Oncogene (2007) 26, 6307–6318
© 2007 Nature Publishing Group All rights reserved 0950-9232/07 $30.00
www.nature.com/onc

ORIGINAL ARTICLE

Loss of the retinoblastoma tumor suppressor: differential action on transcriptional programs related to cell cycle control and immune function

MP Markey[1], J Bergseid[2], EE Bosco[1], K Stengel[1], H Xu[3,4], CN Mayhew[1], SJ Schwemberger[6], WA Braden[1], Y Jiang[2], GF Babcock[5,6], AG Jegga[3,4], BJ Aronow[3,4], MF Reed[5], JYJ Wang[2] and ES Knudsen[1]

[1]Department of Cell and Cancer Biology, University of Cincinnati, Cincinnati, OH, USA; [2]Department of Medicine, Moores Cancer Center, University of California San Diego, La Jolla, CA, USA; [3]Department of Pediatrics, University of Cincinnati, Cincinnati, OH, USA; [4]Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA; [5]Department of Surgery, University of Cincinnati, Cincinnati, OH, USA and [6]Shriners Hospital, Cincinnati, OH, USA

Functional inactivation of the retinoblastoma tumor suppressor gene product (RB) is a common event in human cancers. Classically, RB functions to constrain cellular proliferation, and loss of RB is proposed to facilitate the hyperplastic proliferation associated with tumorigenesis. To understand the repertoire of regulatory processes governed by RB, two models of RB loss were utilized to perform microarray analysis. In murine embryonic fibroblasts harboring germline loss of RB, there was a striking deregulation of gene expression, wherein distinct biological pathways were altered. Specifically, genes involved in cell cycle control and classically associated with E2F-dependent gene regulation were upregulated via RB loss. In contrast, a program of gene expression associated with immune function and response to pathogens was significantly downregulated with the loss of RB. To determine the specific influence of RB loss during a defined period and without the possibility of developmental compensation as occurs in embryonic fibroblasts, a second system was employed wherein *Rb* was acutely knocked out in adult fibroblasts. This model confirmed the distinct regulation of cell cycle and immune modulatory genes through RB loss. Analyses of *cis*-elements supported the hypothesis that the majority of those genes upregulated with RB loss are regulated via the E2F family of transcription factors. In contrast, those genes whose expression was reduced with the loss of RB harbored different promoter elements. Consistent with these analyses, we found that disruption of E2F-binding function of RB was associated with the upregulation of gene expression. In contrast, cells harboring an RB mutant protein (RB-750F) that retains E2F-binding activity, but is specifically deficient in the association with LXCXE-containing proteins, failed to upregulate these same target genes. However, downregulation of genes involved in immune function was readily observed with disruption of the LXCXE-binding function of RB. Thus, these studies demonstrate that RB plays a significant role in both the positive and negative regulations of transcriptional programs and indicate that loss of RB has distinct biological effects related to both cell cycle control and immune function.

Oncogene (2007) **26,** 6307–6318; doi:10.1038/sj.onc.1210450; published online 23 April 2007

Keywords: RB; E2F; transcriptional repression; inflammation; cell cycle

Introduction

The retinoblastoma tumor suppressor (RB) is a potent inhibitor of cell cycle progression that was initially identified based on bi-allelic inactivation in the pediatric cancer retinoblastoma (Sherr and McCormick, 2002; Cobrinik, 2005). Many subsequent studies have demonstrated that RB function is compromised in a large fraction of human cancers, this is consistent with the model that deregulation of cell cycle control is a requisite component of tumorigenesis (Nevins, 2001; Sherr and McCormick, 2002; Cobrinik, 2005). Owing to widespread inactivation of RB in cancer, the mechanism underlying tumor suppression has been the subject of intense scrutiny.

A critical function of RB is the regulation of gene expression. The RB protein has weak, non-specific, DNA-binding activity. However, RB interacts strongly with a number of sequence specific DNA-binding transcription factors (Morris and Dyson, 2001; Frolov and Dyson, 2004). Among these factors, the best understood is the E2F family of transcription factors (Morris and Dyson, 2001; Cam and Dynlacht, 2003; Frolov and Dyson, 2004; Cobrinik, 2005; Dimova and Dyson, 2005). Generally, E2F functions as a heterodimer comprised of E2F and DP subunits. These factors interact with DNA elements in the promoters of a large number of cell cycle regulatory genes as determined by

Correspondence: Dr E Knudsen, Department of Cell and Cancer Biology, University of Cincinnati College of Medicine, Vontz Center for Molecular Studies, 3125 Eden Avenue, Cincinnati, OH 45267-0521, USA.
E-mail: erik.knudsen@uc.edu
Received 14 December 2005; revised 17 January 2007; accepted 17 January 2007; published online 23 April 2007

microarray-based gene expression (Ishida *et al.*, 2001; Markey *et al.*, 2002; Vernell *et al.*, 2003) and chromatin immunoprecipitation (ChIP) analyses (Ren *et al.*, 2002; Cam *et al.*, 2004). Specifically, E2F complexes have been attributed to the appropriate control of DNA replication, DNA repair, apoptosis and G2/M regulation. Most of the E2F family members harbor transcriptional activation domains and stimulate the expression of target genes (Cam and Dynlacht, 2003; Blais and Dynlacht, 2004; Dimova and Dyson, 2005). By interacting with E2F proteins, RB masks the transcriptional activation domains of these proteins (Qin *et al.*, 1995). Additionally, RB functions to recruit corepressor activities to functionally repress E2F-mediated gene expression (Trouche *et al.*, 1997; Brehm *et al.*, 1998; Strobeck *et al.*, 2000). Thus, activation of RB represses the expression of E2F-regulated genes and mediates the inhibition of cell cycle progression.

To mediate physiological proliferation, RB activity is regulated during cell cycle progression. Mitogenic signaling cascades stimulate the activity of cyclin-dependent kinase (CDK) complexes, which phosphorylate RB (Cobrinik, 2005). This phosphorylation attenuates the interaction of RB with E2F family members (Knudsen *et al.*, 1998). Thus, as cells progress through the cell cycle, RB-mediated repression is alleviated, and it is believed that this event is critical for cell cycle progression. During tumorigenesis, RB is functionally inactivated through a multitude of mechanisms. Strikingly, these disparate mechanisms all impinge on RB-mediated repression of transcription by disrupting the interaction with E2F and corepressors.

While the targeting of E2F activity represents a well-appreciated aspect of RB function, there are a number of additional proteins that interact with RB. For example, RB interacts with an excess of 30 additional transcription factors (Wang *et al.*, 1994; Morris and Dyson, 2001). Many of these proteins and additional transcriptional modulators interact with the RB A/B pocket through an LXCXE motif. There are also data that RB can function as a transcriptional activator in specific settings. For example, it has been previously observed that RB-null cells have reduced expression of major histocompatibility complex class I and II proteins, and that restoration of RB induces the expression of these genes (Lu *et al.*, 1994; Zhu *et al.*, 1999; Eason *et al.*, 2001). Additionally, RB can bind to JunD, NeuroD1, CBFA1, PU.1 and the androgen receptor to mediate transcription factor activation (Thomas *et al.*, 2001; Xin *et al.*, 2003; Iavarone *et al.*, 2004; Batsche *et al.*, 2005). Given the prevalence of RB inactivation in cancer, it is important to understand the range of transcriptional changes occurring through RB loss.

Results

Chronic RB loss modifies the expression of known E2F-regulated genes

To initially probe the influence of RB loss on gene expression programs, we utilized murine embryonic fibroblasts (MEFs). As expected, *Rb*−/− MEFs are deficient in the expression of the RB protein (Figure 1a). These cells exhibit relatively similar cell cycle distributions (Figure 1b) and proliferation rates (Figure 1c). However, these asynchronously proliferating *Rb*−/− MEFs harbor increased expression of specific gene products that are under E2F control (Figure 1d). For example, the protein levels of PCNA, MCM7 and Cyclin A were upregulated in the *Rb*−/− MEFs.

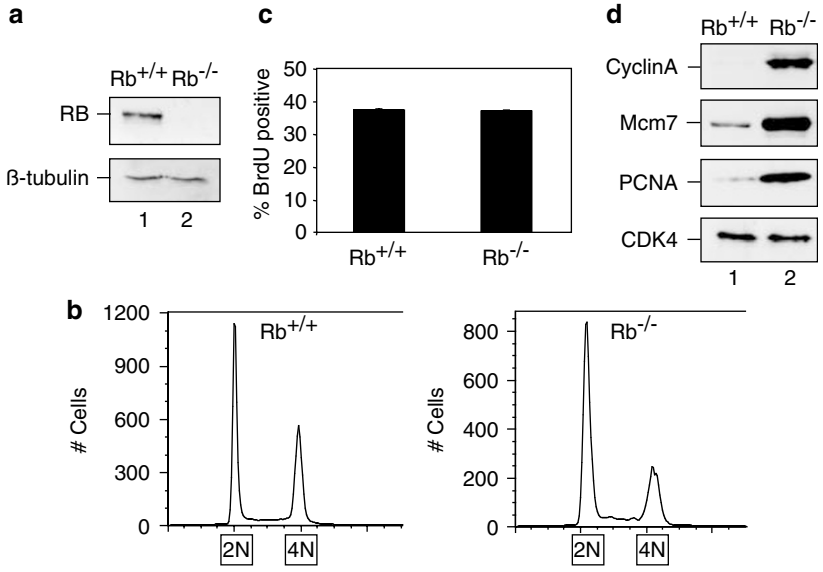

Figure 1 Model of chronic RB loss. (**a**) Cell lysates from *Rb*+/+ and *Rb*–/– MEFs were utilized for immunoblotting. β-Tubulin was used as a loading control. (**b** and **c**). The cell cycle behavior of asynchronously proliferating *Rb*+/+ and *Rb*−/− MEFs was assessed by flow cytometry (**b**) and by immunofluorescent analyses of BrdU incorporation (**c**). (**d**) *Rb*+/+ and *Rb*−/− MEF cell lysates were immunoblotted for known RB target genes cyclin A, MCM7 and PCNA. CDK4 levels were used as a loading control.

Chronic RB loss causes significant changes in gene expression

To delineate target genes deregulated via RB loss in an unbiased manner, we used microarray analysis to identify differentially expressed genes in asynchronously proliferating *Rb*+/+ and *Rb*−/− MEFs. Independent cultures were utilized to isolate RNA from matched passage MEFs. This RNA was then subjected to microarray analyses on the Affymetrix GeneChip Mouse Genome 430A 2.0 Array platform. The resultant intensity data were analysed with GeneSpring software (Agilent Technologies, Palo Alto, CA, USA) to retain only those genes that provide a statistically significant ($P<0.02$) behavioral change between the two conditions. Additionally, a minimum-fold change of 1.3 was used to narrow the list of genes modified by the loss of RB. From these analyses, 248 individual Affymetrix entries were defined that demonstrated highly reproducible behavior (Figure 2a). Inspection of the up-regulated genes (119 in total) showed a striking abundance of genes that have been previously associated with the E2F family of transcription factors (Supplementary Data 1). Ontology-based analysis showed that the preponderance of upregulated genes was involved in cellular proliferation, DNA metabolism (e.g., DNA replication) and transcriptional processes (Figure 2b and Supplementary Table 1).

Strikingly, we found a significant fraction of genes (129 in total) were downregulated in the *Rb*−/− MEFs (Figure 2a). These genes are not involved in cell cycle progression and have not been identified previously on microarrays using the overexpression of either RB or E2F proteins (Ishida *et al.*, 2001; Markey *et al.*, 2002; Vernell *et al.*, 2003). Analysis with the gene ontology programs demonstrated that most of the genes are associated with processes related to immune responses, particularly those induced by pathogens or injuries (Figure 2c and Supplementary Table 1), including a wide spectrum of chemokines, complement factors, cell surface antigens and receptors (Supplementary Data 1). To confirm that the RNA levels of these genes were reduced in the absence of RB, a selected set of these transcripts were analysed by reverse transcriptase polymerase chain reaction (RT–PCR). As shown in Figure 3a, we confirmed that the levels of Rb RNA were

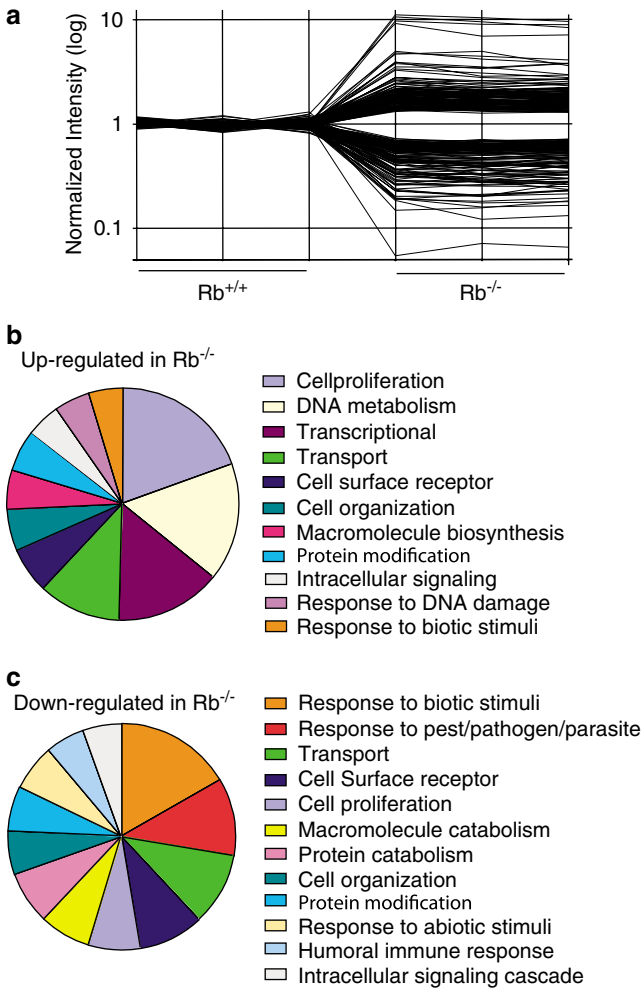

Figure 2 Chronic RB loss causes significant changes in gene expression. RNAs from asynchronously proliferating *Rb*+/+ and *Rb*–/– MEFs were used as substrates for microarray analysis. (**a**) Genes with a statistically significant change in expression were determined as described in Materials and methods. Two hundred and forty-eight targets demonstrating highly reproducible behavior are shown. (**b** and **c**). Simplified ontology of these 248 targets.

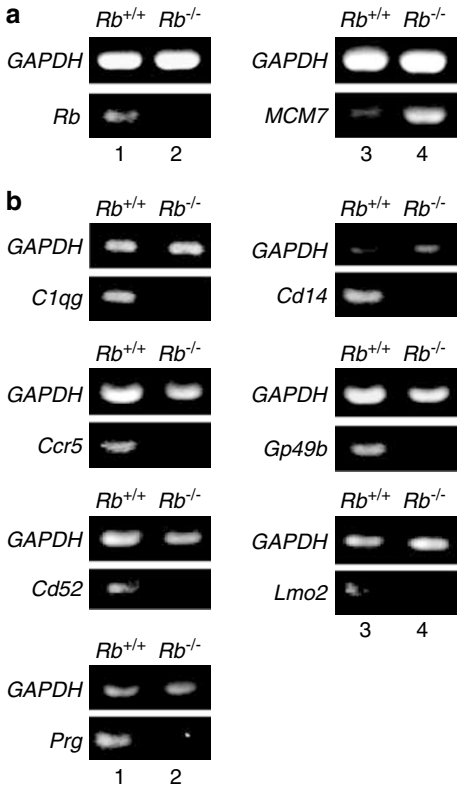

Figure 3 Chronic RB loss attenuates a subset of genes. Semiquantitative RT–PCR analysis was carried out using GAPDH levels as controls in *Rb*+/+ and *Rb*−/− MEFs. (**a**) The levels of RB and known RB target gene MCM7 are decreased in *Rb*–/– MEFs. (**b**) The genes *C1qg*, *Cd14*, *Ccr5*, *Gp49b*, *Cd52*, *Lmo2* and *Prg* are decreased in *Rb*−/− MEFs, consistent with results from microarray analysis.

reduced, while conventionally E2F-regulated genes such as MCM7 exhibit increased RNA levels in *Rb*−/− MEFs. In contrast, the RNA levels of the immune/pathogen response-related genes were reduced in *Rb*−/− MEFs relative to *Rb*+/+ MEFs (Figure 3b).

Acute RB loss deregulates gene expression

The above results indicate that loss of RB in primary fibroblasts can both enhance and attenuate the expression of multiple genes involved in disparate biological processes. However, this finding could be due in part to adaptive changes that occur during development of the *Rb*−/− embryos, for example, upregulation of other pocket proteins (Sage *et al*., 2003). Therefore, we utilized a cell culture system wherein RB can be inactivated acutely. In this system, adult fibroblasts of $Rb^{loxP/loxP}$ mice are isolated and cultured (Mayhew *et al*., 2004). Infection of these cells with adenoviruses encoding Cre recombinase led to a rapid and efficient recombination of the *Rb* gene (Figure 4a). As in the MEF system, the loss of RB results in minimal change in cell cycle distribution (Figure 4b and c), but leads to the accumulation of known E2F gene products (Figure 4d). Thus, this system can be utilized as a model for acute RB loss. Asynchronous $Rb^{loxP/loxP}$ cells were infected with either green fluorescent protein (GFP) or GFP and Cre-recombinase (GFP-Cre) encoding adenoviruses and harvested for microarray analyses at 72 or 144 h post-infection. The primary microarray data were referenced to GFP 72 h controls and queried for genes that were significantly altered using a Welch *t*-test and 5% Benjamani Hochberg false discovery rate using GeneSpring. This analysis resulted in a list of 528 genes upregulated or downregulated by the loss of RB (Figure 5a and Supplementary Data 2). Many of the upregulated genes (273 total) confirmed that loss of RB resulted in the increased expression of known E2F target genes. Thus, the upregulation of E2F-regulated genes occurs both acutely and as a chronic response to RB. Using gene ontology analyses, we found that genes upregulated by acute RB loss were involved in processes virtually identical to those found with the chronic loss of RB (Figure 5b and Supplementary Table 2). We also observed a significant downregulation of specific target genes with the acute loss of RB (Figure 5c and Supplementary Table 2). These genes were observed to be largely involved in immune response to pathogen. The regulation of these targets was further confirmed by real-time quantitative PCR (Figure 5d). Thus, the acute loss of RB recapitulates the results with chronic loss in the MEFs, confirming that RB loss deregulates gene expression programs involved in both cell cycle control and immune function.

Combinatorial analyses define genes tightly regulated in multiple contexts

To provide a comprehensive account of genes with expression modified by the loss of RB, all of the data from both models were combined and evaluated for significant patterns using several different approaches. For this, data sets were combined from the separate experiments using a normalization and referencing strategy in which the gene expression level for each gene in an experimental series was examined relative to the mean of its expression in control samples within its series. Regulated genes were identified in each series using a Welch *t*-test, $P<0.05$, and then ranked according to highest fold-regulated within condition-specific comparisons. Four different gene lists were obtained (597, 331, 528 and 243), which when pooled generated a list of 1166 genes/EST entries reproducibly modified via the loss of RB (Supplementary Data 3). These data were

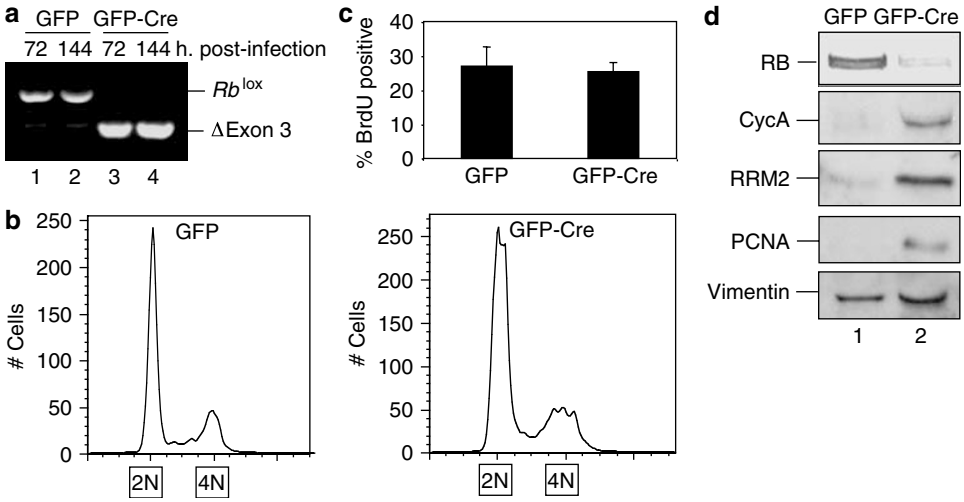

Figure 4 *Model of acute RB loss.* (**a**) *Rb* exon $3^{loxP/loxP}$ cells were cultured for 72 h (lanes 1 and 3) or 144 h (lanes 2 and 4) postinfection with GFP (lanes 1 and 2) or GFP-Cre-encoding (lanes 3 and 4) adenovirus. DNA from these cells was used for PCR with primers spanning *Rb* exon 3. GFP-Cre mediates the efficient recombination of *Rb*, resulting in a truncated transcript. (**b** and **c**) The cell cycle behavior of asynchronously proliferating *Rb* exon $3^{loxP/loxP}$ MAFs was assessed by flow cytometry (**b**) and immunofluorescent analyses of BrdU incorporation (**c**). (**d**) Acute loss of RB in the GFP-Cre-expressing cells results in the overexpression of the known RB target genes cyclin A, ribonucleotide reductase M2 and PCNA. Vimentin was used as a loading control.

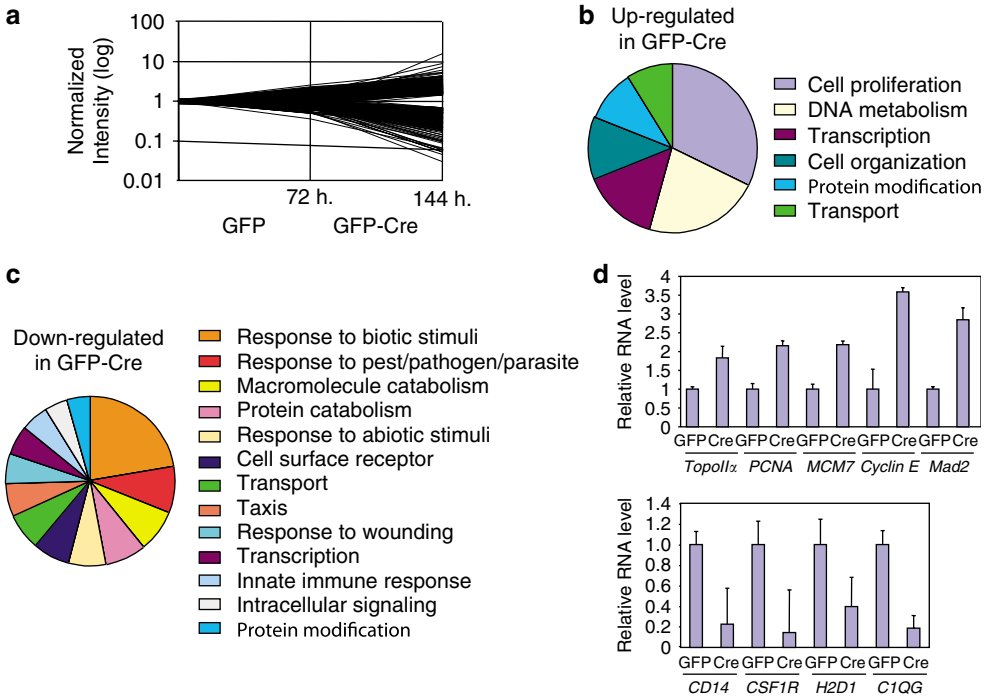

Figure 5 Acute RB loss deregulates gene expression. *Rb* exon $3^{loxP/loxP}$ cells were cultured for 72 h postinfection with GFP-encoding adenovirus or 72 or 144 h postinfection with GFP-Cre-encoding adenovirus. RNA was harvested and used for microarray analysis. (**a**) 528 targets on the array with statistically significant, consistent changes in expression were determined as described. (**b** and **c**). Simplified ontology of these targets. (d) Specific targets upregulated (top panel) and downregulated (bottom panel) with RB loss were verified by real-time PCR.

subjected to hierarchical clustering (Figure 6a). From these analyses, there were two main clusters that exhibited highly conserved changes in behavior. Genes in clusters 1 and 2 were upregulated with RB loss and contained a total of 310 entries. The behavior of all genes in each cluster is depicted in Figure 6b. The majority of the genes in clusters 1 and 2 are involved in cell cycle regulation and transcriptional control. A summary listing of genes in major categories is shown in Figure 7. There were also a large number of genes downregulated by RB loss. The overall behavior of these genes was relatively heterogeneous, being downregulated preferentially in one model. For example, in cluster 3 (composed of 268 genes), virtually all of the genes are downregulated as a result of acute RB loss. However, only 97 genes within cluster 3 were downregulated in both models (denoted by green bars). An abbreviated list of the genes from this cluster is shown in Figure 7. Strikingly, while heterogeneity was observed at the level of individual genes, the overall function of genes within the downregulated cluster and subcluster is related to immune response or response to pathogen/injury, including cell surface molecules, complement factors and genes involved in interferon signaling.

Distinct cis*-element composition of genes differentially regulated with RB loss*

The finding that RB loss differentially regulated gene expression led us to interrogate *cis*-elements that govern distinct gene behavior. Specifically, genes from clusters 1–3 were analysed. The murine and human sequences were aligned using the Trafac server (Jegga *et al.*, 2002) and the 500 base pairs upstream and downstream of the transcriptional start site were probed for presence of conserved E2F sites using the CisMols server (Jegga *et al.*, 2005). Initially, we determined the average number of conserved E2F sites present in each gene. These analyses demonstrated that E2F-binding sites are highly prevalent in activated genes, representing the 9th and 12th most prevalent transcription factor binding sites in clusters 1 and 2, respectively, with an average of approximately four E2F sites per gene (Figure 8a). In contrast, in cluster 3 genes, E2F sites were significantly underrepresented (29th) and the relative number of E2F sites was significantly reduced (Figure 8a). Recent evidence suggests that E2F elements are often dependent on neighboring sites for functional activity (Markey *et al.*, 2002). Therefore, we analysed each conserved E2F site for other binding sites within a window of 200 base pairs. Using this approach, we observed that a number of transcription factor binding sites cooccur with E2F sites and these sites were preferentially observed with clusters 1 and 2 (Figure 8b). Importantly, several of these elements have been previously implicated in cooperating with E2F for gene regulation (e.g., CDE, MYB and AHR), suggesting that there is a significant difference in the promoter composition between genes upregulated and downregulated with RB loss.

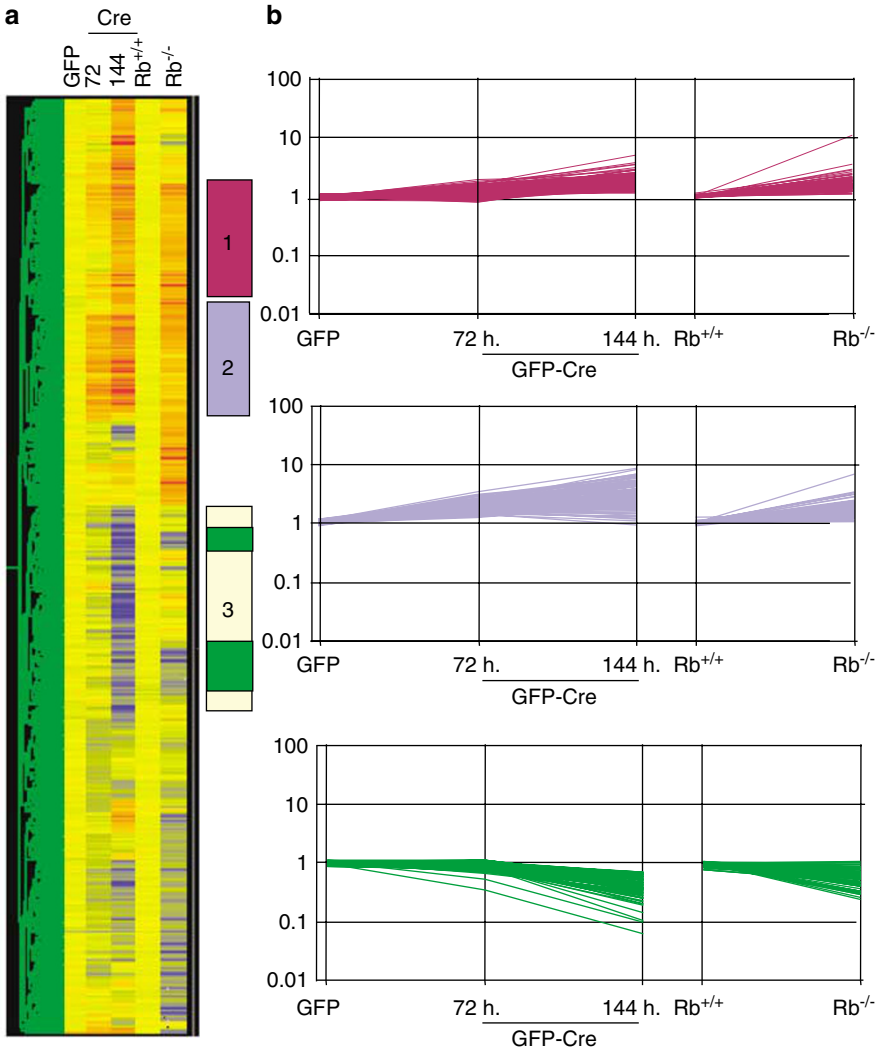

Figure 6 Combinatorial analyses define tightly regulated genes. (**a**) Hierarchical clustering of 1166 genes and expressed sequence tags (ESTs) divided into three clusters, which we examined further. Genes with increased expression are indicated in red, while genes with decreased expression are shown in blue. Yellow indicates no change compared to the control condition (presence of RB). (**b**) Graphical representation of the behavior of the genes falling into these three clusters. Clusters 1 and 2 consist of genes with increased expression in the RB-null condition. Cluster 3 contains genes with decreased expression with RB loss in both MEF and MAF models.

To determine if the difference in proximity to the E2F sites was indicative of overall differences in promoter composition, the 1 kb regions surrounding the transcription start sites were analysed for the presence of over 30 different conserved transcription factor binding sites (Figure 8c). This analysis indicated that the two gene clusters upregulated with RB loss exhibited similar promoter compositions, wherein both common (SP1, MAZ and ETS) and relatively specific (CDE, HIF, ECAT and PCAT) binding sites were observed. In contrast, genes downregulated with RB loss exhibited a distinct profile of transcription factor binding sites. For these genes, a number of unique sites were observed, such as GATA, OCT1 and IRF that are involved in regulating genes involved in immune function. Therefore, these analyses demonstrate not only the paucity of E2F sites, but also an overall difference in promoter constitution between the two classes of genes. Combined, these data suggest that RB loss deregulates gene expression by acting at distinct classes of promoters.

Distinct RB functional domains are associated with upregulation versus downregulation of gene expression

The data described above suggest that distinct regulatory interactions are responsible for the upregulation versus downregulation of gene expression. To explore this possibility, we employed two additional models of RB dysfunction. First, we utilized adenovirus E1A. This viral oncoprotein disrupts the association of RB with both the E2F family of transcription factors and a number of additional regulatory proteins that interact with the RB A/B pocket through an LXCXE motif. Second, we employed a new knock-in mouse model that harbors the N750F allele of RB (Bergseid and Wang, in preparation). This mutation completely compromises

Cluster 1

REPLICATION

Tk1	thymidine kinase 1
Mcm2	minichromosome maintenance deficient 2 (S. cerevisiae)
Tyms-ps	thymidylate synthase, pseudogene
Ris2/CDT1	retroviral integration site 2/CDT1
Mcm5	minichromosome maintenance deficient 5 (S. cerevisiae)
Mcm7	minichromosome maintenance deficient 7 (S. cerevisiae)
Dnajc9	DnaJ (Hsp40) homolog, subfamily C, member 9
Pold1	polymerase (DNA directed), delta 1, catalytic subunit
Lig1	ligase I, DNA, ATP-dependent
Prim1	DNA primase, p49 subunit
Pola1	polymerase (DNA directed), alpha 1
Rfc5	replication factor C (activator 1) 5
Cdca7	cell division cycle associated 7
Orc6l	origin recognition complex, subunit 6-like (S. cerevisiae)
Gmnn	geminin
Cdc45l	cell division cycle 45 homolog (S. cerevisiae)-like
Rrm1	ribonucleotide reductase M1
Rrm2	ribonucleotide reductase M2
Top2a	topoisomerase (DNA) II alpha
Cdc6	cell division cycle 6 homolog (S. cerevisiae)
Rev3l	REV3-like, catalytic subunit of DNA polymerase zeta RAD54
Ccna2	cyclin A2

MITOSIS/CYTOKENSIS

Kif22	kinesin family member 22
Brrn1	barren homolog (Drosophila)
Anapc5	anaphase-promoting complex subunit 5
Stmn1	Stathmin 1
Ckap2	cytoskeleton associated protein 2
Tmpo	Thymopoietin
Plk1	polo-like kinase 1 (Drosophila)
Cks2	CDC28 protein kinase regulatory subunit 2
Cdc20	cell division cycle 20 homolog (S. cerevisiae)
Kif23	kinesin family member 23
Incenp	inner centromere protein
Kif11	kinesin family member 11
Kif4	kinesin family member 4
Cenpa	Centromere autoantigen A
Kif20a	kinesin family member 20A
Cdca3	cell division cycle associated 3
Cdca5	cell division cycle associated 5
Cdca8	cell division cycle associated 8
Prc1	protein regulator of cytokinesis 1
Kif20a	kinesin family member 20A
Mki67	antigen identified by monoclonal antibody Ki 67
Bub1	budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)
Kif2c	kinesin family member 2C
Anln	anillin, actin binding protein (scraps homolog, Drosophila)
Nusap1	nucleolar and spindle associated protein 1
Plk4	polo-like kinase 4 (Drosophila)
Nek2	NIMA (never in mitosis gene a)-related expressed kinase 2
Ccnb1	cyclin B1
Cks2	CDC28 protein kinase regulatory subunit 2
Cdc25c	Cell division cycle 25 homolog C (S. cerevisiae)
Cks1	CDC28 protein kinase 1
Cdc2a	cell division cycle 2 homolog A (S. pombe)
Ccnb2	cyclin B2

TRANSCRIPTIONAL CONTROL/CHROMATIN

Dnmt1	DNA methyltransferase (cytosine-5) 1
Chaf1b	chromatin assembly factor 1, subunit B (p60)
Tfdp1	transcription factor Dp 1
Tcf19	transcription factor 19
E2f1	E2F transcription factor 1
Asf1b	ASF1 anti-silencing function 1 homolog B (S. cerevisiae)
histone 1, H2ab	histone 1, H2ab
H2av	H2A histone family, member V
Hmgn2	high mobility group nucleosomal binding domain 2
Hmgb3	high mobility group box 3
Cbx5	chromobox homolog 5 (Drosophila HP1a)
Cbx5	chromobox homolog 5 (Drosophila HP1a)
Hmgb2	high mobility group box 2
Phc1	polyhomeotic-like 1 (Drosophila)
Ezh2	enhancer of zeste homolog 2 (Drosophila)
Hat1	histone aminotransferase 1
Nap1l1	nucleosome assembly protein 1-like 1
Hmgn1	high mobility group nucleosomal binding domain 1
Smc2l1	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)
Trip13	thyroid hormone receptor interactor 13
Foxm1	forkhead box M1

APOPTOSIS

Casp8ap2	caspase 8 associated protein 2

DNA REPAIR

Brca1	breast cancer 1
Brca2	breast cancer 2
Rad51	RAD51 homolog (S. cerevisiae)
Rad51ap1	RAD51 associated protein 1
Rad21	RAD21 homolog (S. pombe)

Cluster 2

REPLICATION

Recc1	replication factor C 1
Fen1	flap structure specific endonuclease 1
Dtymk	deoxythymidylate kinase
Dck	deoxycytidine kinase
Ris2/CDT1	retroviral integration site 2/CDT1
Mcm3	minichromosome maintenance deficient 3 (S. cerevisiae)
Mcm3	minichromosome maintenance deficient 3 (S. cerevisiae)
Pcna	proliferating cell nuclear antigen
Tyms /// Tyms-ps	thymidylate synthase /// thymidylate synthase, pseudogene
Topbp1	topoisomerase (DNA) II beta binding protein
Mcm4	minichromosome maintenance deficient 4 homolog (S. cerevisiae)
Rpa1	replication protein A1
Rfc2	replication factor C (activator 1) 2
Cdk2	cyclin-dependent kinase 2

MITOSIS/CYTOKINESIS

Mtap4	microtubule-associated protein 4
Anapc11	anaphase promoting complex subunit 11 homolog (yeast)
Mad2l1	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)
Tmpo	thymopoietin
Kif1c	kinesin family member 1C
Kifc5a	kinesin family member C5A

TRANSCRIPTIONAL CONTROL/CHROMATIN

Rbbp4	retinoblastoma binding protein 4
Smc4l1	SMC4 structural maintenance of chromosomes 4-like 1
Dek	DEK oncogene (DNA binding)
Tfdp1	transcription factor Dp 1
Lbr	lamin B receptor
Rbl1	retinoblastoma-like 1 (p107)
Hat1	histone aminotransferase 1
E2f1	E2F transcription factor 1
Hmga2	high mobility group AT-hook 2
H2afz	H2A histone family, member Z
Klf4	Kruppel-like factor 4 (gut)
Cbx2	chromobox homolog 2 (Drosophila Pc class)
H1fx	H1 histone family, member X
Pml	promyelocytic leukemia
Egr1	Early growth response 1

APOPTOSIS

APAF1	apoptotic protease activating factor 1
Tnfrsf6	tumor necrosis factor receptor superfamily, member 6
Pdcd6ip	programmed cell death 6 interacting protein
Wig1	wild-type p53-induced gene 1
Sfpq	splicing factor proline/glutamine rich
Tnfrsf6	tumor necrosis factor receptor superfamily, member 6
Perp	PERP, TP53 apoptosis effector

DNA REPAIR

Atm	ataxia telangiectasia mutated homolog (human)
Xrcc1	X-ray repair complementing defective 1
3300001P08Rik	cisplatin resistance-associated overexpressed protein
Ei24	etoposide induced 2.4 mRNA
Msh2	mutS homolog 2 (E. coli)
Mre11a	meiotic recombination 11 homolog A (S. cerevisiae)
Chek1	checkpoint kinase 1 homolog (S. pombe)
Ddit4	DNA-damage-inducible transcript 4
Mdm2	transformed mouse 3T3 cell double minute 2
Ercc5	excision repair cross-complementing, complementation group 5

Figure 7 Genes categorized by ontology. Genes in the upregulated clusters 1 and 2 include genes involved in the control of cell growth and division (DNA replication, mitosis, chromatin remodeling, apoptosis and so on). In contrast, genes in cluster 3, which were downregulated with RB loss, include genes involved in the immune response and complement systems.

the binding of RB to LXCXE-containing proteins, without influencing binding to E2F proteins (Chen and Wang, 2000). MEFs either wild type for RB and transduced with E1A or homozygous for the N750F allele were utilized to analyse changes in gene expression by microarray analyses on an Agilent platform (see Supplementary Information). As shown in Figure 9a, E1A resulted in a significant induction of genes that were upregulated by RB loss. This effect of E1A was not recapitulated by the N750F allele (Figure 9a). Thus, the

Cluster 3

SURFACE ANTIGENS	
Cd53	CD53 antigen
Cd72	CD72 antigen
Cd14	CD14 antigen
Cd52	CD52 antigen
Cd68	CD68 antigen
IMMUNOGLOBULIN RELATED	
H2-T23	histocompatibility 2, T region locus 23
Igsf7	Immunoglobulin superfamily, member 7
Fcgr2b	Fc receptor, IgG, low affinity IIb
Fcgr1	Fc receptor, IgG, high affinity I
Pira1 /// Pirb	paired-Ig-like receptor A1 /// paired-Ig-like receptor B
Lilrb4	leukocyte immunoglobulin-like receptor, subfamily B, member 4
Fcer1g	Fc receptor, IgE, high affinity I, gamma polypeptide
Lilrb4	leukocyte immunoglobulin-like receptor, subfamily B, member 4
Pirb	paired-Ig-like receptor B
Sema4d	sema domain, immunoglobulin domain (Ig)
COMPLEMENT	
Pfc	properdin factor, complement
C1qg	complement component 1, q subcomponent, gamma polypeptide
C1qb	complement component 1, q subcomponent, beta polypeptide
C1qa	complement component 1, q subcomponent, alpha polypeptide
C1qr1	complement component 1, q subcomponent, receptor 1
C5r1	complement component 5, receptor 1
Pla2g7	phospholipase A2, group VII (platelet-activating factor)
HEMOPOIETIC/LYMPHOCYTE FACTORS	
Hck	hemopoietic cell kinase
Mpeg1	macrophage expressed gene 1
Hcph	hemopoietic cell phosphatase
Hcls1	hematopoietic cell specific Lyn substrate 1
Lcp1	lymphocyte cytosolic protein 1
Lcp1	lymphocyte cytosolic protein 1
Ncf1	neutrophil cytosolic factor 1
INTERFERON RESPONSE	
Icsbp1	interferon consensus sequence binding protein 1
Ifi16	Interferon, gamma-inducible protein 16
Irf5	interferon regulatory factor 5
Ccl4	chemokine (C-C motif) ligand 4
Ccr1	chemokine (C-C motif) receptor 1
CATHEPSINS	
Ctsc	cathepsin C
Ctss	cathepsin S
SIGNALING	
Sfpi1	SFFV proviral integration 1 (PU.1 transcription factor)
Csf1r	colony stimulating factor 1 receptor
Lmo2	LIM domain only 2
Plxna1	plexin A1
Rab7	RAB7, member RAS oncogene family
Rab8b	RAB8B, member RAS oncogene family
Ptpns1	protein tyrosine phosphatase, non-receptor type substrate 1
Rbbp9	retinoblastoma binding protein 9
Emr1	EGF-like module containing, mucin-like, receptor-like 1
Egfr	epidermal growth factor receptor
Tgfbi	transforming growth factor, beta induced
Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b
Msr1	Macrophage scavenger receptor 1
Scap2	src family associated phosphoprotein 2

Figure 7 *Continued.*

upregulation of defined RB target genes is dependent on the disruption of interactions with E2F, but not LXCXE-containing proteins.

As discussed earlier, there is significant heterogeneity among genes downregulated with RB loss, while functional roles in immune function are largely conserved. When analysing genes associated with immune function, we found that E1A significantly downregulated gene expression (Figure 9b). However, distinct from the upregulated genes, the N750F allele influenced gene expression in a manner consistent with E1A (Figure 9b). Thus, the LXCXE-binding function of RB plays a critical role in facilitating the expression of this class of genes.

Discussion

The activity of RB is compromised in the majority of human tumors (Nevins, 2001; Sherr and McCormick, 2002; Cobrinik, 2005). The influence of this event on tumorigenesis is largely viewed as an effect on cell cycle progression, wherein the subsequent activation of E2F-regulated genes drives hyperplastic proliferation (Cam and Dynlacht, 2003; Blais and Dynlacht, 2004). However, it is becoming increasingly clear that RB also plays positive roles in mediating additional responses that also contribute to tumor suppression (Cam and Dynlacht, 2003; Thomas *et al*., 2003; Blais and Dynlacht, 2004).

Role of RB in attenuation of E2F-regulated genes

It is well appreciated that in specific contexts, RB can function to repress E2F-mediated gene expression (Nevins, 2001; Cobrinik, 2005). The paradigms for this activity are based on ectopic expression of active alleles of RB or the use of p16ink4a, which activates endogenous pocket proteins. In both these contexts, a significant repression of E2F-regulated genes is observed (Markey *et al*., 2002; Vernell *et al*., 2003). Such results however must be interpreted with caution since the ectopic expression of RB could usurp the physiological targets associated with the RB-related proteins, p107/p130. Additionally, while the activation of RB and associated members is potentially important from the standpoint of specific anti-mitogenic signaling cascades, the pathological importance of RB represents those situations in which its activity is compromised. Here, we utilized two primary cell systems to specifically evaluate the influence of RB loss on gene expression. In both of these systems, there is a marked stimulation of E2F-regulated gene expression. Before microarrays utilizing overexpression of E2F proteins have defined targets that functionally contribute to DNA replication, DNA repair, G2/M progression, apoptosis and chromatin/transcription. With the loss of RB in both model systems, each of these classes of E2F-regulated genes is clearly represented. Thus, these data indicate through the specific loss of only RB, numerous E2F-target genes are deregulated. Such a result has been reported in the MEF system on a similar platform (Black *et al*., 2003; Iovino *et al*., 2006), although here we define additional genes that are upregulated with RB loss. Importantly, we also demonstrate that this effect on gene expression occurs within the span of a few days and is thus not dependent on chronic loss of RB. Our studies also indicate that the interaction with E2F proteins is critical for the modulation of these upregulated genes, as both complete RB loss and expression of E1A lead to the induction of such genes. In contrast, the LXCXE-binding motif of RB that is implicated in recruitment of corepressors and association with other transcriptional regulators had minimal influence on these target genes.

RB loss and downregulation of gene expression

While the E2F family of transcription factors are the best known targets of RB, a large number of additional transcription factors interact with RB (Kouzarides, 1995; Morris and Dyson, 2001). Interestingly, RB functions as a co-activator for some of these transcription factors (Kouzarides, 1995; Morris and Dyson,

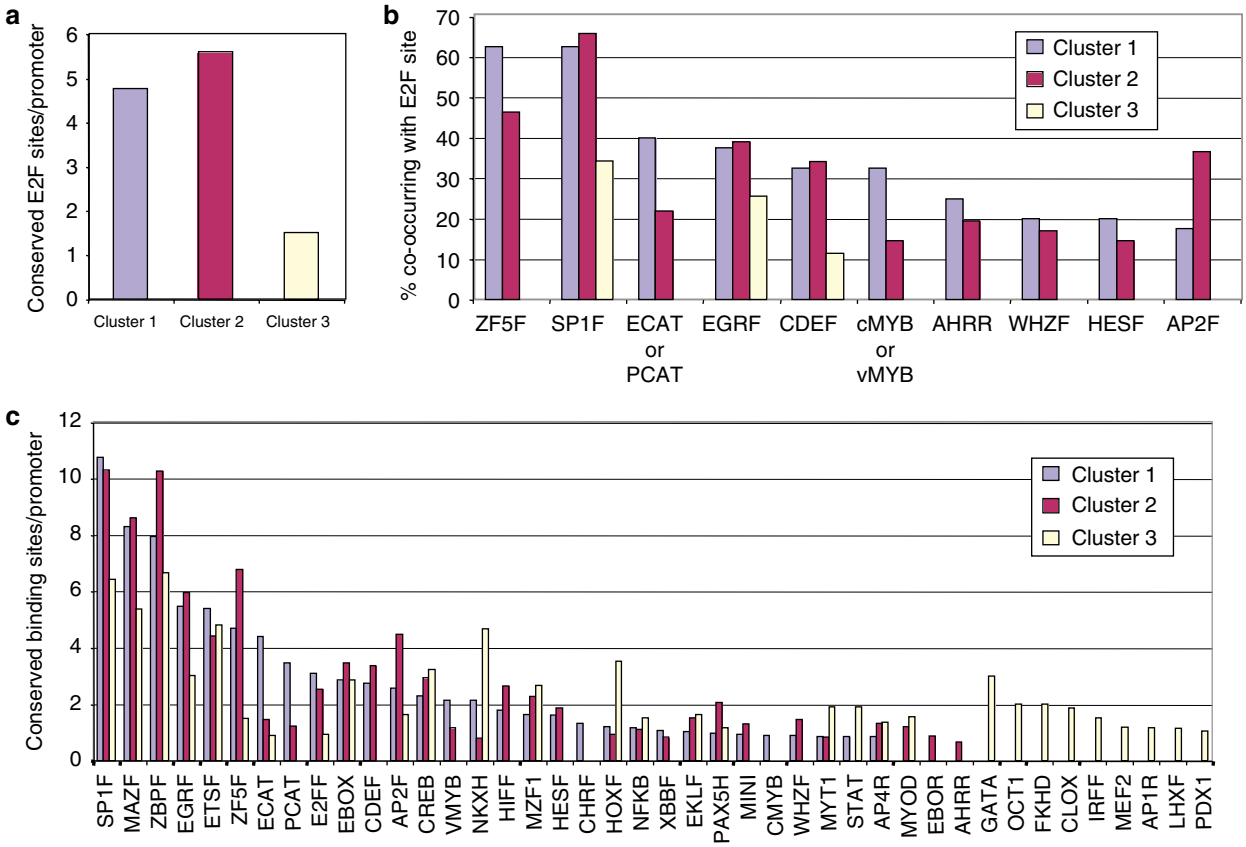

Figure 8 Distinct promoter composition of genes differentially regulated with RB loss. Aligned murine and human sequences (500 base pairs upstream and downstream of the transcriptional start site of a given gene) were probed for the presence of conserved transcription factor binding sites using the program CisMols Analyzer. (**a**) The average number of conserved E2F sequences for the genes in clusters 1–3. (**b**) Analysis of the *cis*-regulatory elements of the genes in each cluster shows that the upregulated genes have common promoter sequences co-appearing with E2F sites, while the down-regulated genes containing E2F sites tend not to hold them in the same context. (**c**) Average number of 42 different transcription factor binding sites appearing in the promoters of the genes in clusters 1–3.

2001). This has been demonstrated for a variety of different classes of transcription factors that regulate discrete biological processes (e.g., MyoD, Androgen Receptor, Runx2 and NeuroD1). Therefore, a positive effect of RB on gene expression has not been reported in microarray analyses. Here, we found that RB loss had a significant effect in downregulating gene expression. Interestingly, among the three different systems utilized, there was significant heterogeneity between the individual genes that are downregulated with RB loss. This finding suggests that the function of RB in maintaining gene expression is highly context dependent, wherein even differences between fibroblast models yield significant changes in gene expression. However, despite the heterogeneity in the models utilized, the downregulated genes were highly enriched to function in the immune system and in response to pathogen.

The influence of RB loss on immune response has been previously proposed. In studies investigating interferon γ-regulated gene expression, there is a deficit in the induction of specific interferon-inducible genes in RB-deficient cells (Lu *et al.*, 1994; Osborne *et al.*, 1997; Zhu *et al.*, 1999; Eason *et al.*, 2001). This change in gene expression has been attributed to changes in chromatin structure and regulation of Oct-1 function (Osborne *et al.*, 1997, 2004). Consistent with this mode of regulation, we find that Oct-1-binding sites are, in fact, observed at higher frequency in the promoter of genes that are downregulated with the loss of RB. Additionally, it has been observed that HPV-E7 (which downregulates RB activity) has a similar influence on gene expression (Arany and Tyring, 1996; Le Buanec *et al.*, 1999). This finding is consistent with our observations with adenovirus E1A. Thus, in the context of viral oncogenes, it is formally possible that targeting RB serves an advantage both by stimulating proliferation through E2F-regulated genes and impeding immune detection. Importantly, the effects of RB on maintaining target gene expression are dependent on the LXCXE-binding function of RB. This result is consistent with our *in silico* analyses that RB is utilizing distinct interactions to maintain the expression of genes, as opposed to mediating repression. Our studies indicate that such gene activation is presumably mediated through interactions between the RB A/B pocket and proteins harboring LXCXE motifs.

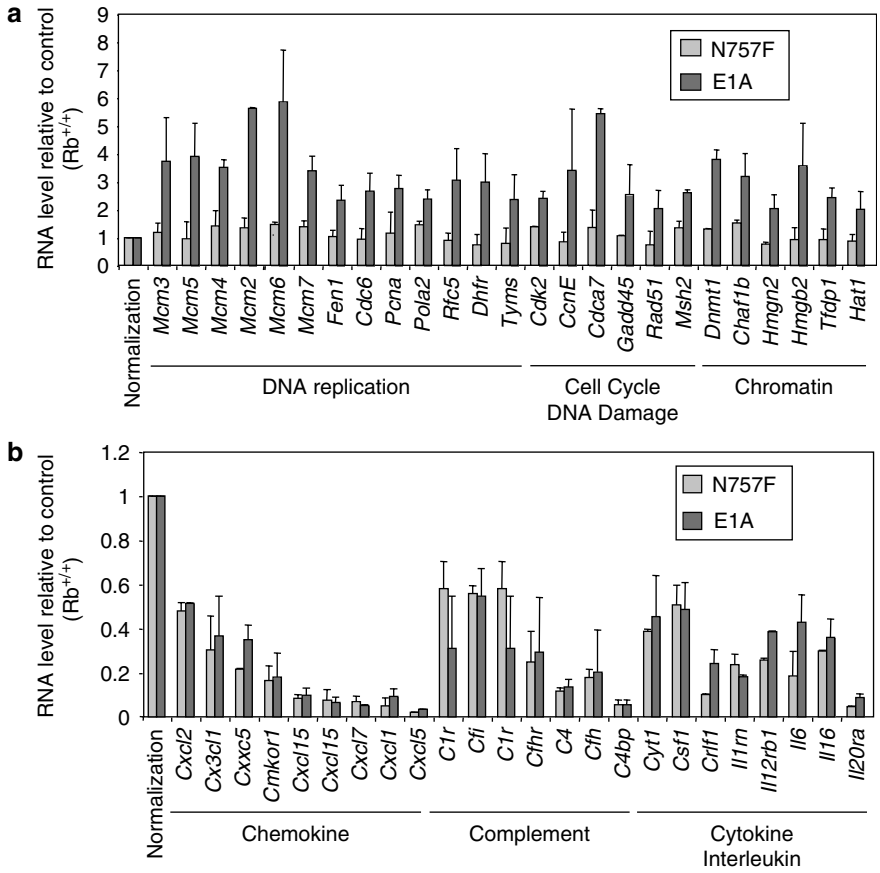

Figure 9 Distinct functional domains of RB are associated with upregulation versus downregulation of gene expression. (**a** and **b**) Relative RNA levels of the specified genes were determined by microarray analyses of the respective model: E1A or N750F.

In summary, these studies describe the influence of compromised RB function on gene expression. As expected, genes involved in cell cycle regulation and conventionally regulated via the E2F family of transcription factors were upregulated concomitantly with the loss of RB/E2F interactions. However, a large class of genes are downregulated as a result of RB deficiency and these genes are involved in immune functions. Combined, these data underscore the complexity of RB function and indicate that loss of RB compromises distinct facets of gene regulation.

Materials and methods

Cell culture
MEFs from wild-type, $Rb^{-/-}$ or $Rb^{N750F/N750F}$ mice were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 1% glutamine and 0.001% β-mercaptoethanol. The $Rb^{N750F/N750F}$ mice were generated using standard gene targeting approaches and expression of the allele confirmed (Bergseid and Wang, in preparation). Adult fibroblasts from *Rb* exon $3^{loxP/loxP}$ mice were cultured as previously described (Mayhew *et al.*, 2004). MAFs were grown to passage three, infected with adenovirus encoding either GFP or GFP-Cre and cultured for 72 or 144 h postinfection. To achieve stable expression of E1A, viral supernatant was harvested from BOSC23 cells, added to plates that contained MEFs at 50% confluence, and 24 h postinfection, puromycin selection was introduced (4 μg/ml).

Microarray and cis*-element analyses*
A detailed elaboration of the methods employed in the microarray and *cis*-element analyses is included in the supplemental section of this manuscript.

Antibodies
Immunoblotting was carried out with the following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA): cyclin A (C-19), β-tubulin (D-10), MCM7 (141.2), PCNA (PC10), RNR-M2 (I-15). Also utilized was anti-topoisomerase II-alpha (Topogen, Port Orange, FL, USA, 2011-1), RB (Becton Dickenson, Franklin Lakes, NJ, USA, 554136) and Vimentin (gift of Dr Wallace Ip).

Flow cytometry and bromodeoxyuridine incorporation
Flow cytometry (Welch and Wang 1995) and bromodeoxyuridine (BrdU) incorporation (Knudsen *et al.*, 1998) analyses were carried out as previously described.

RT–PCR
Total RNA was harvested with Trizol (Life Technologies Inc., Carlsbad, CA, USA) according to the manufacturer's suggested protocol. RNA (1 μg) was reverse transcribed and

amplified using a ThermoScript RT–PCR kit and random hexamers according to the manufacturer's protocol (Invitrogen Life Technologies, Carlsbad, CA, USA). Primers designed to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and primers against each target gene were included per reaction, amplifying products of different sizes. Primer sequences are provided in the Supplementary Information.

Real-time quantitative PCR
Total RNA (1 μg) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). For each sample, 2 μl of cDNA was used as a template in real-time quantitative PCR using the Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). Samples were subjected to 40 cycles of real-time quantitative PCR on the 7500 Fast Real-time PCR System (Applied Biosystems) and data analysed with SDS Software version 1.3.1. Reactions for each target gene as well as the endogenous control, β-actin, were performed in triplicate. Primer sequences are provided in Supplementary Information.

Acknowledgements

We are grateful to Dr George Blanck, Dr Karen Knudsen and Dr Lisa Morey for critical commentary on the manuscript. We also thank Dr Julien Sage and Dr Tyler Jacks for the provision of mouse strains.

References

Arany I, Tyring SK. (1996). Status of local cellular immunity in interferon-responsive and -nonresponsive human papillomavirus-associated lesions. *Sex Transm Dis* **23**: 475–480.

Batsche E, Moschopoulos P, Desroches J, Bilodeau S, Drouin J. (2005). Retinoblastoma and the related pocket protein p107 act as coactivators of NeuroD1 to enhance gene transcription. *J Biol Chem* **280**: 16088–16095.

Black EP, Huang E, Dressman H, Rempel R, Laakso N, Asa SL *et al*. (2003). Distinct gene expression phenotypes of cells lacking Rb and Rb family members. *Cancer Res* **63**: 3716–3723.

Blais A, Dynlacht BD. (2004). Hitting their targets: an emerging picture of E2F and cell cycle control. *Curr Opin Genet Dev* **14**: 527–532.

Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature* **391**: 597–601.

Cam H, Balciunaite E, Blais A, Spektor A, Scarpulla RC, Young R *et al*. (2004). A common set of gene regulatory networks links metabolism and growth inhibition. *Mol Cell* **16**: 399–411.

Cam H, Dynlacht BD. (2003). Emerging roles for E2F: beyond the G1/S transition and DNA replication. *Cancer Cell* **3**: 311–316.

Chen TT, Wang JY. (2000). Establishment of irreversible growth arrest in myogenic differentiation requires the RB LXCXE-binding function. *Mol Cell Biol* **20**: 5571–5580.

Cobrinik D. (2005). Pocket proteins and cell cycle control. *Oncogene* **24**: 2796–2809.

Dimova DK, Dyson NJ. (2005). The E2F transcriptional network: old acquaintances with new faces. *Oncogene* **24**: 2810–2826.

Eason DD, Coppola D, Livingston S, Shepherd AT, Blanck G. (2001). Loss of MHC class II inducibility in hyperplastic tissue in Rb-defective mice. *Cancer Lett* **171**: 209–214.

Frolov MV, Dyson NJ. (2004). Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. *J Cell Sci* **117**(Part 11): 2173–2181.

Iavarone A, King ER, Dai XM, Leone G, Stanley ER, Lasorella A. (2004). Retinoblastoma promotes definitive erythropoiesis by repressing Id2 in fetal liver macrophages. *Nature* **432**: 1040–1045.

Iovino F, Lentini L, Amato A, Di Leonardo A. (2006). RB acute loss induces centrosome amplification and aneuploidy in murine primary fibroblasts. *Mol Cancer* **20**: 5:38.

Ishida S, Huang E, Zuzan H, Spang R, Leone G, West M *et al*. (2001). Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. *Mol Cell Biol* **21**: 4684–4699.

Jegga AG, Gupta A, Gowrisankar S, Deshmukh MA, Connolly S, Finley K *et al*. (2005). CisMols Analyzer: identification of compositionally similar *cis*-element clusters in ortholog conserved regions of coordinately expressed genes. *Nucleic Acids Res* **33**: W408–W411.

Jegga AG, Sherwood SP, Carman JW, Pinski AT, Phillips JL, Pestian JP *et al*. (2002). Detection and visualization of compositionally similar *cis*-regulatory element clusters in orthologous and coordinately controlled genes. *Genome Res* **12**: 1408–1417.

Knudsen ES, Buckmaster C, Chen TT, Feramisco JR, Wang JY. (1998). Inhibition of DNA synthesis by RB: effects on G1/S transition and S-phase progression. *Genes Dev* **12**: 2278–2292.

Kouzarides T. (1995). Transcriptional control by the retinoblastoma protein. *Semin Cancer Biol* **6**: 91–98.

Le Buanec H, D'Anna R, Lachgar A, Zagury JF, Bernard J, Ittele D *et al*. (1999). HPV-16 E7 but not E6 oncogenic protein triggers both cellular immunosuppression and angiogenic processes. *Biomed Pharmacother* **53**: 424–431.

Lu Y, Ussery GD, Muncaster MM, Gallie BL, Blanck G. (1994). Evidence for retinoblastoma protein (RB) dependent and independent IFN-gamma responses: RB coordinately rescues IFN-gamma induction of MHC class II gene transcription in noninducible breast carcinoma cells. *Oncogene* **9**: 1015–1019.

Markey MP, Angus SP, Strobeck MW, Williams SL, Gunawardena RW, Aronow BJ *et al*. (2002). Unbiased analysis of RB-mediated transcriptional repression identifies novel targets and distinctions from E2F action. *Cancer Res* **62**: 6587–6597.

Mayhew CN, Perkin LM, Zhang X, Sage J, Jacks T, Knudsen ES. (2004). Discrete signaling pathways participate in RB-dependent responses to chemotherapeutic agents. *Oncogene* **23**: 4107–4120.

Morris EJ, Dyson NJ. (2001). Retinoblastoma protein partners. *Adv Cancer Res* **82**: 1–54.

Nevins JR. (2001). The Rb/E2F pathway and cancer. *Hum Mol Genet* **10**: 699–703.

Osborne A, Tschickardt M, Blanck G. (1997). Retinoblastoma protein expression facilitates chromatin remodeling at the HLA-DRA promoter. *Nucleic Acids Res* **25**: 5095–5102.

Osborne AR, Zhang H, Fejer G, Palubin KM, Niesen MI, Blanck G. (2004). Oct-1 maintains an intermediate, stable state of HLA-DRA promoter repression in Rb-defective cells: an Oct-1-containing repressosome that prevents NF-Y

binding to the HLA-DRA promoter. *J Biol Chem* **279**: 28911–28919.

Qin XQ, Livingston DM, Ewen M, Sellers WR, Arany Z, Kaelin Jr WG. (1995). The transcription factor E2F-1 is a downstream target of RB action. *Mol Cell Biol* **15**: 742–755.

Ren B, Cam H, Takahashi Y, Volkert T, Terragni J, Young RA *et al*. (2002). E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. *Genes Dev* **16**: 245–256.

Sage J, Miller AL, Perez-Mancera PA, Wysocki JM, Jacks T. (2003). Acute mutation of retinoblastoma gene function is sufficient for cell cycle re-entry. *Nature* **424**: 223–228.

Sherr CJ, McCormick F. (2002). The RB and p53 pathways in cancer. *Cancer Cell* **2**: 103–112.

Strobeck MW, Knudsen KE, Fribourg AF, DeCristofaro MF, Weissman BE, Imbalzano AN *et al*. (2000). BRG-1 is required for RB-mediated cell cycle arrest. *Proc Natl Acad Sci USA* **97**: 7748–7753.

Thomas DM, Carty SA, Piscopo DM, Lee JS, Wang WF, Forrester WC *et al*. (2001). The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Mol Cell* **8**: 303–316.

Thomas DM, Yang HS, Alexander K, Hinds PW. (2003). Role of the retinoblastoma protein in differentiation and senescence. *Cancer Biol Ther* **2**: 124–130.

Trouche D, Le Chalony C, Muchardt C, Yaniv M, Kouzarides T. (1997). RB and hbrm cooperate to repress the activation functions of E2F1. *Proc Natl Acad Sci USA* **94**: 11268–11273.

Vernell R, Helin K, Muller H. (2003). Identification of target genes of the p16INK4A–pRB–E2F pathway. *J Biol Chem* **278**: 46124–46137.

Wang JY, Knudsen ES, Welch PJ. (1994). The retinoblastoma tumor suppressor protein. *Adv Cancer Res* **64**: 25–28 5..

Welch PJ, Wang JY. (1995). Abrogation of retinoblastoma protein function by c-Abl through tyrosine kinase-dependent and -independent mechanisms. *Mol Cell Biol* **15**: 5542–5551.

Xin H, Pramanik R, Choubey D. (2003). Retinoblastoma (Rb) protein upregulates expression of the Ifi202 gene encoding an interferon-inducible negative regulator of cell growth. *Oncogene* **22**: 4775–4785.

Zhu X, Pattenden S, Bremner R. (1999). pRB is required for interferon-gamma-induction of the MHC class II abeta gene. *Oncogene* **18**: 4940–4947.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).