Genome-Wide Analysis of Drosophila RBf2 Protein Highlights the Diversity of RB Family Targets and Possible Role in Regulation of Ribosome Biosynthesis

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ABSTRACT RBf2 is a recently evolved retinoblastoma family member in Drosophila that differs from RBf1, especially in the C-terminus. To investigate whether the unique features of RBf2 contribute to diverse roles in gene regulation, we performed chromatin immunoprecipitation sequencing for both RBf2 and RBf1 in embryos. A previous model for RB–E2F interactions suggested that RBf1 binds dE2F1 or dE2F2, whereas RBf2 is restricted to binding to dE2F2; however, we found that RBf2 targets approximately twice as many genes as RBf1. Highly enriched among the RBf2 targets were ribosomal protein genes. We tested the functional significance of this finding by assessing RBf activity on ribosomal protein promoters and the endogenous genes. RBf1 and RBf2 significantly repressed expression of some ribosomal protein genes, although not all bound genes showed transcriptional effects. Interestingly, many ribosomal protein genes are similarly targeted in human cells, indicating that these interactions may be relevant for control of ribosome biosynthesis and growth. We carried out bioinformatic analysis to investigate the basis for differential targeting by these two proteins and found that RBf2-specific promoters have distinct sequence motifs, suggesting unique targeting mechanisms. Association of RBf2 with these promoters appears to be independent of dE2F2/dDP, although promoters bound by both RBf1 and RBf2 require dE2F2/dDP. The presence of unique RBf2 targets suggest that evolutionary appearance of this corepressor represents the acquisition of potentially novel roles in gene regulation for the RB family.

KEYWORDS retinoblastoma RBf1 RBf2 Drosophila ribosomal protein gene

Retinoblastoma (RB) tumor suppressor proteins, including vertebrate RB, p130, and p107, are important regulators of the cell cycle, apoptosis, differentiation, genomic stability, and metabolism (Weinberg 1995; Dyson 1998; Norton et al. 1998; Fan and Steer 1999; Zheng and Lee 2002; Hernando et al. 2004; Giacinti and Giordano 2006; Nicolay et al. 2013; Reynolds et al. 2014; and references therein). These proteins function as transcriptional corepressors that bind to E2F and DP proteins and control transcription of a diverse set of target genes, in many cases in a cell cycle—dependent manner (reviewed in Weinberg 1995; Classon and Harlow 2002; Du and Pogoriler 2006; Van den Heuvel and Dyson 2008; and references therein). The Drosophila retinoblastoma family members RBf1 and RBf2 are structurally similar to the vertebrate proteins and possess functionally conserved activities in control of cell cycle and developmental genes (reviewed in Du and Pogoriler 2006). The RB–E2F pathway is conserved in most eukaryotic lineages, especially in multicellular organisms (Cao et al. 2010). Most arthropod genomes encode a single RB gene, which is easily distinguishable by conserved sequences encoding the core “pocket domain” essential for E2F interaction. Interestingly, the genus Drosophila contains an additional retinoblastoma family member, RBf2 (Stevaux et al.
The RB2 protein possesses a conserved pocket domain, similar to that of RB1. It also contains a distinct C-terminus that lacks the conserved instability element, which has been shown to control both stability and activity of RB1 (Acharya et al. 2010; Raj et al. 2012). Both RB1 and RB2 mediate transcriptional repression; however, these proteins have different inherent ability to interact with E2F proteins; RB1 has been found to functionally interact with both the activator dE2F1 as well as the repressor dE2F2, whereas RB2 is found to interact with dE2F2 but not dE2F1 (Frolov et al. 2001; Stevaux et al. 2002). Cell-based in vitro assays suggested RB1 acts as a strong repressor of dE2F1 targets. By contrast, the action of RB2 appears to be weaker and requires coexpression of dE2F2 for maximal repression (Stevaux et al. 2002).

RB1 and RB2 are coexpressed at many points in development, but there are important differences. In contrast to the relatively stable expression of RB1 during embryonic development, the RB2 protein levels vary considerably, with a peak at early stages (Stevaux et al. 2002; Keller et al. 2005). In contrast to broadly overlapping patterns early in embryogenesis, the proteins show tissue-specific expression in the developing central nervous system. The RB1 and RB2 proteins are coexpressed in larval imaginal discs, but RB1 is the main family member expressed in adults with the exception of the ovary, where RB2 is also expressed at high levels (Stevaux et al. 2002; Keller et al. 2005). Consistent with its expression profile, RB2 was found to repress differentiation markers in embryos and ovaries. Although unlike RB1 mutants, RB2 null flies are viable, RB2 mutant females laid eggs at a fourfold greater rate than wild-type individuals (Stevaux et al. 2005). Interestingly, this phenotype was not seen in de2f2 mutant flies (Stevaux et al. 2005), although dE2F2 has been suggested to be the mediator of RB2 interactions with DNA (Stevaux et al. 2002).

The genome binding profile of RB1 has been characterized in both Drosophila embryos and larvae, and both studies revealed that RB1 interacts with numerous genes related to cellular signaling pathways, in addition to previously characterized cell cycle genes (Acharya et al. 2012; Korenjak et al. 2012). Although the genome-wide binding of RB2 has not been reported previously, chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR) of individual target genes has revealed that RB2 is present at RB1-bound loci, suggesting that these proteins may regularly co-occupy promoter regions (Korenjak et al. 2012). Considering the evolutionary conservation within the genus Drosophila of RB2 and the pervasive co-occupancy of RB1 and RB2, the modest phenotype of RB2 mutants presents a conundrum regarding the selection pressure for this gene over large evolutionary periods within Drosophila.

Previous studies suggested that RB1 and RB2 targeting is mediated via dE2F/dDP (Stevaux et al. 2002). Biochemical as well as genetic information supports this view; the larval lethality phenotype of RB1 inactivation can be rescued by a mutation in dE2F1 that disrupts this protein’s activation domain (Du 2000). In the larva, a dDP-null mutation abolishes the genome-wide association of RB1, as well as the association of RB2 to several tested target genes (Korenjak et al. 2012). In contrast, the mammalian RB protein does not interact exclusively with E2F family proteins but also physically and functionally interacts with diverse transcription factors and regulatory proteins (as reviewed in Classon and Dyson 2001; Morris and Dyson 2001; Chinnam and Goodrich 2011), as well as components of the RNA polymerase I and III basal transcription machinery (Cavanaugh et al. 1995; Larminie et al. 1997; White 1997; Hirsch et al. 2000, 2004; Gijenoda and Henry 2013). RB proteins in flies, worms, and vertebrates frequently are complexed with additional promoter-associated regulatory factors, including components of the evolutionarily conserved (dREAM) complex, which has been shown to regulate developmental gene expression (Korenjak et al. 2004; Lewis et al. 2004). In Drosophila, a majority of the RB1-bound regions also are occupied by one or more proteins of this multiprotein complex (Acharya et al. 2012). Genetically, the dREAM complex functions not only as a repressor but also appears to recruit insulator proteins to block enhancer activity on divergently transcribed genes (Bohla et al. 2014; Korenjak et al. 2014).

In mammals, individual RB family proteins have distinct molecular targets. This targeting is influenced by structural differences in the RB proteins, particularly in the C-terminus, which allow them to bind preferentially to distinct E2F factors (Rubin et al. 2003; Julian et al. 2008; Cecchini and Dick 2011; Dick and Rubin 2013). In Drosophila, the C-terminus of RB2 is structurally divergent from that of RB1, which affects the regulation of this protein, and potentially influences promoter targeting (N. Raj and R.W. Henry, unpublished data). To determine how this structurally divergent protein interacts with genomic targets, we carried out parallel chromatin immunoprecipitation sequencing (ChIP-seq) analysis of RB1 and RB2 in developing embryos, followed by bioinformatic and functional analysis of target genes. Here, we discuss how distinct genome-wide interactions of RB2 point to possible diversification in functions for these RB proteins. Ribosomal protein genes are one class not previously considered as RB targets, pointing to a potentially important role in growth control as well as cell cycle regulation. Analysis of newly identified RB1 targets suggest that the canonical RB-E2F model may not describe the full spectrum of interactions found for the derived RB2 protein.

MATERIALS AND METHODS

Chromatin immunoprecipitation followed by lambda exonuclease (ChIP-exo)

ChIP-exo was conducted using 12- to 18-hr-old yw Drosophila melanogaster embryos (strain yw2o) collected and aged at room temperature. Fixing and chromatin preparation was conducted as described previously (Acharya et al. 2012). Immunoprecipitations and sequencing were carried out by Peconic LLC (State College, PA), using highly specific polyclonal rabbit anti-RB1 (226.5) or anti-RB2 (4.7) serum raised against C-terminus of RB1 or RB2 as described (Keller et al. 2005).

Read mapping, peaking finding, visualization, and annotation

We obtained 13,453,984 reads for RB1 ChIP-exo and 12,596,328 reads for RB2 ChIP-exo. Read mapping was conducted by Peconic LLC. using Drosophila melanogaster genome version R5/dm3. To identify RB1 and RB2 bound regions, assign these peaks to nearest genes, and classify these peaks to specific genomic regions, Hypergeometric Optimization of Motif EnRichment (HOMER) v3.12 software (http://homer.salk.edu/homer/) was used, with default settings for peak calling and annotation (Heinz et al. 2010). The peaks were visualized using IGV browser v2.2.5. We identified 2356 peaks for RB1 ChIP-exo, which were mapped to 1955 genes, and 4708 peaks for RB2 ChIP-exo, which were mapped to 3945 genes. The RB1 and RB2 peaks are shown in Supporting Information, File S1, and associated annotations in File S2. To compare with dE2F1 and dE2F2 targets, peak information from dE2F1 and dE2F2 ChIP-chip in Drosophila melanogaster larvae (Korenjak et al. 2012) was annotated with HOMER. To compare RB1 and RB2 peaks with BEAF-32 peaks, raw bed file data for binding of BEAF-32 protein in 0- to 8-hr-old Drosophila melanogaster embryos was obtained from Yang et al. (2012) and peaks were calculated by HOMER using default settings. To
compare RBf1 and RBf2 targets with human RB targets, human RB and p130 ChIP-seq peaks (Chicas et al. 2010) were annotated using HOMER with the hg18 genome, and their association with human ribosomal protein gene promoters was inspected manually by browsing the peak-calling files in IGV browser with hg18 genome. To analyze the association of Caenorhabditis elegans RB homolog Lin-35 with ribosomal protein gene promoters, peak-calling file for Lin-35 (Latorre et al. 2015) was visualized in IGV browser with WS220 genome, and the ribosomal protein gene promoters were manually inspected for Lin-35 binding. To compare overlapping peaks between different data sets, HOMER was used with overlapping threshold set at 100 bp.

**De novo motif searching**

To identify motifs associated with RBf1 and RBf2 targets indicated in Figure S4, the sequences of RB1 and RB2 binding regions that associate with TSS/promoter (by HOMER default, -1 kb to +100 bp) were extracted from Drosophila genome R5/dm3 on the UCSC Genome Browser and subjected to *de novo* motif searching using MEME-ChIP with default settings (Machanick and Bailey 2011).

**Validation of ChIP-exo peaks**

To validate the enrichment of RB1 and RB2 on their canonical and noncanonical target genes, several genes were selected and association with RBf proteins tested by ChIP-qPCR. Then, 12- to 18-hr *yw* Drosophila melanogaster embryos were used to prepare chromatin for the immunoprecipitation, and three biological replicates were conducted as previously described (Acharya et al. 2012). Preimmune sera RBf1-2260 and RBf2-4.0 were used for negative controls (Keller et al. 2005). Oligonucleotides used for qPCR are listed in File S7. To directly compare RB1, RB2 targets with de2F1, de2F2, and dDP at the same developmental stage, ChIP-qPCR analysis was performed using the RB1, RB2 antibodies and preimmune sera as described above, along with de2F1, de2F2, and dDP antibodies (gifts from Dr. Nicholas Dyson lab), in 12- to 18-hr embryos.

**Gene ontology (GO) analysis**

RB1 and RB2 associated genes identified using HOMER were subjected to GO analysis. The enrichment of GO terms was performed using the online tool DAVID (Huang et al. 2009a,b) with KEGG_PATHWAY and SP_PIR_KEYWORDS. Eleven annotation clusters were identified for RBf1 and RBf2 targets, three were identified for RBf1-only targets, and 17 were identified for RBf2-only targets. The enrichment scores of the top five annotation clusters for RBf1 and RBf2 targets, and RBf2-only targets were plotted as shown in Figure 2A. The automated gene assignments by HOMER can arbitrarily assign peaks to one of two divergently transcribed genes, although the distance of RBf peak to the more distal TSS may be close enough to be functionally important. Therefore, to identify all genes that may be likely transcriptional targets of the RBf proteins, and to calculate the percentage of genes bound by RBf1 or RBf2 in different functional groups, RBf1 and RBf2 binding regions were manually inspected in the promoter regions of 81 selected cell-cycle genes, 394 signaling pathway genes (Acharya et al. 2012), 94 cytoplasmic ribosomal protein (CRP) genes, and 75 mitochondrial ribosomal protein (MRP) genes (Marygold et al. 2007). A few additional genes were therefore added to the dataset of RBf1 or RBf2 potential targets from HOMER; the manually inspected results are shown in File S3.

**Reporter constructs and luciferase assay**

To analyze RBf1 and RBf2 activity on ribosome protein gene promoters, promoter regions of *RpL37A* -788 to +132, *RpS29* -369 to +60, *mRpS12* -1074 to +155, and *mRpL22* -478 to +79, and 2401 to 1544 were extracted from Drosophila genome R5/dm3 on the UCSC Genome Browser and subjected to *de novo* motif searching using MEME-ChIP with default settings (Machanick and Bailey 2011).

**RNA interference and ChIP**

Double-stranded RNA (dsRNA) for *lacZ*, *de2F1*, *de2F2*, and *dDP*, and BEAF-32 were prepared as described previously (Ullah and Buckley 2007) using primers listed in File S7. A total of 40 million Drosophila Kc cells were treated with dsRNA at concentration of 10 μg/mL for 4 d. ChIP from Kc cells was performed as described (Hirsch et al. 2004). For qPCR analysis shown in Figure 4C, 1 million Drosophila S2 cells were treated with dsRNA for *lacZ*, RBf1, RBf2, RBf1+RBf2, and RBf2+de2F2 at a concentration of 10 μg/mL for 4 d. Total RNA was isolated using TRIzol (Invitrogen), cDNA was prepared using ABI High Capacity cDNA RT Kit (Life Technologies) following the manual with 2 μg of total RNA. Primers for real-time PCR analysis are listed in File S7.

**RNA-seq**

The UAS-RBf1 fly line was constructed as previously described (Zhang et al. 2014), *P Wendelina-Gal4* driver line (Stock Number: 113920) and UAS-GFP line (Stock Numbers: 35786) were obtained from Bloomington Stock Center. Then, 100—150 wing imaginal discs were dissected from third-instar larvae of *PenGal4 > UAS RBf1* and *PenGal4 > UAS GFP* flies. Total RNA was isolated using TRIzol (Invitrogen) followed by cleanup steps using RNEasy Mini kit (QIAGEN).
Then, 1–4 μg of total RNA from three biological replicates was collected. Library preparation and sequencing was conducted by the Research Technology Support Facility (Michigan State University) using an Illumina HiSeq2500. All standard libraries were created using Illumina TruSeq kits and reagents following the manufacturer’s protocols. In brief, polyA mRNA was isolated from total RNA, chemically fragmented, and then reverse transcribed to form double-stranded cDNA. The cDNA was then end repaired, A-tailed, adapter ligated, and amplified, and then reverse transcribed to form double-stranded cDNA.

In brief, polyA mRNA was isolated from total RNA, chemically fragmented, and then reverse transcribed to form double-stranded cDNA. The cDNA was then end repaired, A-tailed, adapter ligated, and amplified to create the final library. A bead-based size selection was performed to target final library molecules with a mean size of 500 base pairs. All libraries were then quantified on a Qubit Fluorometer (Life Technologies) and run on an Agilent BioAnalyzer to determine final size and purity of the library. Final concentration was then determined by qPCR using the KAPA Illumina Library Quantification Kit (KAPABiosystems). Libraries were appropriately diluted and loaded onto the flow cell for sequencing on the Illumina HiSeq2500 following the manufacturer’s protocols. RNA-seq reads were mapped using TopHat v2.0.13 and analyzed using Cufflinks v2.2.1 (Trapnell et al. 2012). Analyzed results were visualized using R v2.15.3 with CummeRbund package as described (Trapnell et al. 2012).

**Data set preparation for STAP analysis**

For all (15,829) D. melanogaster genes, their locations and DNA sequences from 500 bp upstream to transcription start site (TSS) were retrieved from Flybase and UCSC database (dmel-5.48 Flybase release). For the four functional groups: CRP genes, MRP genes, cell-cycle genes, and signaling pathway genes, the same data were extracted and processed separately. The quantitative ChIP enrichments were calculated from the .wiggle files computed by MACS v1.4.2 (Zhang et al., 2008) by taking a maximum average signal over a sliding window within the 500bp upstream of the TSS both for RBf1 and RBf2 ChIP experiments. Position weight matrices of 127 motifs of transcription factors binding sites (TFBS) compiled from literature were used as motif information and provided in File S8.

**Testing for motif association with ChIP enrichment**

The STAP program was used to test which TFBS are associated with ChIP enrichment for the DNA sequences upstream of the TSS (He et al. 2009). For individual motif analysis, STAP was run with default parameters (sequence file, data file, and motif file) with the option of co-operative binding set to 0 for each of the 127 motifs. The Pearson correlation between predicted binding and observed binding scores. (B) Promoters of cell cycle–related genes (annotated by flybase.org, The Interactive Fly), signaling pathway genes (Acharya et al. 2012), cytoplasmic ribosomal protein genes, and mitochondria ribosomal protein genes (Marygold et al. 2007) were manually inspected for RBf1 and RBf2 binding sites. Genes indicated in File S3. The signaling pathways include insulin, target of rapamycin (i.e., TOR), PI3K/Akt, S’ adenosine monophosphate-activated protein , Janus kinase/signal transducers and activators of transcription, transforming growth factor-β, Notch, Wnt, hedgehog, Hippo, nuclear factor-kB, c-Jun N-terminal protein kinases (i.e., JNK), and Ras/Egfr pathways (Acharya et al. 2012). (C) To validate ChIP-Seq results, manual chromatin immunoprecipitation of RBf1 and RBf2 on chromatin from 12- to 18-hr embryos was carried out on selected cell-cycle (PCNA, DNApoly-50), signaling pathway (InR), cytoplasmic ribosomal protein (Rpl37a, Rps29, Rps19b, Rps27, Rpl7), and mitochondrial ribosomal protein (mRpS12/tdo, mRpL22, mRpL1) targets using anti-RBf1, anti-RBf2, and pre-immune serum. An intergenic region (INT) was used as negative control.
we used ChIP-exo analysis to measure binding pro-
To identify the genomic targets of RB family proteins in
RESULTS
the threshold of one-sided p-value
Mann-Whitney U test was performed on the observed two groups with
ized primarily to promoter-spe-
that there were substantially more peaks identi-
The canonical RBf
(Frolov et al. 2001; Stevaux et al. 2002). Therefore, it was surprising that there were substantially more peaks identified for RBf2 (4708) than for RBf1 (2356); this corresponds to 3945 and 1955 genes, re-
respectively. As noted previously for RBf1, RBf2 binding also was localized primarily to promoter-specific regions (Acharya et al. 2012; Figure S1 and Figure S2).
To measure the overlap between the RBf and E2F genomic binding profiles, we compared the RBf genomic targets to those associated with dE2F1 and dE2F2 previously identified in larvae (Korenjak et al. 2012). In the larvae, dE2F2 was found to have nearly 4000 binding sites, compared with dE2F1, which only has less than 300 binding sites (Korenjak et al. 2012). We mapped the dE2F1 and dE2F2 peaks to the
nearest genes and compared with genes bound by RBf1 and RBf2. More than half of RBf1 target genes were bound by dE2F1 or dE2F2, whereas less than half of RBf2 target genes were bound by any E2F factor (Figure 1A). The discrepancy between RBf protein binding and E2F factor binding may reflect the two different developmental stages used for measuring binding, although many individual genes are sim-
larly bound in both stages (Acharya et al. 2012; Korenjak et al. 2012). To directly compare RBf and E2F targets at the same developmental stage, we conducted ChIP-qPCR analysis using RBf1, RBf2, dE2F1, dE2F2, and dDP antibodies in 12- to 18-hr embryos. We checked selected targets that were previously found bound or not bound by RBf1, RBf2 and dE2F2 (Korenjak et al. 2012, and this study) (Figure S3). We noticed weak dE2F2 and dDP bindings on some ribosomal protein gene promoters that were previously shown to be bound by RBf2, but not dE2F2 (Korenjak et al. 2012). However, these ChIP signals also were close to signals from nonspecific promoters that were unlikely to be targeted by RBf or E2F (Figure S3). Thus whether these RBf2-only targets are bound by dE2F2 or dDP needs to be determined by the global background of the dE2F2 and dDP antibodies. But it is possible that some RBf2 binding is directed by E2F-independent mechanisms, which we explore below.

RBf1 and RBf2 were found to co-occupy many genes, either through simultaneous binding to multiple transcription factors on a given promoter, or perhaps in a competitive manner (Figure 1B). A small number of promoters were bound only by RBf1 (Figure 1C), whereas others featured significant RBf2 binding and no trace of RBf1, suggest-
ing that these promoters may recruit RBf factors in a different fashion from the genes bound by both RBf1 and RBf2 (Figure 1C). Indeed, motif searches of RBf1/RBf2 peak areas compared to RBf2-only peaks showed that E2F-like sequences were enriched in those areas co-bound by RBf1/RBf2. Motifs enriched under RBf2-alone peaks did not contain E2F-like sequences, but instead contained distinct sequences (Figure S4).

RBf2 alone targets include most ribosomal protein genes
We analyzed the nature of genes bound by RBf1, RBf2, or both RBf1/ RBf2 using the DAVID gene ontology annotation database (Huang et al. 2009a,b). Consistent with the known importance of RB proteins for cell-cycle regulation, genes bound by both RBf1/RBf2 were signif-
icantly enriched for this category. In contrast, cell cycle—related genes were not enriched in the set of genes bound solely by RBf2; instead, the most significantly enriched category was that of ribosomal proteins (Figure 2A). The RBf1-only group showed no significant enrichment of any gene class in this analysis (data not shown). To further charac-
terize this enriched feature, we manually inspected RBf1 and RBf2 peaks on each of the 94 CRP gene promoters and 75 MRP gene promoters, observing that RBf2 associated with a majority of the ribo-
somal protein gene promoters (Figure 2B). We also compared our results with the previous DREAM complex ChIP-chip study (Georlette et al. 2007) and found that some, but not all, DREAM complex com-
ponents co-occupy with RBf1/RBf2 on ribosomal protein gene promoters (Figure S5). In our earlier study (Acharya et al. 2012), RBf1 was found to bind multiple genes encoding components of conserved signaling pathways. In the current study, we found that RBf2 also associates with a significant number of signaling pathway gene promoters (Figure 2B). ChIP-qPCR assays were performed on selected cell cycle, signaling pathway, and ribosomal protein targets, confirming the enrichment found in the ChIP-exo experiments (Figure 2C). Thus, RBf2 appears to occupy a greater fraction of noncanonical targets such as signaling pathway and ribosomal protein genes, compared to RBf1, which is present together with RBf2 at many canonical cell cycle—related genes.

RBf2 shows differential repression activity on ribosomal protein gene promoters
To determine the regulatory significance of RBf1 and RBf2 binding at ribosomal protein promoters, we selected several genes for further
functional characterization. Six promoter-proximal regions from cytoplasmic and mitochondrial ribosomal protein genes were cloned into a luciferase reporter, and the effects of RBf1, RBf2, dE2F2, or a combination of RBf2 plus dE2F2 were tested in *Drosophila* S2 cells. As expected, transcription from the *PCNA-luc* reporter was repressed by RBf1, dE2F2, and RBf2/dE2F2 (Figure 3). In contrast, none of the ribosomal protein gene promoters were repressed by RBf1, even though these particular promoters have robust RBf1 signals in the embryo. Notably, overexpression of RBf2 alone repressed the *mRpS12/tko* promoter ~25%, with repression increasing to ~50% with coexpression of dE2F2. Overexpression of dE2F2 alone decreased *Rpl37a* promoter activity by about one-third, with a modest but reproducible ~15–20% repression observed on *RpS29* and *mRpL22* promoters. These latter promoters were not sensitive to RBf1 or RBf2 overexpression alone. The *mRpl1* or *mRpS14b* promoters were not repressed to any extent by any of the overexpressed proteins, and in fact transcription of these reporters was mildly stimulated. Thus, unlike the classical RB cell-cycle target *PCNA*, whose expression dynamically varies during cell growth, regulation of these non-canonical ribosomal protein gene promoters is more restrained. This behavior is consistent with the similarly modest but reproducible regulation of these genes under growth-limiting or stress conditions (Gasch et al. 2000; Causton et al. 2001; Gershman et al. 2007; Miller et al. 2011). As central mediators of global protein expression, small changes in ribosomal protein expression are predicted to have significant and pleiotropic effects (Steffen et al. 2012; Xue and Barna 2012; Woolford and Baserga 2013; Hasygar and Hietakangas 2014).

**RBf1 represses ribosomal protein gene expression in vivo**

To further examine the significance of RBf1 association with ribosomal protein gene promoters, we performed RNA-seq of larval wing discs of third instar larvae under control of the Pen > Gal4 driver; three biological replicates were conducted and analyzed by RNA sequencing (RNA-seq, as described in the section Materials and Methods). Ribosomal protein gene expression increased upon RBf1 RNA interference (RNAi). S2 cells were treated with dsRNA for *lacZ* (negative control), RBf1, RBf2, RBf1+RBf2, and RBf2+dE2F2. Gene expression for selected cell-cycle genes (*PCNA* and *Mcm5*) and ribosomal protein genes was detected by quantitative reverse transcription polymerase chain reaction. Consistent with the RNA-seq data for RBf1 overexpression, *Rpl13*, *Rpl37A*, *Rpl24*, *RpS8*, *mRpl39*, and *mRpS18C* gene expressions increased upon RBf1 or RBf1+RBf2 RNAi. We also detected modest derepression of ribosomal protein genes *tko*, *Rpl37a*, *RpS29*, *mRpL22*, *mRpl1*, and *mRpS14b*, even though luciferase reporters with these promoters were not repressed by ectopic RBf1 overexpression in transfected cells (Figure 3).
in expression, with only a few showing an increase (Figure S6). Six ribosomal protein genes were significantly repressed by RBf1 in this developmental context, showing decreases of 20–35% (Figure 4A), similar to the repression observed on cell cycle genes, including PCNA, DNApolα-50, and Mcm5 (Figure 4B). Consistent with the RBf1 overexpression data, knocking-down RBf1 alone, or RBf1 with...
RBf2 in cell culture significantly increased cell cycle genes expression, and widely induced ribosomal protein genes expression (Figure 4C). Knocking-down RBf2 alone or together with de2f2 did not have much impact on the ribosomal protein genes, although some of these gene promoters were significantly repressed by RBf2/dE2F2 in vitro (Figure 3). Interestingly, among this set of ribosomal protein genes, only Rpl13 was bound by RBf1 and RBf2 in embryos and larvae (Acharya et al. 2012; Korenjak et al. 2012; and this study). We speculate that some of these genes not found to bind the corepressor in the embryo may bind RBf1 specifically in the rapidly proliferating cells of the wing disc, or alternatively, these genes may harbor lower levels of RBf1 that were not called as peaks in our analysis. Indeed, a number of these promoters contain DNA motifs such as DREF and RAM that were also enriched under RBf1 peaks, and which may be diagnostic of RBf1 function (Acharya et al. 2012).

Enrichment of BEAF-32 motifs in RBf-bound promoters

Our analyses of the RBf and E2F genomic binding profiles revealed many RBf2 target genes that were not bound by E2F factors, and therefore we tested whether there was evidence for other transcription factors associated with RBf2 bound regions on target promoters. To identify relevant motifs, we used the STAP program, which correlates ChIP signal intensity with presence of overrepresented motifs for known transcription factors (He et al. 2009). Globally, a few motifs showed strong correlation with RBf1 and RBf2 peaks, including the E2F and DREF motifs that we previously demonstrated to be enriched at RBf1 binding sites (Acharya et al. 2012) (Figure 5). Viewed as separate classes, those genes annotated as “cell cycle related,” “signaling” and “cytoplasmic ribosomal protein” also showed a strong enrichment for the E2F motif (Figure S7, Figure S8, and Figure S9). Promoters from cell cycle and ribosomal protein genes also were enriched in a variety of other motifs, presumably related to their unique regulation (Figure S7, Figure S9, and Figure S10). However, genes representing conserved signaling pathways were not strongly enriched for additional motifs, likely because the divergent promoter sequences have very diverse regulatory properties (Figure S8). Unexpectedly, we found motifs for BEAF-32, an insulator binding protein, significantly correlated with both RBf1 and especially with RBf2 peaks (Figure 5). BEAF-32 binding sites measured in Drosophila 0-8-hr old embryos significantly overlap with RBf1 and RBf2 peaks (Figure 6, A and B), with co-occupancy found for one-third of the RBf2 and just over one-quarter of RBf1 sites (Figure 6B) (Yang et al. 2012). A similar overlapping was also observed for BEAF-32 binding sites in S2 cells (Figure 6B) (Schwartz et al. 2012). Other insulator proteins, such as CP190 also co-occupy RBf2 binding sites similar to BEAF-32, whereas the overlapping between RBf2 and CTCF was less

Figure 6 Correlation between RBf2, RBf1, and BEAF-32 ChIP signals (Yang et al. 2012). (A) Heat map centered on the RBf2 peak summits and sorted by RBf2 peak scores on all genomic regions bound by RBf2. (B) Comparison of RBf2, RBf1, BEAF-32 (embryos) (Yang et al. 2012), BEAF-32 (S2 cells), CP190 (S2 cells), and CTCF (S2 cells) (Schwartz et al. 2012) binding sites. The overlap between RBf2 and BEAF-32 (embryos) was statistically significant (log(p) = −5763). The statistics for significance of other overlap between RBf sites and insulator proteins (BEAF-32, CP190, CTCF, in S2 cells) is shown in File S5. (C) Correlations between positions of binding of RBf2, RBf1, and BEAF-32 are shown in ribosomal protein promoter regions, using heat maps centered on the RBf2 peak summits, and sorted by RBf2 peak scores.
significant (Figure 6B) (Schwartz et al. 2012). Focusing specifically on ribosomal protein gene promoters, BEAF-32 binding sites were significantly enriched, especially on RBf2-bound genes (Figure 6C).

Some ribosomal protein gene promoters exhibit noncanonical RBf2 recruitment

Our discovery of genes uniquely bound by RBf2 but not RBf1, and the lack of E2F-like motifs within these promoter regions, prompted us to test whether RBf2 might be recruited to promoters by alternative factors. We tested whether RBf2 recruitment would therefore be dependent on dE2F/dDP proteins or BEAF-32 in cultured cells. We depleted de2f1, de2f2, dDP, or BEAF-32 in Drosophila Kc cells with double-stranded RNA, followed by ChIP for RBf2. (Figure 7, A and B). The knockdown was sufficient to substantially deplete endogenous gene expression levels, leading to loss of expression of cell cycle genes PCNA and Mcm5 in the cases of de2f1 and dDP knockdown (Figure 7C). We examined promoters from cell cycle genes (DNApol-50, PCNA), signaling pathway genes (InR, Thor), and ribosomal protein targets either bound by both RBf1 and RBf2 or RBf2 alone. Knockdown of BEAF-32 had no effect on RBf2 recruitment on any promoter, even those with the greatest BEAF-32 binding signals (Figure 7, A and B). Thus, RBf2 and BEAF-32 may bind to these promoters independently. By contrast, knockdown of de2f1 or dDP substantially reduced the RBf2 signal on the Rps19b, Rps29, mRpl22, mRps12/tko InR, PCNA, DNApol-50, and Rpl37a promoters (Figure 7A), consistent with the previously described RBf2-dE2F2-dDP recruitment mechanism (Stevaux et al. 2002). Significantly, for the Thor gene and eight other ribosomal gene promoters tested, the de2f2/dDP knockdown showed little to no effect on RBf2 interaction (Figure 7B). It is interesting that most of those promoters were not bound by RBf1, and a previous study also suggested they were not bound by dDP (Ambrus et al. 2013). Interestingly, on a number of promoters, we observed a modest increase of RBf2 signal upon de2f1 knockdown, possibly because of competition between dE2F2/RBf2 and dE2F1 on some RBf targets. We repeated this ChIP experiment in Drosophila S2 cells, and found that RBf2 binding on these RBf2-alone ribosomal protein gene promoters was also not affected by de2f2/dDP knockdown (data not shown). These results suggest that RBf2 interacts with some promoters via an E2F/DP-independent mechanism.

To determine whether ribosomal protein gene promoters bound preferentially by RBf2 may have different TFBS, we analyzed the occurrences and affinities of E2F-, DREF-, and FOXJ2-like motifs that previously had been shown to be enriched on RBf1 bound regions (Acharya et al. 2012). We found that promoters bound uniquely by RBf2 have lower binding scores for E2F, DREF, and FOXJ2 (Figure 8, A–C). Surveying the entire set of sites uniquely bound by RBf2 genome-wide, we found a similar lack of strong E2F sites (Figure 8D).
Association with ribosomal protein gene promoters is a conserved character for the RB family

To determine whether the widespread RBf association with ribosomal protein gene promoters represents conserved regulatory interactions, we surveyed human RB and p130 protein ChIP-seq data in protein gene promoters represents conserved regulatory interactions, To determine whether the widespread RBf association with ribosomal protein gene promoters is a conserved character for the RB family

Association with ribosomal protein gene promoters is a conserved character for the RB family

To determine whether the widespread RBf association with ribosomal protein gene promoters represents conserved regulatory interactions, we surveyed human RB and p130 protein ChIP-seq data in fibroblasts (Chicas et al. 2010), and C. elegans RB homolog protein Lin-35 ChIP-seq in larvae (Latorre et al. 2015). We inspected all human and C. elegans orthologs of Drosophila ribosomal protein genes, observing that a majority of the ribosomal protein gene promoters were bound by RB, p130, or Lin-35 (Figure 9). The high proportion of ribosomal protein genes targeted by these corepressors suggests that there may be a conserved role for these RB family proteins in regulating protein synthesis and growth.

DISCUSSION

RB gene families have undergone diversification in multiple lineages (Cao et al. 2010; Gutzat et al. 2012). In metazoans, the RB family proteins of Drosophila and vertebrates have independently diversified; in the case of flies, RB2 has substantial differences in the C-terminus, which is thought to be a key domain for regulation and binding specificity. In vertebrates, RB similarly exhibits substantial differences in the C-terminus compared with the more ancestral p107 and p130 paralogs (Classon and Dyson 2001). Thus, RB2 and RB represent evolutionary innovations, which may direct the regulation of unique sets of genes or respond to different environmental and developmental signals.

The ChIP-Seq comparison of RBf1 and RBf2 binding profiles revealed several unexpected features, given previous findings that RB2 cooccupies a number of promoters with RB1. First, there were approximately 2000 genes targeted uniquely by RBf2. This pattern either represents the neofunctionalization of RBf2 with acquisition of novel gene targets, or alternatively, many of these genes may be bound by the RBf1 homolog in sister species, with a RBf2 acquiring some of these interactions through subfunctionalization of RBf1. Comparative functional studies will help to clarify this point. The unique binding of RB2 to some promoters runs contrary to an earlier model that suggested that RB2 would only interact with a subset of the genes bound by RBf1, because RB2 was thought to bind preferentially to dE2F2, while RBf1 was more promiscuous. Our bioinformatic analysis indicates that there are indeed distinct patterns of motifs present on RBf2-only regions, including a depletion of strong E2F-like sites, suggesting that other transcription factors may direct RBf2 recruitment. In mammals, RB and p107 specificity is driven partially by differential contacts mediated by the C-terminal regulatory domains (Rubin et al. 2005; Julian et al. 2008; Cecchini and Dick 2011; Dick and Rubin 2013). Likewise, the unique C terminal domain of RBf2 may allow interactions with different types of regulators. Previous genetic experiments showed a genome-wide depletion of RBf1 binding in ddp mutant larvae, as well as loss of RBf1 from select genes. Our results are consistent with these findings, in that those specific genes tested for RBf2 association (such as InRf) are E2F-dependent genes that are also bound by RBf1. Just as mammalian RB has diversified its interactions with the genome through association with non-E2F factors, Drosophila RBf2 may have alternative binding partners whose identities remain to be determined.

Despite the widespread binding of RBf2 in the Drosophila genome, genetic analysis of RBf2 has shown that flies lacking this gene are viable, unlike the lethal phenotype of RBf1 mutants. Why is the RBf2 gene evolutionarily retained throughout the Drosophila lineage, despite the modest phenotype? The genes exhibit similar, although not identical expression patterns, suggesting that both proteins are likely to be present in many tissues. One clue comes from the adult pattern of RBf2 expression, which is concentrated in the ovary (Stevaux et al. 2002; Keller et al. 2005). Although RBf2 nulls were healthy and viable,
the selective regulation of ribosomal protein genes noted in our study, whereby only a subset of promoters was bound or regulated, is consistent with previous findings that the regulation of mRNA levels of some ribosomal protein genes is more dynamic than others, likely because other layers of regulation ensure stoichiometric production of ribosome components (Miller et al. 2011). The heterogeneous composition of activators at ribosomal promoters may contribute to this differential regulation; in mammals, the DRE motif for the DREF factor is found at many but not all ribosomal protein promoters, suggesting that common but not identical levels of regulation are probably at work (Yamashita et al. 2007). It is interesting that mammalian RB has been reported to directly regulate the activity of RNA polymerase I and III, providing a link for this cell-cycle regulatory protein to control the biosynthetic capacity of cells (Cavanaugh et al. 1995; Larminie et al. 1997; White 1997; Hirsh et al. 2000, 2004; Felton-Edkins et al. 2003; Gjidda and Henry 2013). A regulatory connection with ribosomal protein genes would ensure that all facets of ribosome production would be influenced by RB signaling. Just as misregulation of c-Myc, which plays a positive role in ribosome synthesis, is linked to cancer, this model provides a new perspective to the impact of retinoblastoma proteins in cancer, where both disturbances to cell cycle control as well as accumulation of biomass through control of ribosome genes would play critical roles in tumorigenesis (White 2004, 2005).

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LITERATURE CITED
Acharya, P., N. Raj, M. S. Buckley, L. Zhang, S. Duperon et al., 2010 Paradoxical instability-activity relationship defines a novel regulatory pathway for retinoblastoma proteins. Mol. Biol. Cell 21: 3890–3901.
Acharya, P., N. Negre, J. Johnston, Y. Wei, K. P. White et al., 2012 Evidence for autoregulation and cell signaling pathway regulation from genome-wide binding of the Drosophila retinoblastoma protein. G3 (Bethesda) 2: 1459–1472.
Ambrus, A. M., A. B. M. M. K. Islam, K. B. Holmes, N. S. Moon, N. Lopez-Bigas et al., 2013 Loss of dE2F compromises mitochondrial function. Dev. Cell 27: 438–451.
Bailey, T. L., and M. Gribskov, 1998 Methods and statistics for combining motif match scores. J. Comput. Biol. 5: 211–221.
Bohla, D., M. Herold, I. Panzer, M. K. Buxa, T. Ali et al., 2014 A functional insulator screen identifies NURF and DREAM components to be required for enhancer-blocking. PLoS ONE 9: e107765.
Cao, L., B. Peng, L. Yao, X. Zhang, K. Sun et al., 2010 The ancient function of RB-E2F pathway: insights from its evolutionary history. Biol. Direct 5: 55.

Causton, H. C., B. Ren, S. S. Koh, C. T. Harbison, E. Kanin et al., 2001 Remodeling of yeast genome expression in response to environmental changes. Mol. Biol. Cell 12: 323–337.

Cavanaugh, A. H., W. M. Hempel, L. J. Taylor, V. Rogalsky, G. Todorov et al., 1995 Activity of RNA polymerase I transcription factor UBF blocked by Rb gene product. Nature 374: 177–180.

Cecchini, M. J., and F. A. Dick, 2011 The biochemical basis of CDK phosphorylation-independent regulation of E2F1 by the retinoblastoma protein. Biochem. J. 434: 297–308.

Chicas, A., X. Wang, C. Zhang, M. McCurrach, Z. Zhao et al., 2010 Dissecting the unique role of the retinoblastoma tumor suppressor during cellular senescence. Cancer Cell 17: 376–387.

Chinnam, M., and D. W. Goodrich, 2011 RB1, development, and cancer. Curr. Top. Dev. Biol. 94: 129–169.

Classon, M., and N. Dyson, 2001 P107 and P130. Versatile Proteins With Interesting Pockets. Exp. Cell Res. 264: 135–147.

Classon, M., and E. Harlow, 2002 The retinoblastoma tumour suppressor in development and cancer. Nat. Rev. Cancer 2: 910–917.

Dick, F. A., and S. M. Rubin, 2013 Molecular mechanisms underlying RB protein function. Nat. Rev. Mol. Cell. Biol. 14: 297–306.

Drosophila E2F/Rb pathway. Genes Dev. 17: 2308–2320.

Dunlop, W., 2005 Suppression of the rbf null mutants by a de2f1 allele that lacks transactivation domain. Development 127: 367–379.

Dunlop, W., and J. Pogoriler, 2006 Retinoblastoma family genes. Oncogene 25: 5190–5200.

Dyson, N., 1998 The regulation of E2F by pRB-family proteins. Genes Dev. 12: 2245–2267.

Elenbaas, J. S., R. Mouawad, W. R. Henry, D. N. Arnosti, and S. Payankaulam, 2001 Functional antagonism between E2F family members. Genes Dev. 15: 2146–2160.

Eibenbaas, J. S., R. Mouawad, W. R. Henry, D. N. Arnosti, and S. Payankaulam, 2001 Role of Drosophila retinoblastoma protein instability element in cell growth and proliferation. Cell Cycle 14: 589–597.

Fan, G., and C. J. Steer, 1999 The role of retinoblastoma protein in apoptosis. Apoptosis 4: 21–29.

Felton-Edkins, Z. A., N. S. Kenneth, T. R. Brown, N. L. Daly, N. Gomez-Roman et al., 2003 Direct regulation of RNA polymerase III transcription by RB, p53 and c-Myc. Cell Cycle 2: 181–184.

Frolov, M. V., D. S. Huen, O. Stevaux, D. Dimova, K. Balczarek-Strang et al., 2012 The retinoblastoma protein. EMBO J. 16: 2061–2071.

Gutschat, R., L. Borghi, and W. Gruissem, 2012 Emerging roles of RETI-NOBLASTOMA-RELATED proteins in evolution and plant development. Trends Plant Sci. 17: 139–148.

Haghigha, K., and V. Hietakangas, 2014 p53- and ERK7-dependent ribosome surveillance response regulates Drosophila insulin-like peptide secretion. PLoS Genet. 10: e1004764.

He, X., C.-C. Chen, F. Hong, F. Fang, S. Sinha et al., 2009 A biophysical model for analysis of transcription factor interaction and binding site arrangement from genome-wide binding data. PLoS ONE 4: e8155.

Heinz, S., C. Benner, N. Spann, E. Bertolino, Y. C. Lin et al., 2010 Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol. Cell 38: 576–589.

Hernando, E., Z. Nahle, G. Juan, E. Diaz-Rodriguez, M. Alamino et al., 2004 Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. Nature 430: 797–802.

Hirsch, H. A., L. Gu, and R. W. Henry, 2000 The retinoblastoma tumor suppressor protein targets distinct general transcription factors to regulate RNA polymerase III gene expression. Mol. Cell. Biol. 20: 9182–9191.

Hirsch, H. A., G. W. Jawdekar, K.-A. Lee, L. Gu, and R. W. Henry, 2004 Distinct mechanisms for repression of RNA polymerase III transcription by the retinoblastoma tumor suppressor protein. Mol. Cell. Biol. 24: 5989–5999.

Huang, D. W., B. T. Sherman, and R. A. Lempicki, 2009a Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37: 1–13.

Huang, D. W., B. T. Sherman, and R. A. Lempicki, 2009b Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4: 44–57.

Julian, L. M., O. Palander, L. A. Seifried, J. E. G. Foster, and F. A. Dick, 2008 Characterization of an E2F1-specific binding domain in pRB and its implications for apoptotic regulation. Oncogene 27: 1572–1579.

Kuiper, S. A., Z. Ullah, M. S. Buckley, R. W. Henry, and D. N. Arnosti, 2005 Distinct developmental expression of Drosophila retinoblastoma factors. Gene Expr. Patterns 5: 411–421.

Korenjak, M., B. Taylor-Harding, and U. Binné, 2004 Native E2F/RBF complexes contain Myb-interacting proteins and repress transcription of developmentally controlled E2F target genes. Cell 119: 181–193.

Korenjak, M., E. Anderssen, S. Ramaswamy, J. R. Whetstone, and N. J. Dyson, 2012 RBF binding to both canonical E2F targets and noncanonical targets depends on functional de2f/dDP complexes. Mol. Cell. Biol. 32: 4379–4387.

Korenjak, M., E. Kwon, R. T. Morris, E. Anderssen, A. Amzallag et al., 2014 dREAM co-operates with insulator-binding proteins and regulates expression at divergently paired genes. Nucleic Acids Res. 42: 8939–8953.

Krizwinski, M., J. Scheu, I. Birol, J. Connors, R. Gascoyne et al., 2009 Circos: an information aesthetic for comparative genomics. Genome Res. 19: 1639–1645.

Larminie, C. G., C. A. Cairns, R. Mital, K. Martin, T. Kouzarides et al., 1997 Mechanistic analysis of RNA polymerase III regulation by the retinoblastoma protein. EMBO J. 16: 2061–2071.

Latorre, I., M. A. Chesney, J. M. Garrigue, P. Stempor, A. Appert et al., 2015 The DREAM complex promotes gene body H2A.Z for target repression. Genes Dev. 29: 495–500.

Lewis, P. W., E. L. Beall, T. C. Fleischer, D. Georlette, A. J. Link et al., 2004 Identification of a Drosophila Myb-E2F/RBF transcriptional repressor complex. Genes Dev. 18: 2929–2940.

Machnick, P., and T. L. Bailey, 2011 MEME-ChIP: motif analysis of large DNA datasets. Bioinformatics 27: 1696–1697.

Marygold, S. J., J. Roote, G. Reuter, A. Lambertsson, M. Ashburner et al., 2007 The ribosomal protein genes and Minute loci of Drosophila melanogaster. Genome Biol. 8: R216.

Muller, C., B. Schwab, K. Maier, D. Schulz, S. Dümcke et al., 2010 Dynamic transcriptome analysis measures rates of mRNA synthesis and decay in yeast. Mol. Syst. Biol. 7: 458.

Morris, E. J., and N. J. Dyson, 2001 Retinoblastoma protein partners. Adv. Cancer Res. 82: 1–54.

Nicolay, B. N., P. A. Gameiro, K. Tschöp, M. Korenjak, A. M. Heilmann et al., 2013 Loss of RBFI changes glutamine catabolism. Genes Dev. 27: 182–196.

Norton, J. D., R. W. Deed, G. Cragg, and F. S abolish, 1998 Id helix-loop-helix proteins in cell growth and differentiation. Trends Cell Biol. 8: 58–65.

Naj, R. I., J. Zhang, Y. Wei, D. N. Arnosti, and R. W. Henry, 2012 Ubiquitination of retinoblastoma family protein 1 potentiates gene-specific repression function. J. Biol. Chem. 287: 41835–41843.

Reynolds, M. R., A. N. Lane, B. Robertson, S. Kemp, Y. Liu et al., 2014 Control of glutamine metabolism by the tumor suppressor Rb. Oncogene 33: 556–566.
Rhee, H. S., and B. F. Pugh, 2011 Comprehensive genome-wide protein-DNA interactions detected at single-nucleotide resolution. Cell 147: 1408–1419.

Rubin, S. M., A. L. Gall, N. Zheng, and N. P. Pavletich, 2005 Structure of the Rb C-terminal domain bound to E2F1–DP1: a mechanism for phosphorylation-induced E2F release. Cell 123: 1093–1106.

Schwartz, Y. B., D. Linder-Basso, P. V. Kharchenko, M. Y. Tolstorukov, M. Kim et al., 2012 Nature and function of insulator protein binding sites in the Drosophila genome. Genome Res. 22: 2188–2198.

Steffen, K. K., M. A. McCormick, K. M. Pham, V. L. MacKay, J. R. Delaney et al., 2012 Ribosome deficiency protects against ER stress in Saccharomyces cerevisiae. Genetics 191: 107–118.

Stevaux, O., D. Dimova, M. V. Frolov, B. Taylor-Harding, E. Morris et al., 2005 Retinoblastoma family 2 is required in vivo for the tissue-specific repression of dE2F2 target genes. Cell Cycle 4: 1272–1280.

Ullah, Z., and M. Buckley, 2007 Regulation of RNA polymerases I and III by the retinoblastoma protein: a mechanism for growth control? Trends Biochem. Sci. 22: 77–80.

White, R. J., 1995 The retinoblastoma protein and cell cycle control. Cell 81: 323–330.