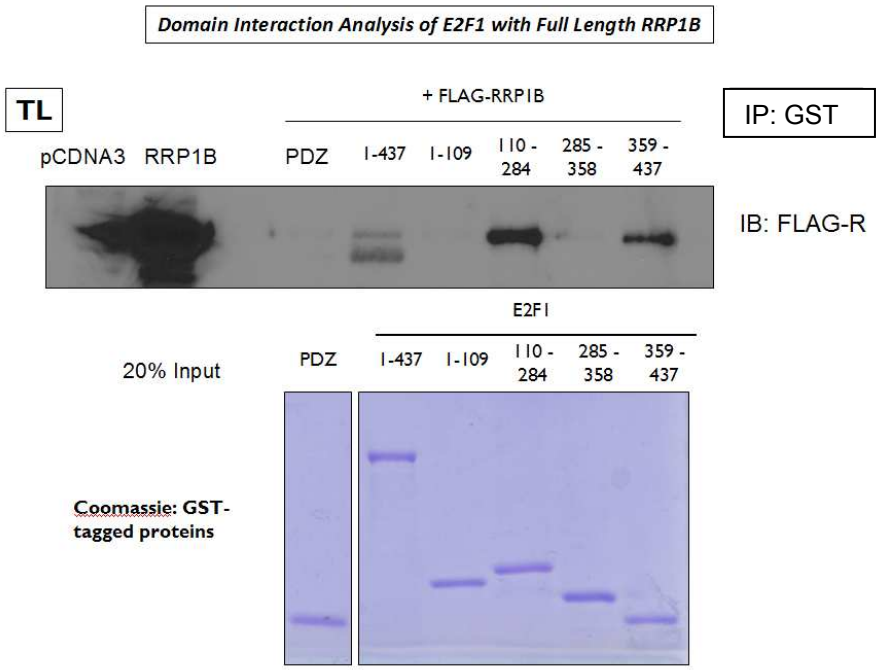
We have abandoned this subaim. Animal experiments would be very useful to show the role of RRP1B in vivo, but it is not believed that it is essential for publication of this work; we also believe that further work on the biochemistry of RRP1B provide the most information at this time prior to publication and graduation for the PI.

Task 2. Characterize the mechanism by which EONR regulates E2F1. (Months 1-36)

a. Perform *in vitro* interaction studies between full length and C-terminal deletion constructs of EONR and full length and N-terminal deletion constructs of E2F1 to identify direct interaction and the minimum interacting domains. Determine *in vitro* whether E2F1 S31D binds to EONR. (Months 1-6)

The previous annual report showed the minimal domains necessary for interaction of RRP1B with E2F1, which showed that two domains, the N terminus and C terminus of RRP1B, could interact independently with E2F1. We now show the minimal domains necessary for E2F1 to interact with RRP1B. We struggled to produce RRP1B proteins in vitro, so instead we produced RRP1B by overexpression in HEK293T cells, then incubated the lysate with in vitro produced E2F1 or E2F1 domain mutants. We were able to show in Figure 2-1 that the DNA binding domain (aa 110-284) and the extreme C-terminal Rb interaction/dimerization domains (aa 359-437) were able to interact independently with RRP1B. We hypothesize that either the N terminus of RRP1B can interact with either the E2F1 DNA binding domain or the Rb dimerization domain, but not the other – further experiments are in progress to determine which RRP1B domain binds to which E2F1 domain by using similar precipitation experiments as in Figure 2-1.

Figure 2-1

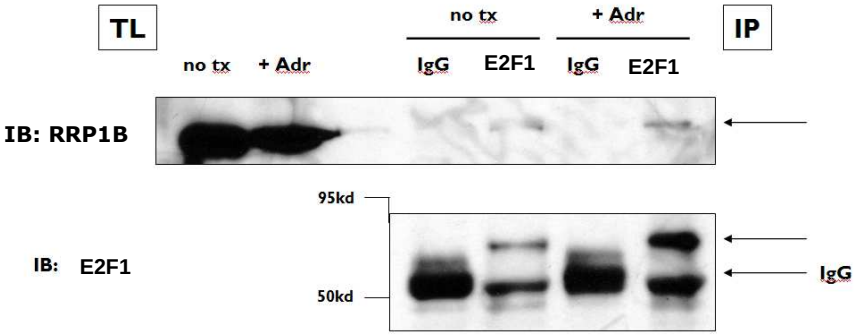


HEK293T cells were transfected by calcium phosphate method with either empty vector or FLAG-tagged RRP1B vector, incubated for 48hr, then lysed and incubated with either full length E2F1 produced *in vitro* and purified via in a glutathione-S-transferase system (Stratagene), or E2F1 domain mutants with the indicated amino acid domains. Incubation was followed by 4 washes and immunoblotting. PDZ is a nonspecific irrelevant protein used as a negative control. Bottom panel shows Coomassie stain to show equality of addition of in vitro produced E2F1 or E2F1 domain mutants.

b. Determine whether induction of DNA damage is able to repress *in vitro* interaction between purified EONR and E2F1 from cells treated with a DNA damaging agent. (Months 6-9)

Though we abandoned this subaim in a previous report, we decided to test the in vivo interaction between RRP1B and E2F1 and whether that interaction could be perturbed by a DNA damaging agent. As shown in Figure 2-2, interaction was increased following treatment with adriamycin, but this was also associated with a concomitant increase in E2F1 levels as well, which is well known [3]. As a result, we do not believe that DNA damage either increases or decreases the ability of RRP1B to interact with E2F1.

Figure 2-1



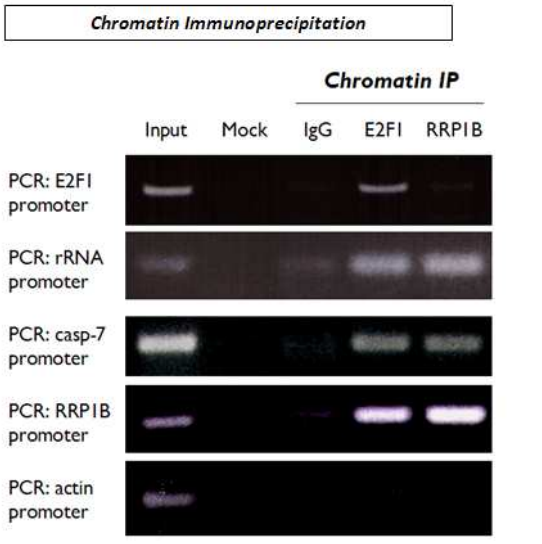
Endogenous Immunoprecipitation

HCT116 cells were nuclear extracted, sonicated, precleared, and incubated with 4ug of the indicated antibodies overnight followed by incubation with protein A+G beads for 3hr, followed by 6 washes with intervening nutation. Beads were immunoblotted and probed with the indicated antibodies.

c. Perform chromatin immunoprecipitation (ChIP) assay to investigate the presence of EONR on E2F1 responsive promoters. Examine the effect of treatment with a DNA damaging agent on binding to the promoter. (Months 12-18)

We further investigated the role of RRP1B to bind to specific E2F1 promoters. The previous annual report described the presence of RRP1B on one specific promoter – we now expand our view to other promoters. In Figure 2-3, we show that not all E2F1 responsive promoters are bound by RRP1B. Chromatin immunoprecipitation experiments now show that RRP1B is able to bind to caspase-7 promoter, the rRNA promoter, but not the E2F1 promoter, even when E2F1 itself is bound to that promoter. The beta-actin promoter does not bind either E2F1 or RRP1B as a negative control. Caspase-7 plays an important role in E2F1 induced apoptosis and its expression is knocked down after RRP1B knockdown (see previous annual report.

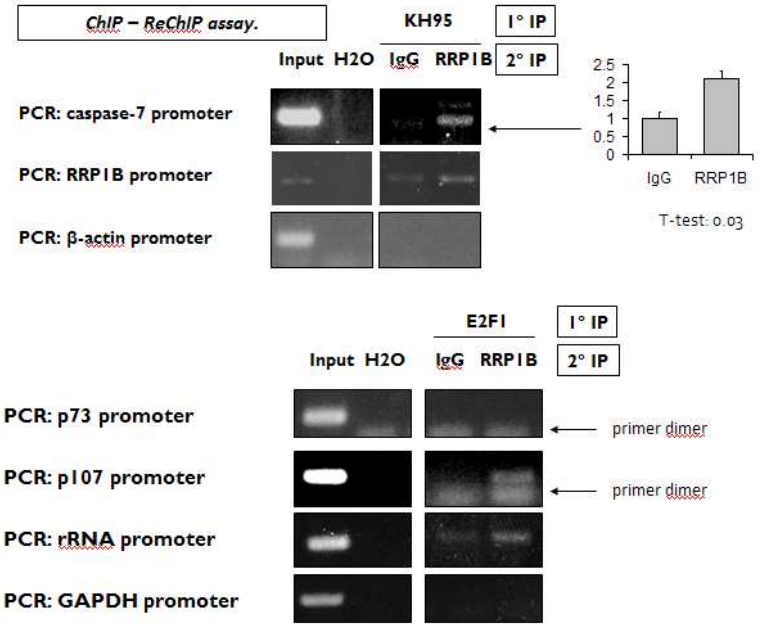
Figure 2-3



U2OS cells, crosslinked, nuclear extracted, sonicated, lysed, and immunoprecipitated with **5ug** indicated antibodies

ChIP assay

U2OS cells were crosslinked, nuclear extracted, sonicated, precleared twice, and incubated with 4ug of the indicated antibodies overnight followed by incubation with protein A+G beads for 3hr, followed by 5 washes. Beads were decrosslinked, incubated with RNase A and proteinase K, and phenol/chloroform extracted, and reconstituted in 50ul H2O. 2ul was used for each lane.



ChIP ReChIP assay

U2OS cells were crosslinked, nuclear extracted, sonicated, precleared twice, and incubated with 4ug of the indicated antibodies overnight followed by incubation with protein A+G beads for 3hr, followed by 5 washes. Beads were then incubated with 1uM DTT for 30min, followed by second IP with 4ug of indicated antibodies O/N and addition of protein A+G beads for 3hr, followed by washes. Beads were decrosslinked, incubated with RNase A and proteinase K, and phenol/chloroform extracted, and reconstituted in 50ul H2O. 2ul was used for each lane.

We further investigated the copresence *of RRP1B and E2F1 together on the same promoter by using the ChIP-ReChIP assay, a double immunopreciptation for chromatin containing BOTH E2F1 and RRP1B (Figure 2-3). We used KH95 as the first E2F1 immunoprecipitating antibody, then used our lab-produced RRP1B antibody as the second immunoprecipitator. We show that RRP1B and E2F1 were together on the same place in the caspase-7, RRP1B, p107, and rRNA promoters, but notably not the p73 promoter, whose expression is not affected by knockdown of RRP1B (see previous annual report). The caspase-7 promoter was subjected to quantitative PCR; RRP1 promoter was bound together with E2F1 to the caspase-7 promoter about 2 fold above background. Because background is high, we are continuing to tweak these assays to obtain data to better support our hypothesis.*

Taken together this data suggests that RRP1B binds to E2F1 together on the same promoter, which is consistent with the direct protein-protein interaction seen between E2F1 and RRP1B in coimmunoprecipiation assays. Furthermore, not all promoters are observed to have RRP1B bound – the ones that are bound have their expression knocked down upon RRP1B knockdown in previously shown RTPCR assays, and the ones that are not bound by RRP1B have expression unaffected by RRP1B knockdown. This finding shows that RRP1B may place a potential role in transcriptional regulation previously undescribed and highly unusual as a nucleolar protein.

d. Determine recruitment of chromatin modifying proteins in collaboration with Hengbin Wang (UAB). (Months 18-36)

This subaim has not yet been initiated, pending further solidifying evidence for the copresence of RRP1B and E2F1 on a subset of E2F1 promoters.

Key Research Accomplishments:

- RRP1B knockdown was tied to reduced proliferation and cell cycle arrest at G1.
- RRP1B knockdown led to the repression of both DNA damage-induced caspase activation and E2F1-induced apoptosis.
- E2F1 interacts with RRP1B through multiple independent domains.
- RRP1B binds to E2F1 together on specific E2F1-responsive promoters.

Reportable Outcomes:

- Abstract submitted for 2008 Era of Hope conference.

Conclusion:

Over the past year, we have made significant progress in determining the role of RRP1B on E2F1 regulation. Data has solidified the role of RRP1B in cellular proliferation and in DNA-damage induced and E2F1-induced apoptosis. Furthermore we have discovered a specific role for RRP1B to interact with E2F1 on specific promoters for regulation of transcriptional activity. Further experiments will determine a mechanism for how RRP1B regulates E2F1 for regulation of apoptotic and proliferative functions.

Appendix:

Abstract: CDMRP Era of Hope 2008 Conference

https://cdmrpcures.org/ocs/index.php/eoh/eoh2008/paper/view/1090

RRP1B IS A SPECIFIC E2F1 TRANSCRIPTIONAL TARGET

BC060015

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E2F1 belongs to the E2F family of transcription factors important for control of cellular proliferation. Dysregulation of E2F is an event in most, if not all cancers. Interestingly, while E2F1 plays an important role in proliferation, E2F1 can also induce apoptosis, an apparently paradoxical effect. Induction of specific E2F1 targets in response to specific stimuli thus may determine whether E2F1 promotes proliferation or induces apoptosis. Our laboratory is interested in the molecular mechanisms by which a cell responds to DNA damage. E2F1 plays an important role in the response to genotoxic stimuli; DNA damage activates and stabilizes E2F1 and induces transcription of proapoptotic E2F responsive targets, such as p14ARF, p73, Apaf-1, caspase-3, and caspase-7.

To further study the role of E2F1 in control of apoptosis, our laboratory screened for genes that are specifically induced by E2F1 from previously published microarray data. We identified RRP1B as a novel and specific transcriptional target of E2F1. High expression of RRP1B has recently been shown in in vitro studies to reduce the size of both primary tumors and lung metastasis of breast cancer cell lines. 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. 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. Together, this data suggests that RRP1B is an E2F1 specific target that can regulate tumor progression. Future studies will determine the effect and mechanism of RRP1B in regulation of E2F1-induced apoptosis.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-06-1-0708.

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