Characterization of the Retinoblastoma binding protein 2 (RBP2)

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Scientiam servimus et in hoc gaudium nostrum

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Abstract

The retinoblastoma (RB) family of proteins plays a pivotal role in cell cycle regulation. Its members, p105/Rb, p107 and p130, interact with the E2F and DP family of transcription factors to regulate transcription of essential cell cycle and DNA synthesis genes. Several reports have mapped the regulation of E2F by RB family members to the "pocket" domain of these proteins. We demonstrate here that RBP2, a pocket-binding protein that encodes multiple DNA-binding and protein-protein interaction domains, is a transcriptional repressor. Overexpression of RBP2 inhibits E2F-dependent transcription and inhibits cellular growth. The growth suppression activity of RBP2 could not be associated with a single domain within the protein but the transcriptional repression activity can be mapped to a minimal 17 kDa fragment located at the extreme N-terminus of RBP2 that can repress transcription when expressed alone but requires the C-terminus in the context of the full-length protein.

Résumé

Les protéines de la famille du rétinoblastome (RB) jouent un rôle essentiel dans le maintien du cycle cellulaire. Ses membres, p105/Rb, p107 et p130, interagissent avec les protéines des familles de facteurs de transcription E2F et DP pour réguler l'expression de gènes essentiels à la progression du cycle cellulaire et à la synthèse d'ADN. Plusieurs études ont associé la régulation de E2F par les protéines de la famille RB à une région conservée appelée "pocket". Nous démontrons ici que RBP2, une protéine se liant au "pocket" et composée de plusieurs domaines permettant d'interagir avec l'ADN et d'autres protéines, est un répresseur de transcription. La surexpression de RBP2 inhibe la transcription de gènes sous le contrôle de promoteurs E2F ainsi que la prolifération cellulaire. Aucun domaine spécifique de RBP2 n'a pu être associé à la suppression de la croissance cellulaire. Toutefois, le domaine de répression a été localisé sur un fragment de 17 kDa situé à l'extrémité N-terminale de RBP2. Ce dernier peut agir seul comme répresseur de transcription mais l'activité de répression de la protéine entière requiert aussi le domaine C-terminal.

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List of Abbreviations

AD	Adenovirus
APL	Aprotinin – Pepstatin – Leupetptin
ARID	AT-rich interacting domain
BSA	Bovine serum albumen
CAT	Chloramphenicol acetyl transferase
CDK	Cyclin-dependent kinase
CDK-I	Cyclin-dependent kinase inhibitor
CMV	Cytomegalovirus
CNS	Central nervous system
CYC	Cyclin
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E2F	Early region 2 factor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GST	Glutathione-S-transferase
HDA	Histone deacetylase
HSV	Herpes Simplex virus
IPTG	Isopropyl β-D-thiogalactopyranoside
KDA	Kilodalton

LXCXE	Leucine-X-Cysteine-X-Glutamic acid, where X can be any amino acid.
NEO	Neomycin
NLS	Nuclear localization signals
NTP	Nucleotide tri-phosphate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
PNS	Peripheral nervous system
RB	Retinoblastoma
RSV	Rous Sarcoma virus
SDS-PAGE	Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
TBP	TATA-box binding protein
TBS	Tris-buffered saline

Chapter 1: Introduction

Ordered progression through the cell cycle is essential for cellular homeostasis and deregulation of the cell cycle is a well-established hallmark of cancer. Regulatory checkpoint control mechanisms monitor intra-cellular and extra-cellular growth signals to ensure that one phase of the cycle cannot begin until a programmed series of events has occurred in the proper sequence. Periodic alterations in the levels and activity of key regulatory proteins, achieved by the phase-specific activation of gene expression and subsequent regulated degradation, is the means by which the timing of the cell cycle is maintained. Therefore, transcriptional regulation of cell cycle genes becomes a critical event in the control of cellular proliferation and mutations that affect checkpoint control pathways are found in all human cancers. (La Thangue, 1996; Sherr, 1996; Bernards, 1997; Herwig *et al.*, 1997; Sladek, 1997; DePinho, 1998; Helin, 1998; Donnellan *et al.*, 1999; Lavia *et al.*, 1999).

A significant checkpoint is the G_0-G_1-S transition, where a cell leaves its quiescent state in response to growth stimulation. The major regulatory protein families involved in this checkpoint will be reviewed below.

1.1 The RB family of proteins

The *Rb* gene is commonly deleted or mutated in a variety of human cancers, notably bilateral retinoblastoma in which most cases result from a germline mutation in one *Rb* allele and acquisition of somatic mutations in the second (Knudson, 1977).

Rb was cloned using retinoblastoma cells as an aid and subsequent studies with the viral proteins E1A and T antigen let to the cloning of the related family members *p107* and *p130* (Ewen *et al.*, 1991; Hannon *et al.*, 1993; Li *et al.*, 1993). Figure 1 shows all 3 members of the RB family. Amino acid analysis of p105/Rb, p107 and p130 shows that p130 and p107 form a subfamily with higher overall homology to each other when compared with Rb. p130 and p107 show about 50% amino acid identity with each other while only about 30% when individually compared with Rb. The A and B boxes are the regions of highest sequence homology among all the members of this family and sequences outside this region, while conserved between p107 and p130, show little homology with Rb. Furthermore, the spacer regions of p107 and p130 display 44% homology, whereas neither shares any significant sequence similarity with the Rb spacer (Dyson, 1994; Wang, 1997; Mulligan *et al.*, 1998).

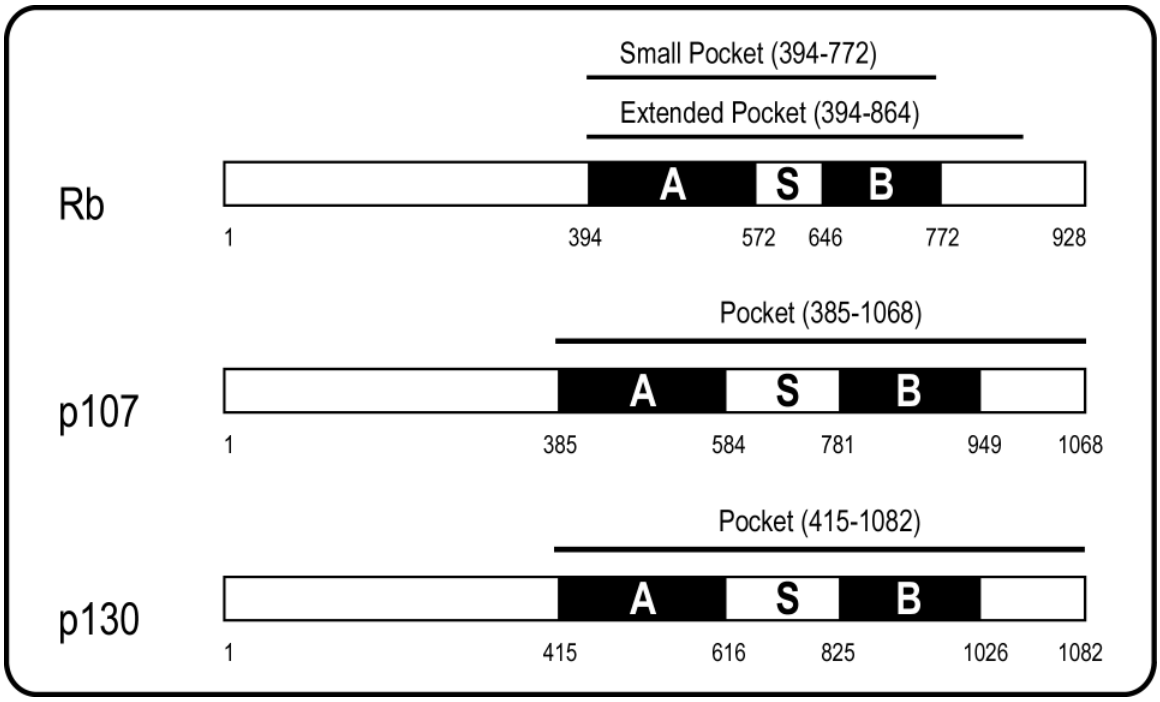


Fig. 1 RB family members. The functional domains are illustrated as boxes. This figure is not to scale. The A and B boxes, separated by the spacer (S) region form the pocket. LXCXE-containing proteins interact with the small pocket. E2F binding occurs in the extended pocket.

Mounting evidence indicates that the portion of RB that is central to its physiological role is the 45 kilodalton (kDa) "pocket" region, which is formed by interactions between A and B boxes. The crystal structure of the RB pocket has recently been resolved and demonstrates that RB-interacting proteins bind to a conserved groove on the B-box portion of the pocket via a conserved leucine-X-cysteine-X-glutamic acid (LXCXE, where X represents any amino acid) motif. The A-box is required and provides a scaffold for proper protein folding. The residues conserved across the family members cluster in 2 structural regions: the LXCXE binding site on the B-box and A-box/B-box interface, confirming that these domains are essential to the cellular functions of the pocket proteins (Lee *et al.*, 1998). Most natural mutations map to the regions of highest homology between the RB family members and involve gross changes such as deletions, frameshift, nonsense, and splice-site mutations that affect the structure of the pocket. Occasional missence mutations have been observed and map to the conserved residues of the A/B interface or the B-box (Yandell, 1989; Hu *et al.*, 1990; Huang *et al.*, 1990; Lee *et al.*, 1998).

Although all the members of the RB family are nuclear phosphoproteins, they can be distinguished by their cell-cycle-dependent expression profile and protein levels. p107 becomes the most prominent member of the RB family from late G_1 to G_2/M (Stiegler *et al.*, 1998) and its expression is strictly regulated by its E2F-dependent promoter. p107 is not found in quiescent (G_0) or differentiated cells but accumulates as cells enter the cell cycle (Nevins, 1998).

p130 shows an opposite expression pattern, as accumulation of p130 is a hallmark of a quiescent or differentiated cell and provides a functional distinction between G_0 cells and cells that arrest transiently in G_1. Although p107 and p130 have reciprocal expression patterns, their regulation mechanism is quite different. As stated previously, p107 expression is regulated through an E2F-dependent promoter while p130 expression levels are controlled post-transcriptionally, as *p130* mRNA levels are relatively constant in growing and quiescent cells. Following G_0 exit, phosphorylation of p130 is concurrent with its cytoplasmic relocalization and proteolytic degradation by the 26S proteosome (Verona *et al.*, 1997; Smith *et al.*, 1998). In contrast to the expression patterns of p107 and p130, which are directly linked to a cellular growth state, the expression level of Rb is relatively constant, although there is a slight increase in mid-to-late G_1. This likely reflects the parallel increase in E2F transcription factor levels and the fact that the Rb promoter is E2F-responsive. (Shan *et al.*, 1994; Smith *et al.*, 1996; Nevins *et al.*, 1997; Smith *et al.*, 1998)

1.2 The E2F transcription factor family

Several viral families have devised mechanisms that promote S-phase entry as a preamble for viral replication. The genomes of SV40 and human papillomavirus type 16 (HPV), members of the papovaviridae family, encode proteins that perform such a task. Both the SV40 large T antigen (TAg) and HPV 16 E7 are essential for viral replication and have been shown to bind and inactivate RB family members through LXCXE-dependent interactions (DeCaprio *et al.*, 1988; Dyson *et al.*, 1989).

The human adenovirus (Ad) type 5 protein E1A has a similar function, among others (Whyte *et al.*, 1988). Studies on adenovirus infection led to the discovery of the E2F transcription factors. Following infection, E1A activates transcription from the Ad E2 promoter by releasing active E2F from inhibitory complexes (Bagchi *et al.*, 1990). The region of E1A required for E2 transactivation mapped to the region that interacted with the RB family members (Raychaudhuri *et al.*, 1991). Although the E2F transcription factor family is central to the G_1/S checkpoint, it is not a frequent target of mutation in human cancers. Loss of Rb function has a broader impact on cellular homeostasis. As such, Rb and its upstream regulators are mutated more frequently than the downstream targets of Rb, such as E2F (Herwig *et al.*, 1997; Helin, 1998; Muller *et al.*, 2000). RB family proteins may bind an array of cellular and viral proteins, but none are more important than members of the E2F family of transcription factors (Bernards, 1997; Dyson, 1998; Helin, 1998).

E2F is found both free and in complex with RB members. E2F is actually a heterodimer of two proteins, E2F and DP. Seven individual E2F and three DP proteins have been identified. E2F/DP heterodimers (henceforth referred to collectively as E2F) can be formed with either DP protein. The 3 DP proteins come from 2 genes as DP2 has 2 splice variants (Lavia *et al.*, 1999). Figure 2 illustrates that the various E2F members can be subdivided into 3 sub-families based on functional characteristics.

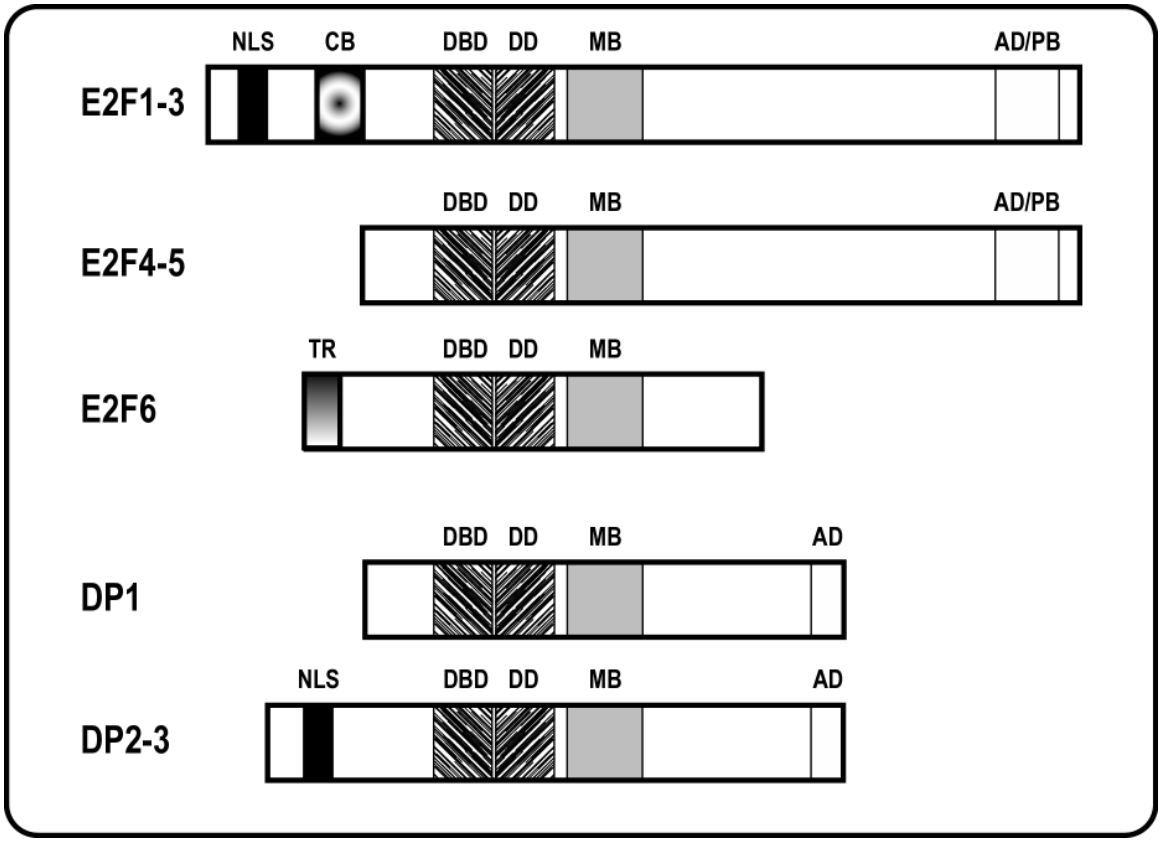


Fig. 2 E2F/DP family. The functional domains are illustrated as boxes. This figure is not to scale. NLS: nuclear localization signal. CB: cyclin A/cdk2 binding site. DBD: DNA binding domain. DD: dimerization domain. MB: marked box, a domain that can interact with RB. AD: acidic activation domain. PB: pocket protein binding domain. TR: transcriptional repression domain. In E2F1-5, the PB domain overlaps with the AD domain. E2F3 refers to the two splice variants, E2F3a and E2F3b, unless noted otherwise. Adapted from Lavia *et al.*, 1999.

E2F1-5 contain a C-terminal transactivation domain that is lacking in E2F6 (Morkel *et al.*, 1997; Trimarchi *et al.*, 1998). Heterodimerization results both in sequence-specific DNA binding derived from conserved DNA binding domains (DBD) in both E2F and DP, and potentiation of the activation domain.

Protein levels vary among the members of the E2F family. E2F4-5 are relatively constant throughout the cell cycle while E2F1-3a expression is absent in G_0 and induced in G_1.

This reflects an autoregulatory mechanism as the promoters of E2F1-3a are E2F-responsive (Dyson, 1998; Nevins, 1998; Lavia *et al.*, 1999). Interestingly, although the expression profile of E2F3a is under cell-cycle control, that of E2F3b, a product encoded from an intronic promoter within the *E2F3* locus, is similar to the constitutive expression pattern of E2F4-5 (Adams *et al.*, 2000; Leone *et al.*, 2000). E2F localization can be either nuclear or cytoplasmic. E2F1-3 contain nuclear localization signals. E2F4-5 are cytoplasmic but can be targeted to the nucleus by interacting with p107, p130, or with DP2 as some splice variants contain a NLS.

E2F activity is regulated at many levels. Cell cycle expression has already been mentioned. A major level of regulation is complex formation with RB family members. The various E2Fs bind differentially to RB family members, as shown in table 1.

Table 1. RB-E2F complexes (Ikeda *et al.*, 1996; Nevins, 1998; Lavia *et al.*, 1999)

RB family member	Interacting E2F
Rb	E2F1-4, with preference for E2F1-3
p107	E2F4-5
p130	E2F4-5

Association with different members has opposite effects on E2F-dependent transcription. Free E2F1-3 have the intrinsic ability to enter the nucleus and act as transcription activators while E2F4-5 only reach the nucleus and act as activators if they interact with a DP containing a NLS. If they interact with p107 or p130, they become repressors. Subcellular localization provides another level of E2F control. The biological activity of E2F4-5 is critically dependent on regulated relocalization during the cell cycle (Magae *et al.*, 1996; Lindeman *et al.*, 1997; Muller *et al.*, 1997).

E2F activity is also regulated by protein stability and turnover. Free E2F is very labile and rapidly degraded by the ubiquitin pathway, a process that is inhibited by interactions with RB family members (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). Phosphorylation of E2F and DP proteins regulates the DNA binding potential of E2F and its significance will be discussed in greater detail later on. Acetylation by P/CAF and by p300/CBP has been shown to increase the DNA binding ability, activation potential and protein half-life of E2F1 (Martinez-Balbas MA *et al.*, 2000). Finally, CpG methylation provides another level of E2F activity regulation, as methylation of E2F promoter elements can inhibit binding of any or all E2F proteins in a promoter-specific fashion (Campanero *et al.*, 2000). E2F-dependent transcription is regulated by a complex interplay of the processes mentioned above. For example, p130 binds E2F4-5 in G_0, which increases their stability, ensures proper nuclear localization and represses expression of E2F1-3, as well as several other cellular genes (Muller *et al.*, 2000).

1.2.1 E2F-Dependent gene expression

E2F-responsive genes fall into 2 classes: genes involved in DNA biosynthesis and genes encoding cell cycle regulatory proteins. Refer to table 2 for a partial list.

Table 2. E2F Responsive genes

Regulatory Genes		DNA Biosynthesis Genes	
Rb, p107	cycA, cycE	DNA Polymerase α	TK
E2F1, E2F2, E2F3a	B-myb	DHFR	PCNA
cdc2, cdc6,	c-myc	Histone H2A	RRM2
cdc25A, cdc25C		ORC1	SRP20
		TS	

Source: (Sladek, 1997; Helin, 1998; Lavia *et al.*, 1999; Vigo *et al.*, 1999)

These 2 classes show different patterns of regulation by E2F. Genes involved in DNA biosynthesis are all transactivated by E2F in late G_1 while those involved in cell cycle regulation are controlled by distinct mechanisms. For example, cycE is transactivated by E2F in G_1 while cycA and p107 are repressed in G_0 and become derepressed at the G_1/S transition (Lavia *et al.*, 1999).

Footprinting studies have shown that promoter occupancy mirrors the genetic effect of E2F-responsive promoters. E2F promoters silent in G_0 (e.g. cdc2) show *in vivo* E2F binding during G_0 but this binding disappears when the cell enters a proliferative state (Tommasi *et al.*, 1995). In contrast, promoters for genes activated by E2F (e.g. DHFR) become bound by E2F at the G_1/S transition (Wells *et al.*, 1996). The G_1 transactivation of certain E2F promoters might be attributed to E2F1-3a, which are themselves subject to activation in this phase of the cell cycle. Negative control of transcription in G_0 may be dictated by E2F3b-4-5, which act to recruit a transcriptional repression function through their association with Rb and p130. E2F3b/Rb and p130/E2F4-5 complexes are hallmarks of cellular quiescence (Sardet *et al.*, 1995; Smith *et al.*, 1996; Lavia *et al.*, 1999; Leone *et al.*, 2000).

E2F1-5 complexes have been shown to activate overlapping sets of promoters containing minor variants of the consensus E2F binding site that affect the binding affinity of the various E2F family members (DeGregori *et al.*, 1997; Tao *et al.*, 1997).

It is assumed that each has a unique, if redundant, range of specificity that may be enhanced by interactions at the promoter with other transcription factors from other regulatory pathways (Schulze *et al.*, 1995; Karlseder *et al.*, 1996; Lavia *et al.*, 1999). As E2F6 lacks an activation domain, it may function in a dominant negative fashion to block E2F activation by occupying E2F-responsive promoters (Morkel *et al.*, 1997; Cartwright *et al.*, 1998; Trimarchi *et al.*, 1998).

1.3 The RB family and growth regulation

The activity of RB family members is governed by the interplay of many different regulation mechanisms, including cell-cycle dependent expression, localization, stability, interaction with regulatory proteins, post-transcriptional and post-translational modifications.

1.3.1 Cyclin-dependent kinases and the role of phosphorylation

The phosphorylation status of Rb has been investigated in depth and has been shown to change from a hypo-phosphorylated to a hyper-phosphorylated state as the cell cycle progresses. Hypo-phosphorylated Rb exists only in early G_1. Past this point, hyper-phosphorylated forms of Rb accumulate and are maintained in this state until late M phase, where they are dephosphorylated in preparation for the next G_1 phase (Ludlow *et al.*, 1990; Mittnacht *et al.*, 1994; Chew *et al.*, 1998).

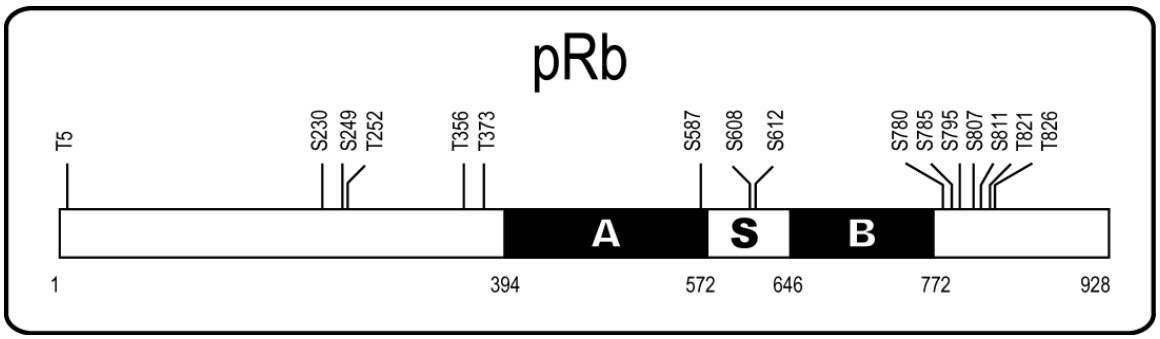


Fig. 3 Phosphorylation sites on Rb. Numbers represent amino acid residues. S: serine. T: threonine. This figure is not to scale. Adapted from Kaelin, 1999.

As illustrated in figure 3, the human Rb protein contains 16 cyclin-dependent kinase (cdk) consensus sites. It is unclear if all sites become phosphorylated but evidence indicates that differentially phosphorylated forms of Rb exists at different times during the cell cycle and regulate the interactions of different proteins with Rb. For example, phosphorylation at T-821 or T-826 disrupts LXCXE interactions, while phosphorylation at S-807, S-811, T-821, T-826 and S-780 disrupt E2F binding (DeCaprio *et al.*, 1992; Mittnacht *et al.*, 1994; Knudsen *et al.*, 1996; Knudsen *et al.*, 1997). It has also been shown that different CDKs phosphorylate Rb at different sites (Kitagawa *et al.*, 1996; Zarkowska *et al.*, 1997; Harbour *et al.*, 1999). Finally, phosphorylation by different CDKs may regulate Rb functions at different stages of the cell cycle.

The importance of Rb at the G_1/S transition has been well established, but recent work has demonstrated that Rb also seems to regulate progression through S-phase by interacting with the DNA synthesis machinery and potentially preventing complete genome replication (Chew *et al.*, 1998; Knudsen *et al.*, 1998).

Only the hypo-phosphorylated forms of the RB family members can bind E2F (Mudryj *et al.*, 1991; Dynlacht *et al.*, 1994). As G_1 progresses, the pocket proteins become hyper-phosphorylated, which is achieved through sequential interactions with multiple cyc/cdk complexes. Binding of cycD/cdk4/6 to Rb is a major initiating event in response to growth stimulation and occurs in early G_1 (Kato *et al.*, 1993; Connell-Crowley *et al.*, 1998; Dyson, 1998). Phosphorylation by cycD/cdk4/6 is required for subsequent phosphorylation and inactivation of Rb (Lundberg *et al.*, 1998). Cyclin D (D1, D2 or D3) binds to Rb via its LXCXE motif (Dowdy *et al.*, 1993) and the associated cdk phosphorylates residues S-807, S-811, T-821 and T-826 at the C-terminus of Rb. This primary phosphorylation initiates a sequence of intramolecular interactions between the C-terminus and the pocket, inhibiting LXCXE-protein binding in the process. This conformational shift not only provides a binding site for cycE/cdk2 but also allows access to S-567, which is normally buried in the A/B-interface. Phosphorylation of this residue disrupts the structure of the pocket and abolishes Rb-E2F binding (Harbour *et al.*, 1999). This sequential loss of function provides a model to explain the differential regulation of genes involved in cell cycle progression.

In this scenario, cycD expression is stimulated by mitogenic factors and phosphorylates Rb. This causes the release of protein complexes from the pocket that actively repress transcription of downstream effector promoters, such as the cycE promoter. The increased levels of cycE activate cdk2, which phosphorylates Rb in late G_1 and leads to its release from E2F. The cycA promoter is E2F-responsive and thus becomes active (Sherr, 1996; Knudsen *et al.*, 1999; Zhang *et al.*, 1999).

CycA/cdk2 activity is maximal in S-phase, where it phosphorylates both E2F and DP, causing their release from their DNA cognate elements. This has been shown to be an essential step for S-phase exit (Bernards, 1997; Dynlacht *et al.*, 1997; Dyson, 1998). This elegant cascade exemplifies how cyc/cdk complexes act to both stimulate and then inactivate E2F as the cell passes from G_1 to S-phase. The role of Rb in transcription repression will be expanded later on.

CycA and cycE and their associated cdks, mostly cdk2, can associate with p107 and p130 through a conserved binding site located in the spacer region. The function of such complexes is still unclear, but up to 50% of p107 is found associated with cycA/cdk2 or cycE/cdk2 and different complexes are present at different stages of the cell cycle (Dyson, 1994). Recent data suggest that p107 and p130 recognize, or form by association, a distinct pool of cycA/cdk2 that preferentially phosphorylates RB family members (Hauser *et al.*, 1997). Phosphorylation of p130 by these complexes in mid-to-late G_1 targets it to degradation by the 26S proteosome and is the mechanism by which p130 disappears after growth stimulation (Smith *et al.*, 1998).

CDKs are present at constant levels throughout the cell cycle. CDK kinases, such as cycH/ckd7, and phosphatases, such as cdc25A or cdc25C, can activate and inhibit CDK activity via reversible phosphorylation on specific subsets of residues (Hengstschlager *et al.*, 1999). CDK inhibitors (CDK-I) provide an additional level of regulation.

Two families of CDK-Is exist, those that can inhibit a broad range of cyclin/cdk complexes, such as $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$, and those that only inhibit cdk4 and cdk6, namely the proteins of the INK4 locus (Roussel, 1999; Sherr *et al.*, 1999). Association with a cyclin regulatory subunit also modulates CDK activity. Cyclin expression is cell-cycle dependent and provides a mechanism to synchronize and regulate various cellular events. For example, cyclin D is expressed following mitogenic stimulation to allow G_0 exit (Connell-Crowley *et al.*, 1998), cyclin E allow R-point transition in mid-to-late G_1 (Harbour *et al.*, 1999) while cylin A is essential for S-phase exit (Krek *et al.*, 1995). Cyclins have a short half-life and are readily degraded by the ubiquitin degradation pathway. The interplay of these regulatory processes is presumed to be responsible for the sharp transitions between the individual cell cycle phases (Hengstschlager *et al.*, 1999; Lavia *et al.*, 1999).

1.3.2 RB and E2F in the cell cycle

Figure 4 recapitulates the expression patterns of the RB and E2F family members, while Figure 5 provides a more detailed account of Rb/E2F complexes at the G_1/S checkpoint. Complexes containing mainly E2F4/5 bound primarily to p130 and E2F3b/Rb to a lesser extent characterize cells in G_0. Growth stimulation induces cycD expression, which initiates the phosphorylation cascade of Rb and p130 and coincides with the appearance of cycE. The phosphorylation of Rb by cycE/cdk2 is termed the restriction (R)-point, prior to which the cell can still exit the cell cycle under the influence of anti-mitogenic signals.

Past this point, the cell is committed to the cell cycle and no longer requires continual mitogenic stimulation, although other regulatory checkpoints exist downstream such as the G_2/M checkpoint that monitors genomic integrity before mitosis (Clarke *et al.*, 2000; O'Connell *et al.*, 2000). Phosphorylated p130 is translocated out of the nucleus and subsequently degraded while the phosphorylation of Rb derepresses E2F4/5, which are targeted to the cytoplasm in G_1/S.

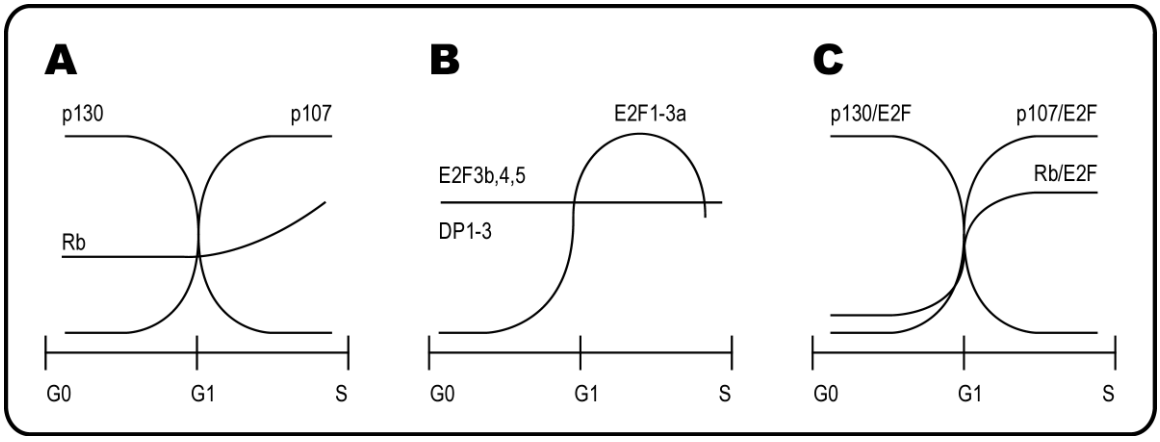


Fig. 4 Expression profile of RB and E2F family members. Panel A illustrates the expression profile of the pocket protein family members. The reciprocal expression pattern of p130 and p107 is well apparent during the G_o-G_1-S transition. Rb levels, although relatively constant, show a slight increase in G_1, which is probably concurrent with increased expression of E2F1. Panel B depicts the expression profile of E2F and DP proteins. As mentioned above, only E2F1-3a show cell-cycle dependent expression. Panel C shows that as the cell progresses through S-phase from G_0, predominantly p130/E2F are replaced with p107/E2F and Rb/E2F complexes. This figure is not to scale and the relative levels pictured above are in no way quantitative. Adapted from Nevins, 1998.

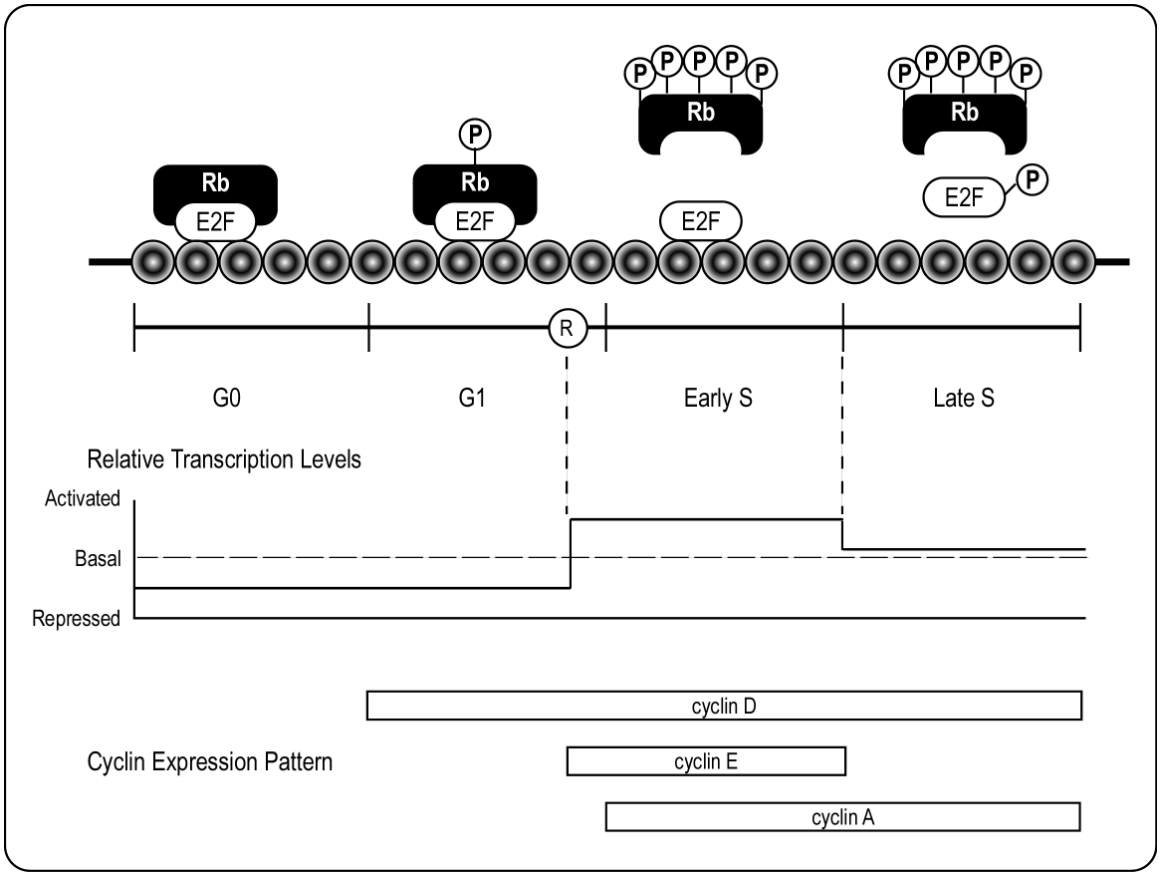


Fig. 5 Rb/E2F interactions at the G_1/S transition. Rb/E2F complex formation is regulated by cell-cycle dependent phosphorylation by cyclin/cdk complexes. For clarity, E2F represents E2F/DP heterodimers. In G_0, Rb/E2F complexes actively repress E2F-dependent genes below basal levels. Cyclin D expression, driven by mitogenic signals, initiates Rb phosphorylation. Expression and activation of cyclin E corresponds to Restriction (R)-point passage, after which mitogenic stimulation is no longer required. Hyper-phosphorylated Rb dissociates from E2F to allow expression of DNA synthetic and other regulatory genes under E2F control. Phosphorylation of E2F and DP by cycA-regulated CDKs releases E2F from its DNA element in late S-phase. Adapted from Kaelin, 1999.

Derepression of E2F4-5 allows E2F-responsive genes to be transcribed in a synergistic fashion as increasing amounts of E2F1-3a are synthesized. p107/E2F/cycE/cdk2 complexes appear in G_1/S and are replaced by p107/E2F/cycA/cdk2 in S-phase. Although quite prevalent, the actual physiological relevance of these complexes remains to be determined (Nevins, 1998; Stiegler *et al.*, 1998).

Dissociation of E2F from its cognate DNA element is required for S-phase exit and is achieved by phosphorylation of residues on E2F and DP by cycA-regulated CDKs. Overexpression of E2F1-5 causes entry into S-phase, presumably by activation of rate-limiting genes (Bernards, 1997; Dyson, 1998; Helin, 1998). However, the unscheduled presence of E2F on critical DNA sequences in S-phase will activate S-phase checkpoints that will either induce cell-cycle arrest or apoptosis. Overexpression of E2F1 will induce S-phase entry but also induces apoptosis, suggesting that S-phase exit requires termination of E2F activity (Krek *et al.*, 1995).

1.3.3 Rb and terminal differentiation

Terminal differentiation describes a state wherein a cell permanently exits the cell cycle and acquires a distinct phenotype by expressing a tissue-specific subset of genes. The importance of Rb in differentiation was observed in knockout mice. *Rb* $^{-/-}$ mice die *in utero* at day 13-15 post-conception owing to aberrant erythropoietic and neural development (Clarke *et al.*, 1992; Jacks *et al.*, 1992), while wild-type mice show a marked increase in Rb levels in the corresponding tissues during this time. Furthermore, loss or inactivation of Rb by oncoviral proteins can reverse terminal differentiation and return the cell to an actively cycling state (Lee *et al.*, 1994; Feddersen *et al.*, 1995; Tiainen *et al.*, 1996). The mechanism of differentiation was determined using neuronal and myeloid cell lines, which can be induced to differentiate. This system demonstrated that cells must first arrest in G_0/G_1 before the differentiation pathway can be initiated and Rb is the key regulatory element (Herwig *et al.*, 1997).

Elevated levels of hypophosphorylated Rb are observed at the onset of differentiation and are maintained by increased transcription of Rb and p21, a cdk inhibitor which blocks cycD/cdk4/6 phosphorylation (Halevy *et al.*, 1995; Herwig *et al.*, 1997). Active Rb plays a dual role at this point: it binds all E2F heterodimers to inactivate E2F-dependent transcription and interacts directly or indirectly with transcription factors, such as MyoD and myogenin in muscle cells, C/EBPβ and NL-IF6 in monocyte/macrophage precursors and NF-IL6 in adipose tissue (Chen *et al.*, 1996; Chen *et al.*, 1996), to enhance transcriptional activity of tissue-specific genes. MyoD and NF-IL6 have been shown to induce p21 expression, which prevents Rb re-phosphorylation and cell-cycle re-entry.

Increased p21 expression is also observed in myeloid and neuronal differentiation, although the mechanism remains unclear (Macleod *et al.*, 1995). The coordinated action of Rb and other transcriptional activators induces a positive feedback loop resulting in an increase in the active form of Rb. This model is believed to illustrate one of the mechanisms by which Rb can couple cell cycle exit and terminal differentiation (Lipinski *et al.*, 1999). As mentioned previously, Rb has the distinct ability to bind to most E2F family members, suggesting a mechanism by which rapid shut-off of E2F-responsive promoters by Rb is required for initiation of differentiation, followed by a shift from Rb/E2F complexes to p130/E2F complexes, which characterize a differentiated cell (Ikeda *et al.*, 1996). The role of p107 and p130 in terminal differentiation is still unclear.

Murine knockouts of *p107* or *p130* do not produce any detectable developmental defects. However, the *p130*$^{-/-}$ *p107*$^{-/-}$ double knockout shows defects in bone formation and dies shortly after birth (Cobrinik *et al.*, 1996). This would implicate p107 and p130 in bone development in a role that cannot be compensated by Rb. Overexpression of p107 or p130 in *Rb* $^{-/-}$ muscle precursors can functionally rescue MyoD transactivation to some extent, however only early markers of muscle differentiation are observed. Rb is required for complete muscle differentiation (Gu *et al.*, 1993).

1.3.3 Rb and E2F in apoptosis

A growing body of evidence suggests a role for Rb in the suppression of cellular apoptosis. As mentioned previously, *Rb* $^{-/-}$ mice die *in utero* and show specific tissue abnormalities further accompanied by increased cell death in the CNS, PNS and in the lens (Mulligan *et al.*, 1998). Additional studies with DNA tumor viruses further demonstrate that the inactivation of Rb through interaction with viral proteins such as Ad5 E1A induce apoptosis (Liu *et al.*, 1996; Putzer *et al.*, 2000). The loss of Rb function has been shown to induce both p53-dependent and p53-independent apoptosis, but the exact mechanisms remain unclear (Macleod *et al.*, 1996). A major consequence of Rb deficiency, through mutation or viral inactivation, is the deregulation of E2F, which may play an important role in apoptosis initiation.

Interestingly, although all E2F family members can induce S-phase entry when overexpressed, only the overexpression of E2F-1 induces apoptosis (Qin *et al.*, 1994; Kowalik *et al.*, 1995; Lukas *et al.*, 1996; Muller *et al.*, 1997). The regulated suppression of E2F-1 DNA binding, mediated through cycA/cdk2 phosphorylation, is required to suppress the apoptotic response and allow S-phase entry (Krek *et al.*, 1995). E2F-1 mutants that no longer posses a transactivation domain but retain their DNA binding ability can still induce apoptosis, indicating that S-phase entry and apoptosis induction are separate functions (Hsieh *et al.*, 1997; Phillips *et al.*, 1997).

This suggests a mechanism by which Rb prevents apoptosis by regulating E2F-1 activity and improper E2F-1 activation may function to induce an apoptotic pathway that is normally blocked. Indeed, *Rb* $^{-/-}$ *E2F-1* $^{-/-}$ double knockout mice show a significant decrease in apoptosis in the lens and CNS when compared to *Rb* $^{-/-}$ mice at the same developmental stage. Surprisingly, the loss of E2F-1 had less effect on apoptosis in the PNS of the double knockout, indicating a possible tissue-specific mechanism (Tsai *et al.*, 1998).

The exact function of E2F-1 in apoptosis remains unclear as both *E2F-1* $^{-/-}$ knockout mice and transgenic mice overexpressing E2F-1 show testicular atrophy as a result of increased apoptosis (Field *et al.*, 1996; Yamasaki *et al.*, 1996; Holmberg *et al.*, 1998). Furthermore, overexpression of E2F-1 can lead to cellular transformation (Miyajima *et al.*, 1996), yet the *E2F-1* $^{-/-}$ mouse phenotype leads to the development of a broad and unusual spectrum of tumors (Yamasaki *et al.*, 1996).

The apparent contradiction that E2F-1 is both a tumor suppressor and a proto-oncogene may be explained by considering that it can transactivate a panel of genes in the absence of Rb and transrepress the same panel when complexed with it (Lavia *et al.*, 1999).

A possible mechanism for E2F-1 mediated p53-dependent apoptosis has been advanced. E2F-1 induces the expression of $p19^{ARF}$, a splice variant of the CDK-I $p16^{INK4A}$ protein. Although both proteins are immunologically and functionally unrelated, they share common exons and both are induced by E2F-1 (Roussel, 1999). Thus, deregulated E2F-1 leads to an increase in $p19^{ARF}$ levels, which abrogate the mdm2-induced degradation of p53 and the mdm2-dependent transcriptional silencing of p53. The stabilization and derepression of p53 could then induce apoptosis (DeGregori *et al.*, 1997; Haines, 1997; Kowalik *et al.*, 1998; Zhang *et al.*, 1998; Muller *et al.*, 2000). However, this model does not explain p53-independent apoptosis, nor does it address why E2F-1 transactivation defective mutants can still induce apoptosis.

Rb has also been implicated in apoptosis by its ability to interact with mdm2 and by its identification as a target for caspases, cysteine proteases that are essential mediators of apoptosis. In the progression of apoptosis, 42 amino acids are cleaved from the C-terminus of Rb, a region associated with mdm2 interactions (Janicke *et al.*, 1996). The binding of Rb to mdm2 is required for Rb to overcome both the anti-apoptotic function of mdm2 and the mdm2-dependent degradation of p53 (Hsieh *et al.*, 1999).

It should be noted that Rb cleavage is not observed in all apoptotic pathways and could be a death signal-specific response (Tan *et al.*, 1997).

The function of p107 and p130 in apoptosis, if any, is unclear. The original studies involving *p107* or *p130* knockout surprisingly produced no detectable phenotype, (Cobrinik *et al.*, 1996; Lee *et al.*, 1996; Mulligan *et al.*, 1998) although recent work has shown that the *p130* $^{-/-}$ mutation is embryonic lethal at day 11-13 post conception with increased apoptosis in the neural tube, brain and dermomyotome and showed increased proliferation in the CNS (LeCouter *et al.*, 1998). Similarly, another recent study showed that *p107* $^{-/-}$ mice are viable but display severe growth defects (LeCouter *et al.*, 1998). The different observations seem to be attributable to the strains of mice used and possibly reflect the existence of modifier genes that can alter their function. Additionally, p107 and p130 may not be targeted by caspases as the cleavage site, while highly conserved across species for Rb, is absent in both protein (Tan *et al.*, 1998). However, while p107 and p130 have not been observed as components in apoptotic pathways, they cannot be discounted as possible contributors.

1.4 Transcription activation and repression by the RB family

1.4.1 Transcriptional activation by Rb or E2F

Transcriptional activation by Rb was observed in terminal differentiation pathways. The mechanisms of Rb-dependent transactivation differ between cell lineages but usually imply an Rb-dependent potentiation of the tissue-specific transcription factor. Direct binding of Rb to NF-IL6 and C/EBPβ improves their DNA-binding activity and enhances transcription from their responsive promoters (Chen *et al.*, 1996). Rb, when complexed with MyoD, considerably enhances its transcriptional activity and is believed to cooperate in the MyoD-dependent activation of MEF2, a transcription factor necessary for downstream gene expression in the muscle differentiation pathway (Gu *et al.*, 1993).

Early work on the role of Rb in E2F-driven transcription lead to a simple model where binding of E2F to RB-family members via the pocket repressed transcription by masking the activation domain of E2F (Helin *et al.*, 1993). Hence, phosphorylation of Rb would relieve Rb-mediated repression and activate E2F. Noteworthy corroboration for this model comes from the fact that E2F site mutations in the DHFR promoter, known to be under E2F control, significantly reduce the levels of protein expression (Wade *et al.*, 1995). E2F has also been shown to interact cooperatively with other transcription factors such as SP-1 (Karlseder *et al.*, 1996), can bind with elements of the basal transcription machinery such as the TATA-box binding protein (TBP) and TFIIH (Pearson *et al.*, 1997) and can also interact with the transcriptional co-activators P/CAF and P300/CBP (Fry *et al.*, 1999; Martinez-Balbas MA *et al.*, 2000). Finally, the minimal activation domain of E2F can efficiently activate transcription when fused with a heterologous DNA binding domain (Pearson *et al.*, 1997). The activation domain also overlaps with the Rb-binding domain, further supporting this simple model Rb action.

1.4.2 Transcriptional repression

Early evidence of a possible transcriptional repression function associated with Rb/E2F complexes was obtained through Rb overexpression studies and reporter assays using synthetic E2F-responsive promoters. In these studies, the addition of E2F-binding sites could not only silence otherwise active promoters but Rb was shown to repress transcription to below-basal levels (Weintraub *et al.*, 1992). Further studies mapped this repression activity to the pocket of Rb, as overexpression of the pocket region fused to a heterologous DBD was sufficient to duplicate the repression function of the wild type protein (Adnane *et al.*, 1995; Weintraub *et al.*, 1995).

Rb-mediated transcriptional repression could be abolished by phosphorylation of Rb, which releases Rb from E2F complexes and also inhibits interactions with basal transcription factors such as TBP, TFIID and $TAF_{II}250$ (Chow *et al.*, 1996a; Chow *et al.*, 1996b; Siegert *et al.*, 1999). A fundamental question remained concerning the mechanism of Rb repression. An early model suggested that, since the Rb binding site on E2F overlaps with the activation domain, Rb could repress E2F-dependent transcription simply by masking the activation domain of E2F (Helin *et al.*, 1993). However, this model could not explain the repression observed in E2F-dependent promoters where the E2F sites act as negative regulatory elements that, when deleted, lead to an increase in gene expression through a relief from repression (Dalton, 1992; Dyson, 1998).

Furthermore, the ability of Rb to bind to E2F does not appear to be sufficient for transcriptional repression, as Rb mutants that interact with E2F but fail to repress E2F-driven transcription efficiently have been identified (Sellers *et al.*, 1998). This strongly argues that Rb can repress transcription in an E2F-independent manner.

Recent progress in the field of transcriptional regulation has highlighted the significant role of histone modification by histone acetyl transferases (HATs) and their antagonists histone deacetylases (HDACs). The yeast Rpd3 protein was the first HDAC to be characterized and has been shown to have 3 mammalian homologs, HDAC1-3. To date, several other families of HDAC families have been identified in yeast, including the HDA and HOS families. Homology searches have revealed the existence of mammalian counterparts for several of these proteins (reviewed in Cress *et al.*, 2000). Several well-characterized DNA binding transcription factors have been shown to repress by recruiting multimeric protein complexes containing HDAC family members. Unliganded human nuclear hormone receptors such as the thyroid hormone receptor (TR) or the retinoic acid receptor (RAR) both silence transcription by recruiting the Sin3-HDAC complex through other linker proteins such as the nuclear hormone receptor co-repressor (NCoR) and the silencing mediator of retinoid and thyroid hormone receptor (SMRT) (Xu *et al.*, 1999). Other transcription factors that interact with HDACs include p53 (Murphy *et al.*, 1999), YY1 (Yang *et al.*, 1997), yeast Ume6 (Kadosh *et al.*, 1997), *Drosophila* co-repressor Groucho (Chen *et al.*, 1999), SP1 (Doetzlhofer *et al.*, 1999), the breast cancer susceptibility gene 1 (BRCA1) (Yarden *et al.*, 1999) as well as several others (Cress *et al.*, 2000).

Although the correlation between histone deacetylation and transcriptional silencing has been established (Kadosh *et al.*, 1998; Struhl, 1998), the question of whether repression is achieved by inhibiting the formation of a transcription initiation complex or by inducing a higher-order DNA conformation that inhibits transcription remains unanswered. Furthermore, although the core histone proteins are the major physiological targets of the HDAC family members through their association with sequence-specific DNA binding proteins, deacetylation of other proteins, such as basal transcription factors, may play a part in the overall regulatory mechanism. Conversely, deacetylation of these proteins may have a synergistic effect with nucleosome remodeling to silence transcription.

A breakthrough was made towards elucidating the mechanism of Rb repression when it was discovered that the Rb pocket interacted with HDAC1 while tethered to DNA via E2F to form an active repression complex that regulates transcription through chromatin remodeling. (Brehm *et al.*, 1998; Ferreira *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Zhang *et al.*, 1999).

It was originally proposed that HDAC 1 and 2 could interact directly with the pocket of Rb via a degenerate IXCXE motif or be recruited through RBAP48/46, two proteins that co-purified with HDACs and did not contain an LXCXE motif but could still interact with the C-terminal portion of the extended pocket of Rb (Qian *et al.*, 1995; Nicolas *et al.*, 2000).

Work from our group has recently demonstrated that Rb predominantly recruits HDAC activity via the retinoblastoma binding protein 1 (RBP1). RBP1, a protein first characterized by its ability to interact with the Rb pocket via its LXCXE motif (Fattaey *et al.*, 1993), has two independent repression domains and can repress E2F-dependent transcription through HDAC-dependent and -independent mechanisms. The R1 domain, whose exact repression mechanism remains unclear, contains an AT-rich interacting domain (ARID) domain. The R2 domain of RBP1 can interact with all of the members of the HDAC family, being one of the first Rb binding proteins to show this potential (Lai *et al.*, 1999).

Covalent modification of histones and other proteins is not the only mechanism by which Rb regulates transcription. As mentioned above, the RBP1 corepressor has an intrinsic HDAC-independent repression domain. BRG1 and hBRM are two proteins that contain LXCXE motifs and bind to the pocket of Rb. They are also components of the mammalian SWI/SNF chromatin-remodeling complex that uses ATP to modify local DNA topology. BRG1 and hBRM have been shown to cooperate with Rb to repress E2F activity through an ATP-dependent mechanism (Trouche *et al.*, 1997; Muchardt *et al.*, 1999). Rb can also bind and inhibit $TAF_{II}250$, a component of TFIID, through domains that do not involve LXCXE motifs. $TAF_{II}250$ possesses both HAT and kinase activities that are required for formation and activation of the pre-initiation complex (Mizzen *et al.*, 1996; Siegert *et al.*, 1999). Finally, the LXCXE-containing Rb-interacting zinc finger (RIZ1) protein has been shown to repress transcription through a PR domain (Xie *et al.*, 1997).

This domain mediates protein-protein interactions and shares homology with SET domain found in a group of transcriptional regulators that modulate chromatin structure (Huang *et al.*, 1998). Therefore, RIZ1 might be recruited to E2F promoters via Rb to act as a binding interface for other transcriptional regulators that affect DNA topology. Figure 6 summarizes the current models of Rb repression.

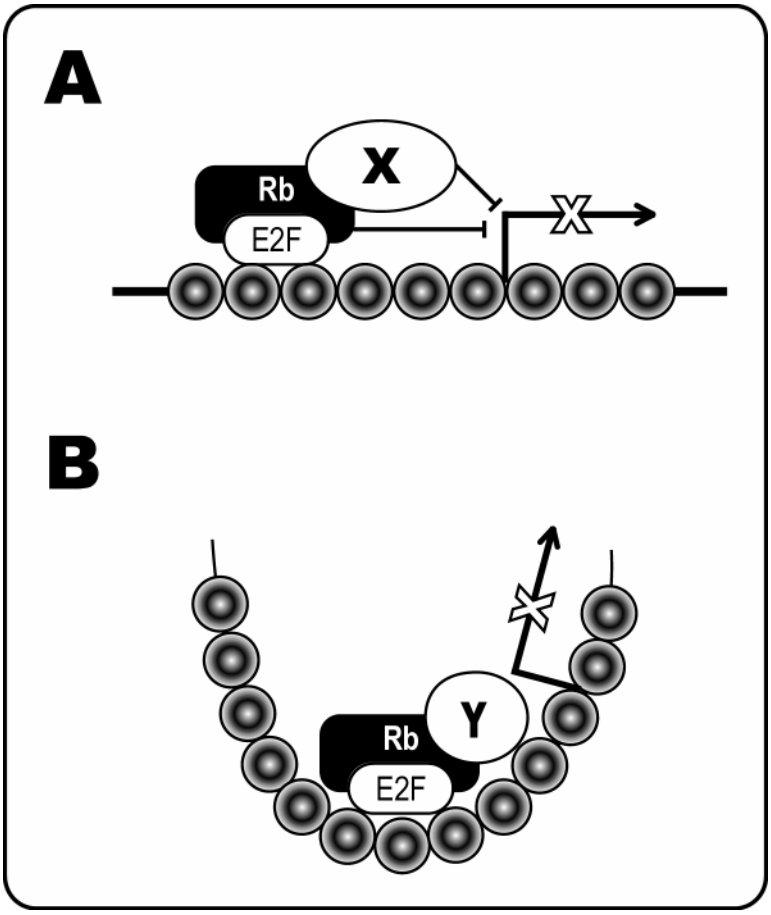


Fig. 6 Rb repression mechanism models. Model A: E2F-bound Rb recruits a corepressor (X) to silence transcription, such as HDAC1-3. This enlistment can be direct or indirect, using RBP1 or RBAP48 as linker proteins. Rb, possibly in conjunction with its corepressor, can also interact with elements of the basal transcription machinery, such as TBP, TFIID and $TAF_{II}250$, to shut off E2F-dependent transcription. Model B: Rb modifies DNA topology at the promoter to a form unsuitable for transcription through the recruitment of the chromatin-remodeling complex (Y), such as SWI/SNF or those possibly recruited by RIZ1. It is noteworthy to mention that both mechanisms are not mutually exclusive. For clarity, E2F represents E2F/DP heterodimers. Adapted from Kaelin, 1999.

1.5 The Retinoblastoma Binding Protein 2 (RBP2)

RBP2 is one of the first two genes that were cloned based on their ability to interact with the Rb pocket domain. A partial cDNA clone was discovered (Defeo-Jones *et al.*, 1991) and used to clone the full-length sequence from a human Nalm-6 pre-B leukemia expression library.

Preliminary characterization of RBP2 showed it to be a 1722 amino acid nuclear phosphoprotein (Fattaey *et al.*, 1993). Further studies illustrated that it possessed differential binding capabilities with members of the RB family and other cellular proteins. RBP2 contains the LXCXE motif and can interact with hypophosphorylated p107 and Rb through pocket-dependent interactions. The binding of RBP2 to p130 has never been assessed, yet the high degree of sequence and structure homology between p107 and p130 makes it likely that RBP2 could also interact with p130. RBP2 can also specifically interact with Rb through a 15 kDa domain near its C-terminus in a mechanism independent of the Rb pocket. As such, RBP2 retains the ability to bind to all naturally occurring Rb point mutants. The spacer region of Rb is required for non-pocket binding (NPB) but other regions are most probably required as simply replacing the p107 spacer with that of Rb could not grant NPB ability to p107. RBP2 can also interact with TBP through the 15 kDa NPB domain (Kim *et al.*, 1994). These interactions were observed *in vitro* as, while RBP2 seems to be expressed in a wide range of tissues, it appears to exist at very low levels (Fattaey *et al.*, 1993; Kim *et al.*, 1994).

The first RBP2-binding protein interaction demonstrated *in vivo* involved the rhombotin-2 (RBTN2), a proto-oncogene involved in erythropoiesis and T-cell leukemogenesis. RBTN2 contains two LIM domains that mediate protein-protein interactions through which RBTN2 is presumed to accomplish its multiple functions (Neale *et al.*, 1995; Mao *et al.*, 1997).

The second LIM domain of RBTN2 was shown to interact with the extreme C-terminus of RBP2 by a yeast two-hybrid screen using the LIM domain as bait and this interaction was later confirmed by coimmunoprecipitation of RBP2 and RBTN2. This interaction is functionally significant, as transcription reporter assays showed that coexpression of both proteins produced increased levels of transcription when compared to those observed in the presence of RBTN2 alone. This could be explained by the fact that RBTN2 is a weak transcriptional activator but has no intrinsic DNA binding activity and recruitment to DNA through RBP2 would naturally enhance its transactivation potential (Mao *et al.*, 1997).

Initial protein homology searches assigned only 3 conserved domains to RBP2, the AT-rich interacting domain (ARID), a zinc finger located near the N-terminus and a region of homology to the homeobox domain of the engrailed family of homeotic genes (Fattaey *et al.*, 1993). This was further refined when RBTN2 was shown to interact with the C-terminus of RBP2 and further homology searches were performed. RBP2 was shown to contain three cysteine- and histidine-rich domains possibly encoding atypical zinc fingers.

These domains were referred to as leukemia-associated protein (LAP) domains as they are found in a wide variety of proteins, including some involved in leukemogenesis (Saha *et al.*, 1995).

To produce a definitive functional domain map of RBP2, its primary amino acid (Genbank reference P29375) was submitted to the latest generation of protein analysis tools available on the Internet, including Pfam (Bateman *et al.*, 2000), Prosite (Hofmann *et al.*, 1999), PRODOM (Corpet *et al.*, 2000), ProfileScan (ISREC, 2000) and BLOCKS (Sonnhammer *et al.*, 1998) to identify domains by sequence homology, which are illustrates in figure 7.

Fig. 7 Functional domains of RBP2. Amino acids are represented in parentheses. ARID: AT-rich interaction domain (81-189), LXCXE: pocket interaction motif (1373-1377), jmjN – jmjC: bipartite jumonji-like DNA binding domain (19-50, 458-595), PHD: PHD Zinc Fingers (1647-1696, 275-341, 1164-1218), NPB: non-pocket binding domain (1457-1558), LZ: predicted leucine zipper (877-898), Y: predicted tyrosine phosphorylation sites (159-167, 430-438), NLS: predicted bipartite nuclear localization signal (804-820, 1539-1556), HLH: predicted helix-loop-helix dimerization domain (1576-1584). Those domains qualified as predicted are unconfirmed as they correspond to low-complexity domains, which could be incorrectly assigned by the search algorithms. More than 50 putative serine-threonine phosphorylation sites were found distributed across the full-length protein. They are not listed here for clarity. All other domains were identified either experimentally or through high sequence homology.

JmjN and jmjC are hallmarks of the jumonji family of transcription factors and form a bipartite DNA binding domain that shows conservation from yeast to humans. Although both motifs are not contiguous, it is possible that protein folding could bring them together to form a single functional domain (Balciunas *et al.*, 2000).

The NBP and the LXCXE motifs have been described above. The NLS, the leucine zipper (LZ) and helix-loop-helix (HLH) dimerization domains are qualified as *predicted* as they correspond to low-complexity domains, which could be incorrectly assigned by the search algorithms. More than 50 putative serine-threonine phosphorylation sites were found distributed across the full-length protein. The tyrosine phosphorylation site predicted within the ARID domain could be physiologically relevant as phosphorylation can modulate DNA binding affinity of certain DNA binding motifs.

The previously described LAP domains were correctly identified as PHD finger domains by the search algorithms. The PHD finger is a C4HC3 zinc-finger-like motif found in nuclear proteins believed to play a role in chromatin remodeling and transcriptional regulation (Aasland *et al.*, 1995). The exact function of this domain remains unknown but, in analogy with the LIM domain, it could be involved in protein-protein interaction and be important for the assembly or activity of multi-component protein complexes involved in transcriptional activation or repression. Other PHD-containing proteins include *Drosophila* trithorax (TRX), p300/CBP and the *Xenopus* homologue of Mi-2, a component of a multi-protein complex containing HDAC and SNF2 ATPase-associated chromatin remodeling activity (Tkachuk *et al.*, 1992; Aasland *et al.*, 1995; Stassen *et al.*, 1995; Wade *et al.*, 1998; Newton *et al.*, 2000). One domain of major interest is the ARID. Originally identified from the analysis of the DNA binding ability of certain regulatory factors in mouse, humans and *Drosophila*, more than 20 ARID-containing proteins have now been identified in several species, from fungi to humans (Gregory *et al.*, 1996; Kortschak *et al.*, 2000).

ARID proteins are characterized by specific or non-specific DNA binding abilities. Indeed, RBP1 and RBP2 have been shown to be retained on immobilized calf thymus DNA under very high salt concentrations (Fattaey *et al.*, 1993).

The ARID has significant physiological relevance. The dead-ringer (DRI) *Drosophila* gene, when mutated, leads to embryonic lethality caused by developmental and patterning defects (Shandala *et al.*, 1999). Mutations in the mouse jumonji (JMJ) gene induce embryonic lethality with defects in neural tube, liver, cardiac, spleen and thymus development (Takeuchi *et al.*, 1995; Motoyama *et al.*, 1997; Toyoda *et al.*, 2000). It is of interest to note that background genetic modulators must exist, as phenotypic pleiotropy is apparent in both DRI and JMJ mutations. The B-cell regulator of IgH transcription (Bright) can specifically binds to DNA sequences associated with nuclear matrix association, a process that has been shown to be mediate alterations in chromatin structure (Herrscher *et al.*, 1995; Pemov *et al.*, 1998). The yeast Swi1 contains the ARID motif and is a component of the SWI/SNF ATP-dependent chromatin remodeling complex, which has been implicated in transcriptional regulation and cell growth control (Cairns *et al.*, 1994; Muchardt *et al.*, 1999). Although many other examples could be listed, it is possible to generalize that ARID proteins are encoded by physiologically important genes involved in many aspects of gene regulation and cellular growth.

Inactivation of RBP2 has never been associated with carcinogenesis. It has been shown to be present at detectable levels in leukemia, lung and colon cancer cell lines (Kim *et al.*, 1994).

Given its interactions with key members of the G_1/S cell cycle checkpoint and RBTN2, a proto-oncogene that can alter the cellular transcription levels, RBP2 mutations could present a plausible target for cell cycle disruption. A newly discovered homologue termed RBP2H1 has been cloned and shares significant homology with several domains of RBP2, including the jumonji-like DNA binding domains (jmjN, jmjC), ARID, PHD fingers and the NPB domain although it does not lave an LXCXE domain (Vogt *et al.*, 1999; Balciunas *et al.*, 2000). RBP2H1, like RBP2, has been shown to be present in normal tissue such as uterus, small intestine, prostate, colon, testes and fetal heart, brain, lung, liver and kidney. Interestingly however, RBP2H1 expression is totally absent in human malignant melanoma and its use as a diagnostic marker for malignancy is being investigated (Vogt *et al.*, 1999).

1.6 Project proposal

This project is based on prior studies of RB family members during terminal differentiation. Rb/E2F complexes formed during differentiation were analyzed by electromobility shit assay (EMSA) using E2F-specific oligonucleotides. These studies showed that while p107/E2F complexes disappeared and Rb/E2F complexes were maintained, p130/E2F complexes were induced in the process of differentiation (Corbeil *et al.*, 1995). A novel p130/E2F complex, termed C7, was of interest due to its large apparent size and its possible role in cell cycle exit (Corbeil *et al.*, 1997).

Further analysis of the C7 complex revealed that an unidentified protein occupied the p130 pocket, most probably through LXCXE-dependent interactions. Mobility supershift assays were done with several antibodies directed against LXCXE-containing proteins and determined that RBP1 was found to be a component not only of the C7 complex but other large Rb/E2F complexes as well. Interestingly, the supershift assays could not rule out the possibility that a subset of C7 might be composed of other LXCXE-containing proteins. Primary characterization of RBP1 showed it to possess growth inhibitory and transcriptional repression activities (Lai *et al.*, 1999).

RBP1 and RBP2 were cloned concurrently and both contain the ARID domain (Fattaey *et al.*, 1993). RBP2 has homology to a known transcriptional repressor that is involved in cellular growth control. RBP2 contains several domains associated with growth control, chromatin remodeling and transcriptional regulation. RBP2 has been shown to interact with members of the RB family of proteins and elements of the basal transcription machinery. The C7 and other p130/E2F or Rb/E2F complexes that do not contain RBP1 could exist in growth arrested and differentiated cells. These facts, taken together, prompted us to investigate the function of RBP2 in transcriptional and cell growth regulation. We hypothesize that, with the body of circumstantial evidence described above, RBP2 could be a transcriptional repressor and could negatively regulate cell growth.

Chapter 2: Experimental Materials and Methods

2.1 Cell Culture

Chinese Hamster Ovary (CHO) cells (ATCC CCL-661) or C33A human cervical carcinoma cells (ATCC HTB-31) were grown in α minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% PSG (Penicillin, Streptomycin, L-Glutamine). H1299 human large cell carcinoma cells (Mitsudomi *et al.*, 1992) and CV-1 African green monkey kidney cells (ATCC CCL-70) were grown in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% FBS and 1% PSG. Some cell prolifreation assays were done using C33A or CV-1 cells grown in the appropriate medium supplemented with G418 750 μg/ml.

2.2 Recombinant Plasmid Generation

Dr. Ed Harlow graciously provided pBS-RBP2, the full-length cDNA encoding RBP2 (Fattaey *et al.*, 1993). A full-length fusion protein was made by PCR amplification using the Vent DNA polymerase (New England Bioloabs) of the pBS-RBP2 cDNA with oligos spanning the first 210 base-pairs (For: 5'-TAG AGG TAC CGT CGA CGG ATG GCG GGC GTG GGG; Rev: 5'-AAA CGA AAG CTT TTT ACT TCA C). The oligo pair introduced a *Sal1* restriction digest site at the 5' end and a *HindIII* at the 3', which were used to clone this fragment in frame with the GAL4 DNA binding domain of pSG424, a vector encoding the GAL4 domain under control of the SV40 early promoter.

The remaining portion of the coding sequence of RBP2 was was joined to the pSG424-RBP2 (210bp) construct by ligating the 6.1 kb *HindIII-Xba1* fragment of pBS-RBP2 to produce a full-length fusion protein, which was confirmed by sequencing using appropriate primers. C-terminal deletion mutants were constructed by digesting the full-length pSG424-RBP2 with *Sfi1* and various other enzymes at the 3' end to generate fragments of various sizes (refer to figure 9). These fragments were blunted with the Vent DNA polymerase following the manufacturer's recommended protocol and sub-cloned in an *EcoRV*-linearized pCDNA3-STOP, a cytomegalovirus (CMV) promoter driven mammalian expression plasmid engineered to contain 3 stop codons after the polylinker region. The constructed plasmids were confirmed by direct sequencing using the *T7* and *SP6* oligos. An internal deletion mutant (ΔARID) was constructed by digesting pSG424-RBP2 with *Sma1* and *Apa1* and re-ligating the isolated fragment. The coding region pSG424-RBP2-ΔARID was sub-cloned in pCDNA3 using the method described above and also served as a template for construction of pcDNA3-RBP2 ΔARID/ΔCT. This plasmid was constructed by ligating the blunted *Sfi1-BamH1* fragment of pSG424-RBP2-ΔARID into the pcDNA3-STOP backbone, as described above. pcDNA3 RBP2-CT was generated by subcloning the appropriate *BamH1* fragment of RBP2 in pSG424 followed by subcloning of the GAL4-RBP2-CT fragment in pcDNA3-STOP. the A full-length HA (influenza hemagglutinin)-tag RBP2 fusion protein was constructed by sub-cloning the *Sma-Xba1* fragment of pSG424-RBP2 into pCAN-HA.2, a vector encoding the HA-tag under control of the CMV promoter. Glutathione-S-transferase (GST)–tagged RBP2 fusion proteins were required for antibody production (discussed below).

2 high potential antigenicity fragments of RBP2 were amplified by PCR and cloned in-frame in pGEX-2TK (Pharmacia). Refer to table 3 for the oligo pairs. Both oligo pairs introduce *EcoR1* and *BamH1* restriction sites, which were used in the sub-cloning process. All constructed plasmids were confirmed by sequencing using appropriate primers.

Table 3. Oligo pairs used in the generation of GST-Fusion proteins

Construct	Forward Oligo	Reverse Oligo
GST-RBP2 (C60)	5'-CGC GGA TCC GAT GAG TGG TTT CAT CGG	5'- CCG GAA TTC CTA ACT GGT CTC TTT AAG ATC
GST-RBP2 (1416-1447)	5'- CGC GGA TCC AAA GGT TCT AGC ACC CC	5'- CCG GAA TTC CTT AGC TCC AGG TGA CAA C

2.3 Other Plasmids

Some studies utilized the following plasmids: the pG_5TkCAT reporter plasmid that expresses the chloramphenicol acetyl transferase (CAT) gene under control of the Herpes Simplex virus (HSV) thymidine kinase (TK) promoter that is flanked upstream by 5 GAL4 cognate DNA binding motifs (Teodoro *et al.*, 1997), the RSV-βGal reporter plasmid expressing the β-Galactosidase gene under control of the Rous Sarcoma virus (RSV) promoter (Popperl *et al.*, 1992) or the E2F-TATA-CAT reporter plasmid expressing the CAT gene under control of the minimal TATA promoter flanked upstream the minimal E2F cognate DNA binding motif (Weintraub *et al.*, 1992). Several plasmids were obtained from other members of our group, including the pSG424-RBP1 plasmid expressing the GAL4-RBP1 fusion protein under control of the SV40 early promoter, the pSG424-VP16 plasmid expressing the GAL4-HSV VP16 activation domain fusion protein under control of the SV40 early promoter.

The pcDNA3-p130, pcDNA3-Rb, pcDNA3-p107 constructs expressing the extended pocket region of each family member under the control of the CMV promoter and pcDNA3-E1A12S, a plasmid expressing the 243 residue E1A product of adenovirus type 5, are all described elsewhere (Lai *et al.*, 1999)

2.4 Transcriptional Reporter Assays

CHO cells were co-transfected at a density of $2x10^5$ cells per 60mm-diameter dish by the calcium phosphate method (Brent, 1988) with 2.5 μg of pG_5TkCAT, 3μg of pRSV-βGal, and 2.5 μg of various GAL4-fusion proteins, including full-length RBP2 and a panel of GAL4-tagged deletion mutants. The GAL4 DBD alone, GAL4-RBP1 and GAL4-VP16 were included as experimental controls. A final concentration of 10 μg of DNA per transfections was achieved by the inclusion of either sonicated salmon sperm DNA or the pGEM4 (Promega) empty vector. Cells were harvested at 40h post-transfection and CAT assays were performed using samples containing equal β-galactosidase activity, an assay used to normalize transfection efficiency (Gorman *et al.*, 1982). CAT activity was quantified following thin layer chromatography using either a BAS2000 (Fuji) or Storm (Molecular Dynamics) phosphoimaging system. Transcriptional repression was determined by comparing CAT activity results obtained in the presence GAL4-RBP2 constructs compared to the CAT activity obtained in the presence of pSG424 alone, arbitrarily set as a 100% baseline.

In other studies, C33A cells were co-transfected at a density of $2x10^5$ cells per 60mm-diameter dish by the calcium phosphate method (as described previously) with 3 units of purified β-galactosidase protein (Sigma), 2.5 μg of pE2F-TATA-CAT and 0.5 μg or 1.5 μg of either HA-RBP2, Rb, p130, p107, E1A 12S or HA-RBP1. A final concentration of 7.5 μg of DNA per transfection was achieved by the inclusion of the pcDNA3 (Stratagene) empty vector. Cells were harvested at 40h post-transfection and CAT assays were performed and analyzed as described above, using the CAT activity obtained in the presence of pcDNA3 as a 100% baseline.

2.5 GST Fusion protein purification

XL-1 Blue *E. Coli* competent bacteria (Stratagene) were transformed with plasmids expressing GST-RBP2 (C60), GST-RBP2 (1416-1447) or GST alone and grown in LB/ampicillin medium at 30°C with agitation to an OD_{600} of 1.5. Cultures were diluted 1:10 in fresh medium, incubated for 1h at 30°C and induced with 0.1 mM Isopropyl β-D-thiogalactopyranoside (IPTG) for 2h. Cells were harvested, resuspended in 25 ml of GST lysis buffer (1X PBS, 1 mM DTT, 0.5 mM PMSF, 1% Triton-X 100) and lysed by sonication in ice. The cell lysate was cleared by centrifugation incubated with 1 ml of a 50% slurry of glutathione sepharose 4 fast flow beads (Pharmacia) for 2h at 4°C. The beads were collected and repeatedly washed with 1X PBS, 1 mM DTT, 0.5 mM PMSF over a period of 1h. The purified GST fusion proteins were eluted from the beads by incubating the beads twice with 1 ml of 1X PBS pH 8.5, 15 mM reduced glutathione for 15 minutes at 4°C.

The eluted proteins were concentrated and dialyzed into 1X PBS by multiple rounds of spin-dialysis using Centricon-30 columns (Millipore, using the manufacturer's recommended protocol). Protein concentration was assayed with the Bradford assay using the Bio-Rad Protein Assay kit and protein purity was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel and subsequent staining with Coomassie Blue.

2.6 Polyclonal Antibody Production

New Zealand White Rabbits were primed with sub-cutaneous injections of 400 μg purified GST-RBP2 (C60) or GST-RBP2 (1416-1447) emulsified in complete Freund's Adjuvant (Gibco Life Technologies) and boosted with 500 μg purified protein emulsified in incomplete Freund's Adjuvant (Gibco Life Technologies) at 3-4 week intervals over a period of 6 months. Immune response was assayed after each boost by ELISA using the purified GST proteins as antigens and an alkaline phosphatase (AP) conjugated α-rabbit IgG antibody (Jackson Immuno Research Laboratories) as a secondary antibody. Para-nitrophenol phosphate (PNPP, Sigma-Aldrich chemicals) was used as a substrate for AP activity. Immune response was assayed by visual color change. After 6 months, the rabbits were exsanguinated and antibody affinity purification was performed as described elsewhere (Harlow *et al.*, 1998) using a column of purified GST-RBP2 fusion protein coupled to NHS-activated sepharose (Pharmacia, according to the manufacturer's recommended protocol).

Rabbit whole serum was diluted 2X in PBS and incubated with glutathione-sepharose bound GST protein in excess to remove any antibodies reactive to the GST moiety. The cleared serum was passed through the column by gravity. The column was washed with alternating cycles of high-salt, low-salt buffers and antibodies were eluted from the column in two fractions, with low pH followed by high pH elution buffers. Refer to Harlow *et al.*, 1998 for a complete protocol description. The eluted fractions were kept separate and were dialyzed against a storage buffer (1X PBS, 0.02% NaN_3).

2.7 Cell proliferation assay

C33A or CV-1 cells were transfected at a density of $7x10^4$ cells per 60mm-diameter dish by the lipofectamine plus method (Gibco Life Technologies, following the manufacturer's recommended protocol) with 1μg of DNA per plate and were put under G418 selection 48h post-transfection. Cells were allowed to grow over a 14-day period with regular media changes every 2 days. The dishes were washed twice with 1X PBS, incubated with 1ml of 0.05% trypsin-EDTA (Gibco Life Technologies) and harvested in a total volume of 2ml. Survival was measured as a function of cell number, as determined using a Beckman Coulter cell counter.

2.8 Western Blot Analysis

Cell extracts were prepared on ice by incubating the cell pellets in 200 µl of lysis buffer (50 mM HEPES pH 7.9, 400 mM KCl, 4 mM NaF, 4 mM $NaVO_4$, 0.2 mM EDTA, 0.2 mM EGTA, 10 % glycerol, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, 1 µg/ml APL). 40 µg of total cell protein, measured with the Bio-Rad Protein Assay kit, was separated by SDS-PAGE using either 6% or 12% polyacrylamide gels. The separated proteins were transferred to Immobilon-P PVDF membranes (Millipore) using a semi-dry transfer apparatus with a current density of 1 mA / cm^2 for 2 hours. Membranes were blocked for 12-16 hours in a solution of 1X TBS, 0.1% Tween-20, 5% powdered milk, 1% BSA, 1% calf serum and probed with either RK5C1 (Santa Cruz) mouse monoclonal antibody directed against the GAL4 DBD, HA.11 (BabCO) mouse monoclonal directed against the HA-tag or the rabbit polyclonal antibodies generated as described above. All antibody dilutions were 1:1000 in 1X TBS, 0.1% Tween-20, 1% BSA, 1% calf serum unless noted otherwise. Goat anti-mouse IgG or donkey anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno Research Laboratories) were used to perform an enhanced chemiluminescence (ECL) reaction to identify target proteins according to the manufacturer's recommended protocol.

2.9 Immunoprecipitation

Cell extracts were prepared on ice by incubating the cell pellets in 500 µl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1 µg/ml APL) for 20 minutes followed by sonication with a small bore probe (3 x 15 seconds).

Following centrifugation, the cell extracts were pre-cleared by incubating 50 µl of 50% protein A-sepharose fast-flow (Pharmacia) in lysis buffer for 2-4 hours with slow rotation. The clarified extracts were incubated with 10µg of antibody for 4-16 hours with slow rotation, following which 30µl of 50% protein A-sepharose fast-flow in lysis buffer was added for 2 hours. Beads were collected by centrifugation and the supernatant was removed. The beads were resuspended in 1 ml of fresh lysis buffer and incubated for 15 minutes with slow rotation, This wash cycle was repeated 5 times, after which the collected beads were resuspended in 25µl of 2X SDS-PAGE sample buffer (Bio-Rad), boiled for 30 seconds and subjected to SDS-PAGE on a 6% polyacrylamide gel. The gel was transferred to PVDF and immunoblotted ad described above.

2.10 Immunofluorescence

H1299 cells were grown in 4-chambered slides (Nunc) and transfected with 1µg of DNA using the lipofectin method (Gibco Life technologies, following the manufacturer's recommended protocol) and fixed 24h post transfection in 0.5ml of 1X PBS, 4% paraformaldehyde (PFA) for 30 min. The cells were permeabilized by adding 0.5ml of 1X PBS, 4% PFA, 0.1% Triton X-100 for 45 min. After 4 rounds of rinsing with 1X PBS, 0.5ml of filtered blocking solution (1X PBS, 50% FBS, 6% milk powder, 3% BSA 0.1% Triton X-100, 0.05% NaN_3) was added for 4h. Cells were washed twice with 1X PBS and the primary antibody, either the mouse monoclonal α-HA.11 (BabCO, 1:1000 dilution) or the rabbit polyclonal α-RBP2 2410 (1:500, 1:1000 or 1:1500), was incubated in buffer A (1X PBS, 3% BSA, 0.1% Triton X-100, 0.05% NaN_3) for 2 hours.

After multiple rounds of rinsing with 1X PBS, cells were incubated with either Alexa Fluor488-conjugated goat α-mouse IgG or Alexa Fluor594-conjugated goat α-rabbit IgG secondary antibodies (Molecular Probes) diluted 1:500 in buffer A for 45 min. Finally, the slides were washed, mounted with the Molecular Probes Slowfade Antifade kit following the recommended protocol and visualized using a scanning laser confocal microscope.

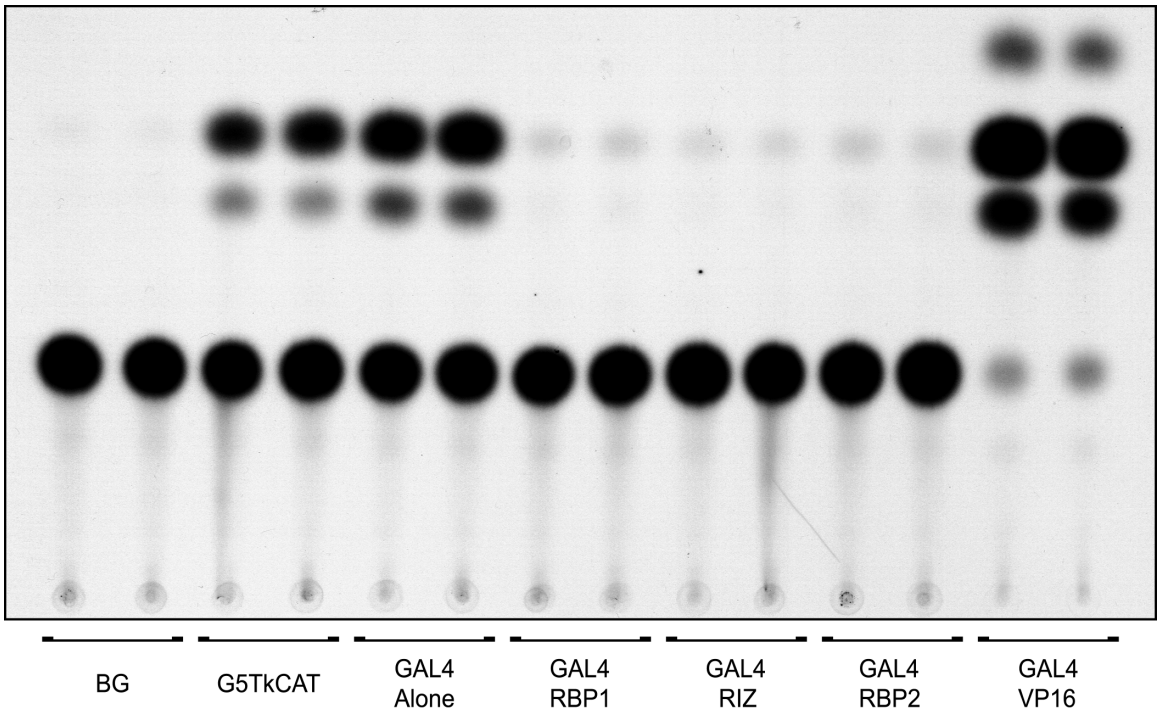
Chapter 3: Experimental Results

3.1 RBP2 is a transcriptional repressor

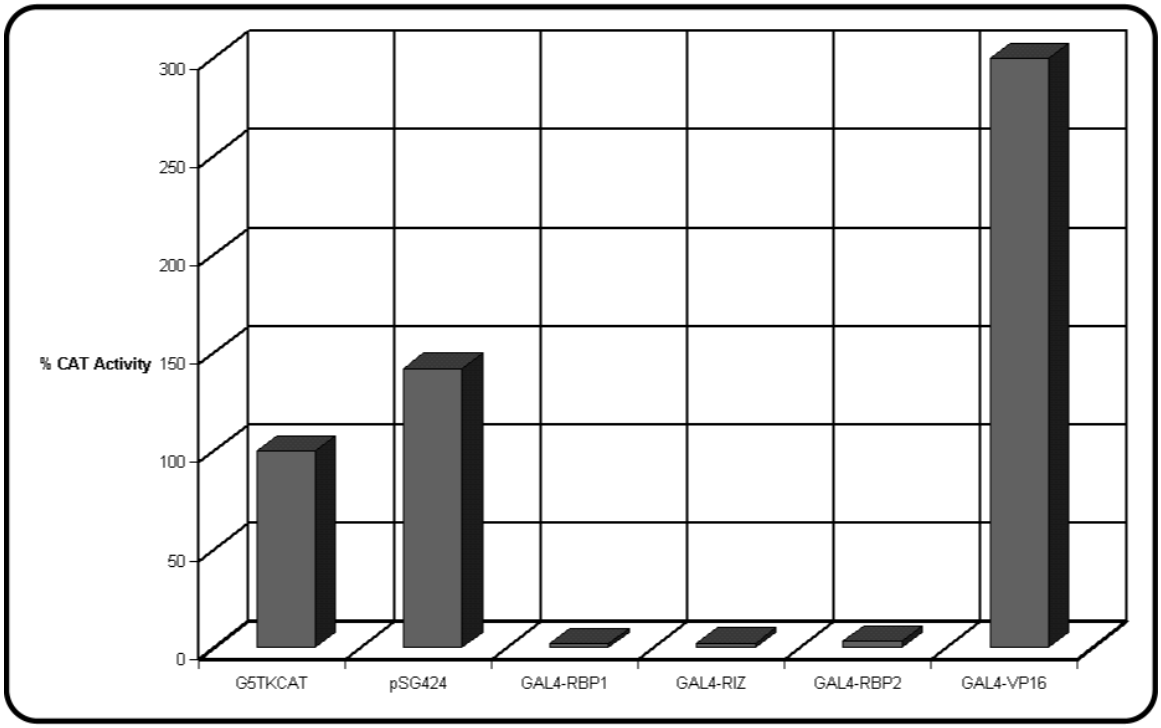
At the beginning of this project, the body of knowledge concerning RBP2 was limited and no clear indication existed regarding its possible function. However, given its homology to a known transcriptional repressor, RBP1, and given that an ever-growing number of Rb-interacting proteins act as transcriptional repressors, we hypothesized that RBP2 would also have transcriptional repression activity. To address this hypothesis, the G_5TkCAT reporter construct was used in conjunction with the full-length RBP2 protein tagged with the GAL4 DBD to assess the transcription repression potential of RBP2. This reporter construct expresses the CAT gene under control of the HSV Tk promoter flanked upstream by 5 GAL4 DNA binding sites. Figure 8 shows the results of one such assay.

Fig. 8 Transcriptional repression by GAL4-RBP2. CHO cells were transfected by the calcium phosphate method with G_5TkCAT, a plasmid expressing the CAT gene under the control of a minimal HSV Tk promoter flanked upstream by 5 GAL4 DNA binding sites, RSV-βGAL and various GAL4-tagged constructs, as indicated. Assays were performed as described in Experimental Materials and Methods. The raw data is shown in panel A. The quantified results are presented in graph form in panel B. Results have been expressed as a percentage of CAT activity relative to pG_5TkCAT alone, arbitrarily set at 100%.

A


BG
G5TkCAT
GAL4 Alone
GAL4 RBP1
GAL4 RIZ
GAL4 RBP2
GAL4 VP16

B


% CAT Activity
300
250
200
150
100
50
0
G5TKCAT
pSG424
GAL4-RBP1
GAL4-RIZ
GAL4-RBP2
GAL4-VP16

CAT expression was elevated in cells transfected with the reporter alone and showed a slight increase when the pSG424 empty vector was co-transfected. This effect was observed in a reproducible fashion in subsequent assays and ranged from a 20% to 50% increase (data not shown). Expression of the HSV VP16 transcriptional activator produced a four-fold elevation in CAT activity and was used as a control for transcriptional activation. Cotransfection with GAL4-RBP1 and GAL4-RIZ, both known transcriptional repressors (Xie *et al.*, 1997; Lai *et al.*, 1999), reduced CAT activity by about 95%. Interestingly, GAL4-RBP2 demonstrated transcriptional repression activity to about the same extent.

3.2 Mapping of the RBP2 repression domain

In order to determine the region of RBP2 involved in transcriptional repression, a panel of C-terminal deletion mutants was generated using the full-length cDNA linked to the GAL4 DBD as a working template. Figure 9 illustrates what domains were removed by deletion, the enzymes used to generate the C-terminal cut and the predicted size of the fusion protein. The deletion mutants were engineered as described in Experimental Materials and Methods.

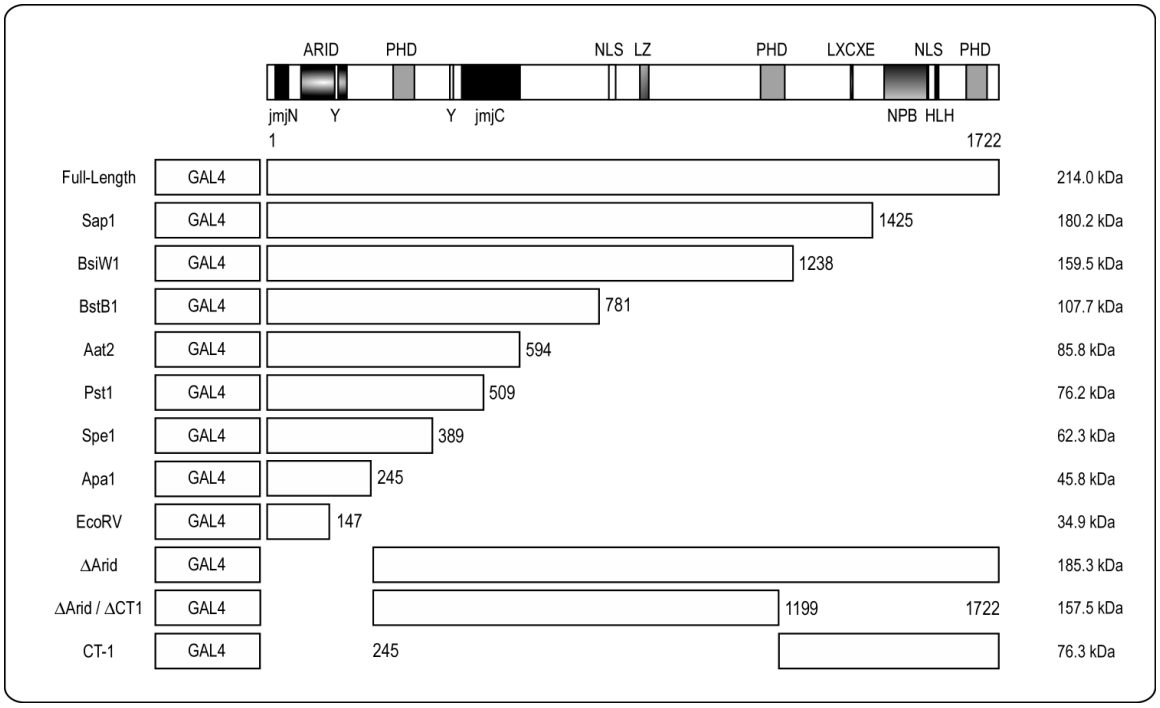


Fig. 9 Deletion mutants of RBP2 fused to the GAL4 DBD. The functional domains of the full-length RBP2 protein have been previously described. The full-length GAL4-RBP2 cDNA was used as a template to create C-terminal deletion mutants as described in Experimental Materials and Methods. Sizes are expressed in kDa and represent the predicted size of the expressed fusion proteins. Numbers represent amino acid positions. This figure is to scale.

3.2.1 Expression and stability of GAL4-RBP2 deletion mutants

Expression levels and stability of the fusion products were investigated by transfecting plasmids expressing the various clones into human H1299 cells by lipofection. Cell extracts were prepared 40 hours post-transfection. Following SDS-PAGE separation and transfer to PVDF membranes, the expression pattern of the GAL4-RBP2 fusion products was analyzed by immunoblotting using an antibody directed against the GAL4 DBD. Results are shown in figure 10.

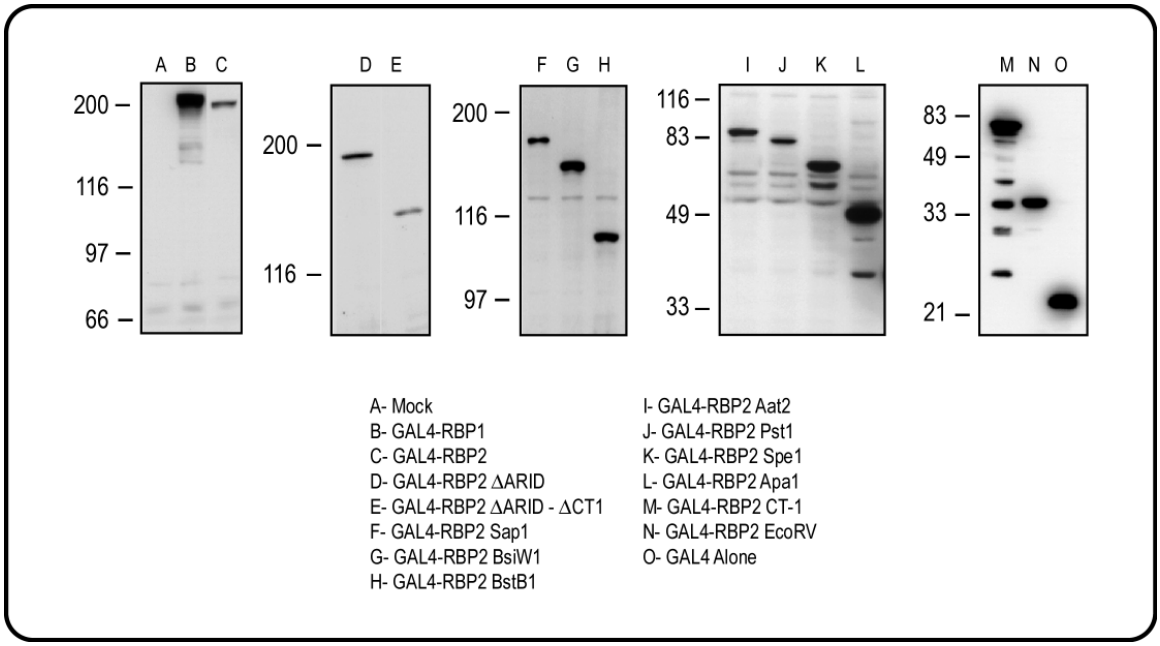


Fig. 10 Expression of RBP2 deletion mutants. H1299 cells were transfected by lipofection with plasmids expressing the various RBP2 deletion mutants fused to the GAL4 DBD and immunoblotted with a mouse monoclonal antibody directed against the GAL4 DBD as described in Experimental Materials and Methods. Sizes are in kDa. Lanes correspond to the deletion mutants as indicated in the figure legend. Mock refers to an untransfected H1299 cell extract.

Referring back to figure 9, it is possible to conclude that all the engineered deletion mutants migrate in a pattern consistent with that predicted. These results indicate that all the mutants are stable and expressed at detectable levels in mammalian cells. GAL4-RBP2 CT1 (lane M) would seem to be less stable that the other mutants, as indicated by the degradation products below the expected band of 76 kDa. Interestingly, when comparing the expression levels of GAL4-RBP1 and GAL4-RBP2 (lanes B and C), RBP2 is always expressed at lower levels than RBP1. This pattern has been observed in a consistent fashion and might indicate that RBP2 has a shorter half-life than RBP1.

3.2.2 Both the N-terminus and C-terminus of RBP2 are involved in repression

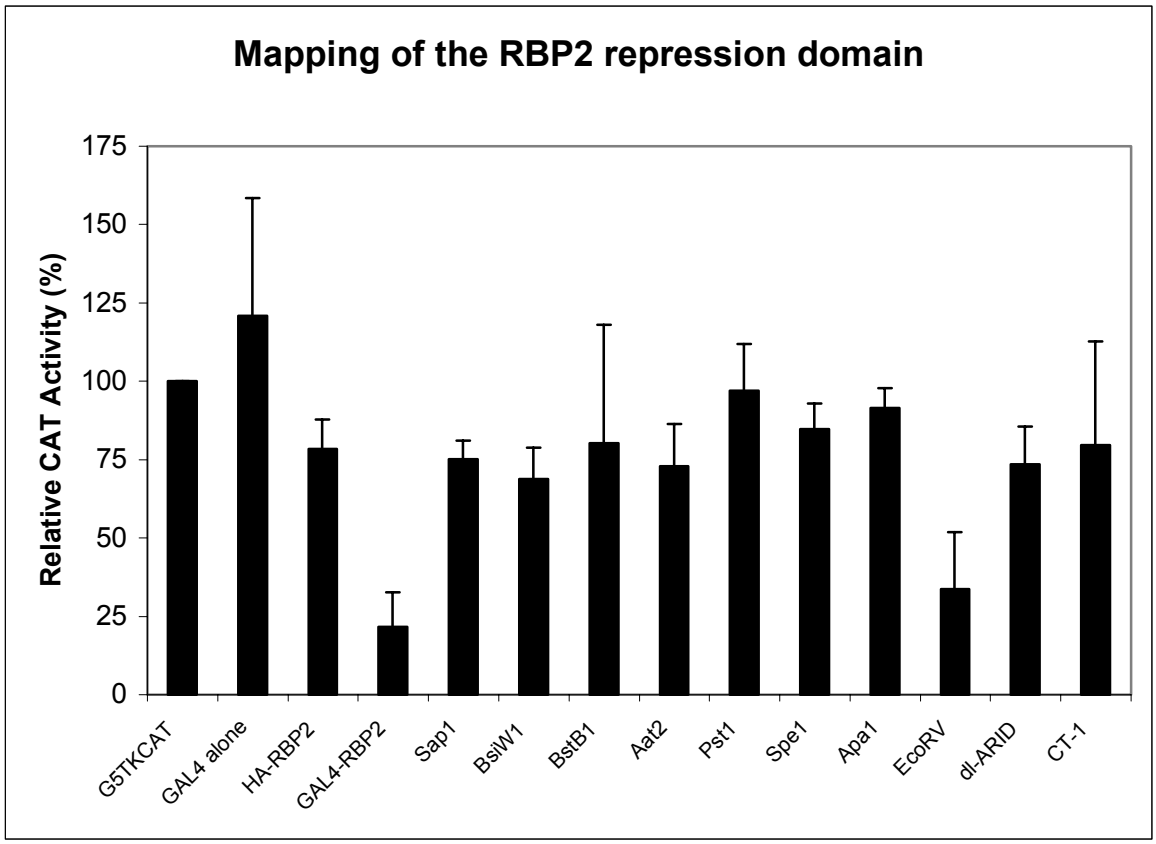


Fig. 11 RBP2 repression domain mapping. CHO cells were transfected by the calcium phosphate method with G_5TkCAT, RSV-βGAL as well as the various GAL4 RBP2 constructs, as indicated. Assays were performed as described in Experimental Materials and Methods. Results have been expressed as a percentage of CAT activity relative to pG_5TkCAT alone, arbitrarily set at 100%. This figure tallies the results of multiple assays.

Figure 11 illustrates the mapping results of multiple CAT transcription assays. CHO cells were cotransfected by the calcium phosphate method with plasmids encoding RBP2 deletion mutants fused to the GAL4 DBD, the G_5TkCAT reporter plasmid and the RSV-βGal plasmid used to normalize the cell extracts. CAT activity was measured on normalized cell extracts as described in Experimental Materials and Methods.

As observed previously, the full-length RBP2 protein is a transcriptional repressor in this system. RBP2 must be tethered to the promoter in order to act as a repressor, as indicated by the fact that GAL4-RBP2 but not HA-RBP2 can repress transcription by more than 75%. HA-RBP2 does not bind to the GAL4 recognition elements of the reporter but still reduced transcription by approximately 20%, probably through squelching (i.e. non-specific interactions with elements of the basal transcription machinery, such as TBP).

The results obtained from the panel of C-terminal deletions were quite surprising. GAL4-RBP2-Sap1, which expresses most of the full-length protein save for the extreme C-terminal 30 kDa fragment, showed a three-fold reduction in its repression activity. This fragment contains the NPB domain, which is responsible for Rb and TBP interactions, as well as the terminal PHD finger implicated in RBTN2 transcription modulation. These results suggest that a region near the C-terminus of RBP2 is required for repression. However, expression of GAL4-RBP2-CT1 indicates that the C-terminal 523 amino acids are insufficient to reproduce wild-type repression activity or that this region requires an additional portion of RBP2 for repression.

Interestingly, the Sap1, BsiW1, BstB1 and Aat2 deletion mutants all retain approximately 25% repression activity yet the next 3 deletion mutants, Pst1, Spe1 and Apa1, show no residual repression activity. This loss of residual repression activity could possibly be ascribed to the deletion of the jmjC domain, as both events occur simultaneously.

Expression GAL4-RBP2-EcoRV, the extreme N-terminus of RBP2, had unexpected results. This mutant can repress transcription almost to the extent of the wild type protein, or by approximately 70%. The removal of a 98 amino acid fragment from the C-terminus of the GAL4-RBP2-Apa1 deletion mutant to produce the GAL4-RBP2-EcoRV deletion mutant almost completely restored the repression activity observed in the wild type protein. It could be concluded that this 98 amino acid fragment inhibits the repression domain located within the 17-kDa region at the extreme N-terminus through an undetermined mechanism.

In summary, these results implicate two regions of RBP2 in transcriptional repression: an extended region at the C-terminus and a 17-kDa fragment at the N-terminus of RBP2 (hereafter referred to as NtR, for N-terminal Repression domain).

3.3 RBP2 represses E2F-dependent transcription

E2F transcription factors are the major physiological targets of RB family members. To assess the role of RBP2 in E2F-dependent transcription, actively cycling C33A human cervical carcinoma cells were assayed for their ability to regulate the minimal E2F-TATA-CAT reporter. C33A cells are deficient both for functional p53 and Rb and display a high expression level of active E2F proteins.

This reporter was chosen because any E2F family member that possesses a transactivation domain can activate it and, as such, will have a high basal activity in C33A cells. Fig 12 shows the results obtained from transcriptional repression assays performed in C33A cells as described in Experimental Materials and Methods.

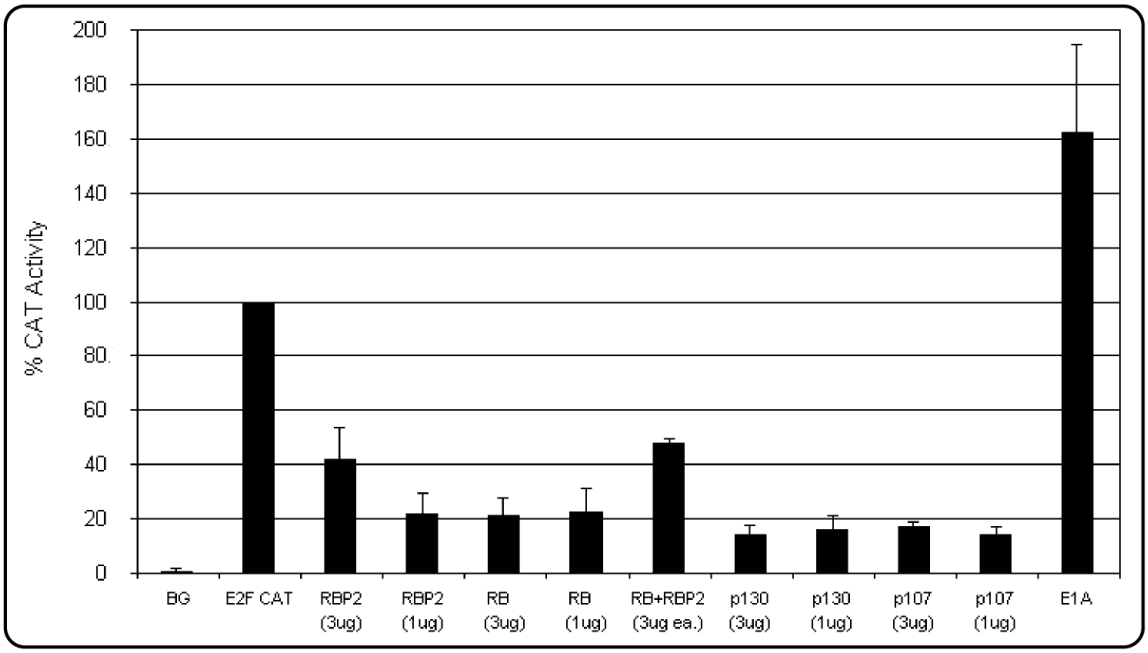


Fig. 12 Transcriptional repression of the E2F-CAT promoter by RBP2. C33A cells were transfected with plasmids expressing the CAT gene under control of a minimal TATA box flanked upstream by E2F DNA binding sites as well as various amounts of HA-RBP2 or RB family members as controls of transcriptional repression. The Ad5 E1A construct was included as a control for transcriptional activation. Assays were performed as described in Experimental Materials and Methods. Results have been expressed as a percentage of CAT activity relative to pE2F-CAT alone, arbitrarily set at 100%. BG: background.

As expected, basal CAT activity was high in the sole presence of the pcDNA3 empty vector, with negligible background (BG) activity in the absence of the reporter. Cotransfection of Rb, p130 or p107 caused an approximate 80% decrease in CAT activity, which is consistent with their known function as repressors of E2F-dependent transcription.

Conversely, cotransfection of E1A 12S showed a 60% increase in CAT activity, which is consistent with its role in cell-cycle activation by disrupting inhibitory p130/E2F and Rb/E2F complexes to drive quiescent cells into S-phase. Cotransfection with 3µg of RBP2 induced a 60% decrease in CAT activity. Interestingly, cotransfection with a lesser amount of RBP2 DNA showed a reduction at par with that observed with Rb, p107 and p130 of about 80%, indicating a dose-dependent effect. The coexpression of Rb with RBP2 did not significantly alter CAT activity when compared to that obtained in the presence of an equivalent amount of RBP2 alone.

3.4 Overexpression of RBP2 inhibits cellular growth

RBP1 and several other Rb binding proteins, such as hBRM and BRG1, have been shown to be involved in cell proliferation control (Lai *et al.,* 1999*;* Muchardt *et al.*, 1999). Furthermore, RBP2 contains domains that have been associated with growth regulation in other proteins, most notably the ARID. Interestingly, RBP2 has homology to several domains found in jumonji, which has recently been shown to negatively regulate cell growth (Toyoda *et al.*, 2000). This led us to believe that RBP2 might play a role in the control in the control of cell proliferation through its ability to repress E2F-dependent transcription and thus initiating or sustaining cellular quiescence.

To verify this hypothesis, CV-1 normal kidney fibroblasts were transfected by lipofection with plasmids encoding RBP1, a known growth suppressor, and several RBP2 constructs.

The cells were then grown under neomycin (neo) selection for a two-week period and proliferation was measured by a direct cell count as described in Experimental Materials and Methods.

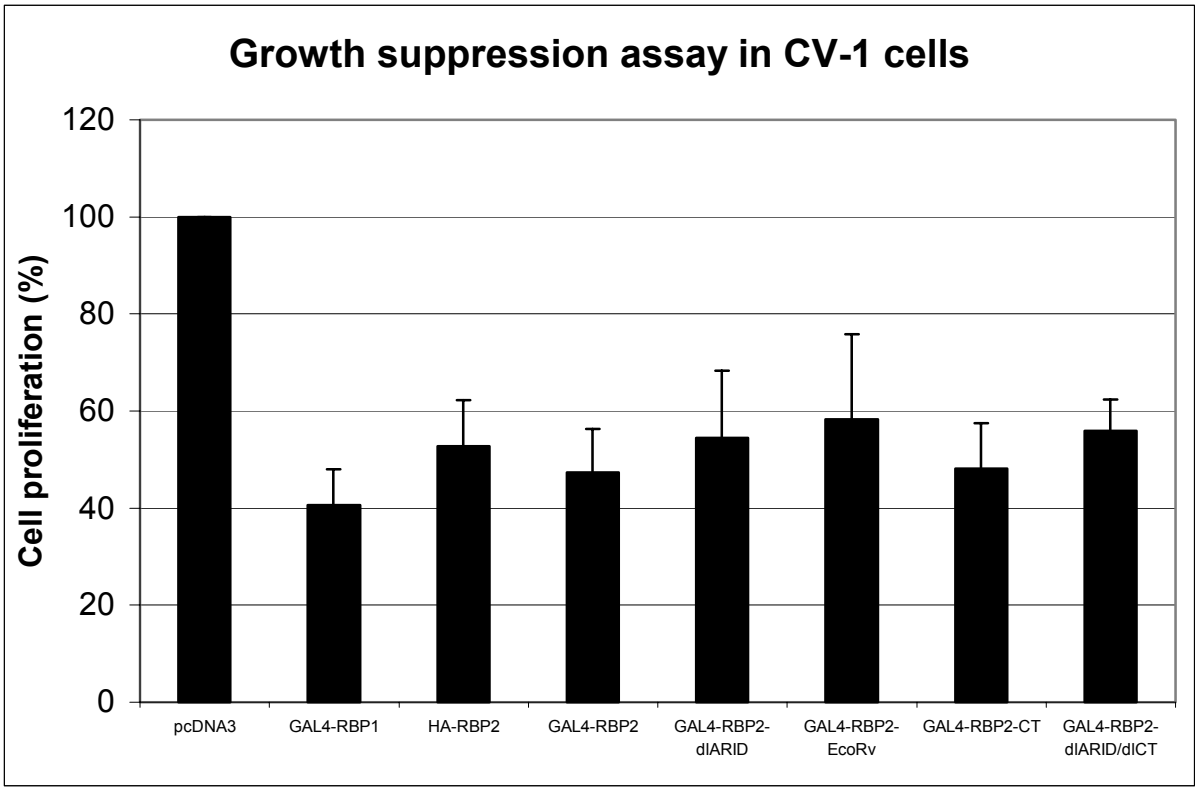


Fig. 13 Growth suppression assay in CV-1 cells. CV1 cells were transfected with plasmids expressing GAL4-RBP1 and various RBP2 constructs, as indicated. Cells were put under G418 selection 48 hours post transfection. After a two-week period, cells were harvested and counted as described in Experimental Materials and Methods. Cellular proliferation is expressed as a percentage of cells counts obtained with cells transfected with pcDNA3 (neo) vector alone, arbitrarily set at 100%. This figure represents triplicate counts in three different assays.

Figure 13 illustrates the results obtained from multiple cell proliferation assays. Cell proliferation is expressed as a percentage of cell counts obtained with cells transfected with pcDNA3, an empty vector expressing the gene for neomycin resistance. No cell counts were obtained for untransfected cells (data not shown). RBP1 was used as a control for growth suppression and showed a 60% decrease in cell proliferation.

Both full-length RBP2 clones showed a two-fold reduction in proliferation. Several RBP2 deletion mutants were assayed to determine if the growth suppression domain correlated with the transcription repression domain. These included RBP2-ΔARID, which expresses the full-length protein minus the extreme N-terminus; RBP2-EcoRV, which expresses the NtR domain; RBP2-CT, which expresses the Rb interaction domains and the C-terminal PHD finger and RBP2-ΔARID/ΔCT, which expresses the middle portion of RBP2 consisting of the two internal PHD finger domains and the jmjC domain. Interestingly, all deletion mutants retained similar growth suppression potential, at par with that observed in the full-length protein. All RBP2 constructs were found to be expressed at similar levels (data not shown). This could possibly indicate that RBP2 regulates cellular growth through several mechanisms that can act independently of each other.

Further cell proliferation assays were done in *Rb* $^{-/-}$ C33A cells in order to determine the role of Rb in RBP2-mediated growth regulation. Figure 14 demonstrates the results of multiple cell selection experiments. As expected, cell survival without neo resistance was insignificant, as observed in the background (BG) lane while transfection with pcDNA3, which has the neo resistance gene, allowed for observable growth. RBP1 significantly inhibited proliferation in these assays. Constitutive expression of HA-RBP2 produced a four-fold reduction in cell proliferation while GAL4-RBP2 produced a seven-fold reduction. Both RBP2 transfected proteins were found to be present at similar levels, with GAL4-RBP1 present at higher levels, as observed previously (data not shown).

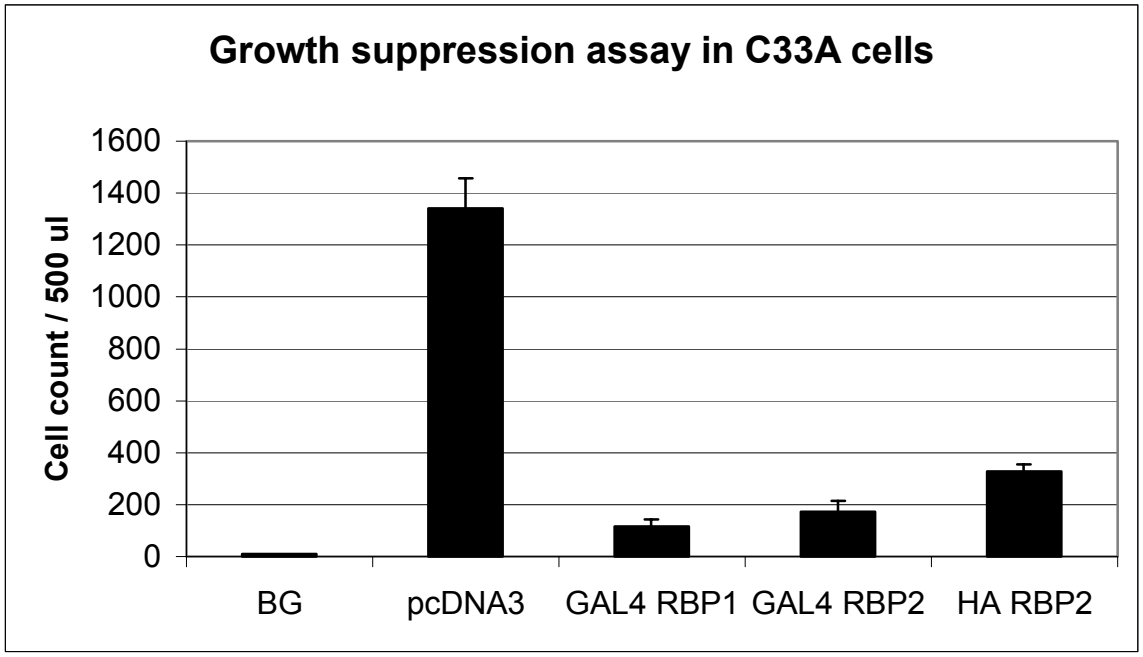


Fig. 14 Growth suppression assay in C33A cells. *Rb* $^{-/-}$ C33A cells were transfected with plasmids expressing differentially tagged RBP2 as well as other control plasmids and put under neomycin (neo) selection. After a two-week period, cells were harvested and counted as described in Experimental Materials and Methods. BG: untransfected cells as a measure of background noise. pcDNA3 encodes the gene for neo resistance. RBP1 is a known growth suppressors and was used as a control for growth inhibition. Cell growth was measured by direct cell counting. This figure represents triplicate counts in two different assays.

The difference in growth suppression between the two RBP2 constructs might be explained by increased nuclear import by GAL4-RBP2, which has one more NLS present in the GAL4 domain.

In summary, these data would suggest that RBP2, like RBP1, could negatively regulate cell proliferation through a mechanism that does not require the presence of Rb but could still involve p107 or p130. Furthermore, the growth regulation domain could not be mapped to a single domain within RBP2.

3.5 α-RBP2 antibody characterization

Although an antibody directed against RBP2 had been previously described (Fattaey *et al.*, 1993), we decided to produce a novel antibody directed against RBP2 as previous members of our group had reported mixed results using the original α-RBP2 antibody (Albert Lai, personal communication). New Zealand white rabbits were immunized with GST-fusion proteins expressing high antigenicity fragments of RBP2. The antigenicity profile of RBP2 was determined using the Protean sequence analysis software (DNAStar suite, Lasergene). Two high antigenicity regions were identified: the last 60 amino acid at the extreme C-terminus and a 31 amino acid fragment located at 1416-1447, between the LXCXE and the NPB domains of RBP2. These regions were subcloned by PCR in pGEX-2TK, a bacterial expression vector encoding the GST domain, and protein purification and dialysis was performed as described in Experimental Materials and Methods. Figure 15 shows the end result of these manipulations.

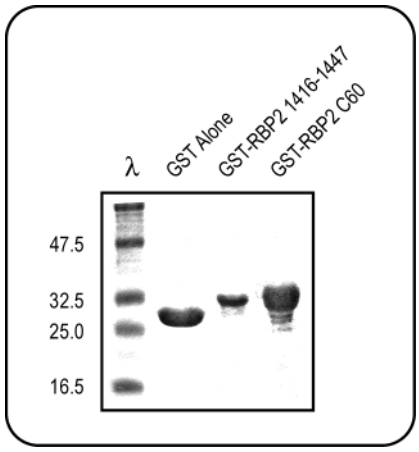


Fig. 15 Expression and purification of GST-RBP2 proteins. XL1-blue E. Coli were transformed with pGEX-2TK-GST-RBP2 plasmids and protein purification was performed as described in Experimental Materials and Methods. 20 μg of protein was loaded in a 15% polyacrylamide gel, separated by SDS-PAGE and stained by coomassie blue. λ: Protein weight marker. Lanes are as indicated in the figure. Sizes are expressed in kDa.

Lane 1 contains a broad-range protein size marker (New England Biolabs). Lane 2 corresponds to the GST protein alone, and migrates to the expected size of ~27 kDa. Lanes 3 and 4 correspond respectively to GST-RBP2(1416-1447) and GST-RBP2(C60) and migrate to their predicted size of 30 kDa and 33 kDa respectively. All proteins were purified to homogeneity with minimal degradation products observed, with GST-RBP2(C60) showing the highest levels of degradation products.

New Zealand white rabbits were immunized with the purified GST-RBP2 proteins and the immunoblotting and immunoprecipitation (IP) potential of the generated antibodies was assayed once the immune response was deemed appropriate by ELISA. H1299 cells were transfected with HA-tagged RBP2 by lipofection in 150mm culture dishes. Cell extracts were harvested 40 hours post transfection and used to test the antibodies. In one case, a whole cell extract was separated by SDS-PAGE in one-well preparative gels. The gels were transferred to PVDF membranes, which were used to test the various antibodies in western blotting by means of a multi-screen apparatus (BioRad). Each antibody was tested at dilutions ranging from 1:100 to 1:2000 of whole serum. Figure 16 shows the obtained results for one such screen.

The first two lanes of the multi-screen apparatus were blotted with a commercial α-HA antibody as a control for HA-RBP2 expression and position (see panel II). Antibodies 2410 and 2411, directed against the 1416-1447 fragment of RBP2 recognized a predominant band corresponding to the transfected HA-RBP2 with other non-specific background bands of low intensity.

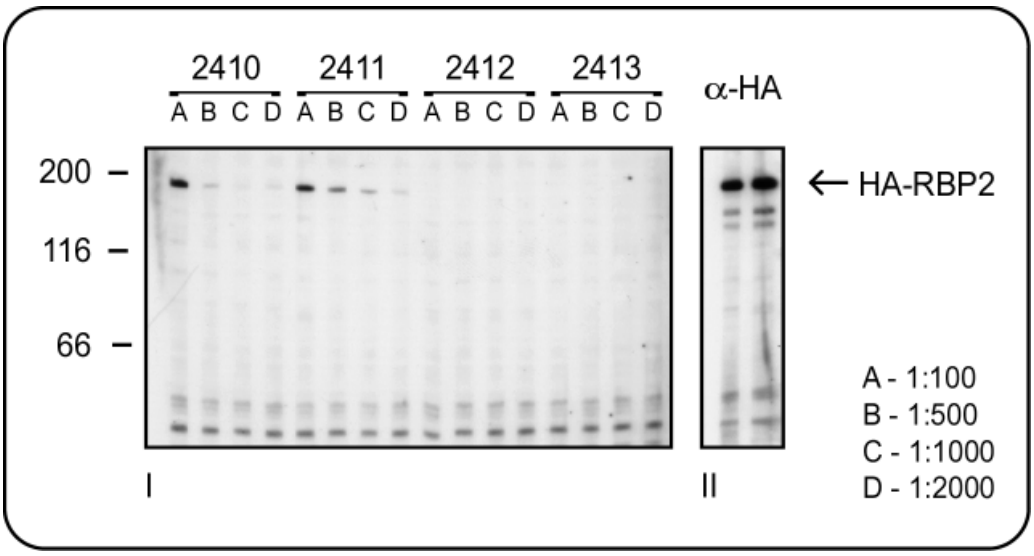


Fig. 16 Western blotting with α-RBP2 antibodies. One-well preparative gels were used to separate 400μg of HA-RBP2 transfected H1299 whole cell extract. α-RBP2 antibodies were tested at 4 different dilutions ranging from 1:100 to 1:2000. Panel I was blotted with rabbit α-RBP2 antibodies at the noted dilutions. Antibodies 2410 and 2411 recognize RBP2 (1416-1447) while antibodies 2412 and 2413 recognize RBP2 (C60). Panel II was blotted with the mouse monoclonal α-HA.11 antibody (BabCo) as a control for HA-RBP2 expression. Both panels were derived from the same membrane. Appropriate HRP-conjugated secondary antibodies were used to visualize recognized proteins by ECL. Immunoblotting was done as described in Experimental Materials and Methods. Sizes are expressed in kDa.

Antibody 2411 produced a recognizable signal at dilutions of up to 1:2000 while the signal from antibody 2410, although quite specific at a dilution of 1:100, quickly diminished in intensity at higher dilutions. Antibodies 2412 and 2413, directed against the C60 fragment of RBP2, produced no detectable signal in this assay.

To investigate the immunoprecipitation capabilities of the antibodies, HA-RBP2 transfected H1299 cell extracts were prepared as described above and lysed in 1ml of RIPA buffer. The cell extracts were split in 200 μl aliquots and subsequently diluted to 1ml with fresh RIPA buffer. Two aliquots were used per antibody characterization. One was incubated with 10μl of whole serum and immunoprecipitated as described in Experimental Materials and Methods.

The other aliquot was subjected to the same manipulations save that no antibody was added as a control for background noise. Immunoprecipitates were separated by SDS-PAGE and blotted with a commercial α-HA antibody. Figure 17 shows the results of the IP characterization and demonstrate that antibodies 2410 and 2411, but not 2412 and 2413, were found to immuno-precipitate RBP2 very efficiently.

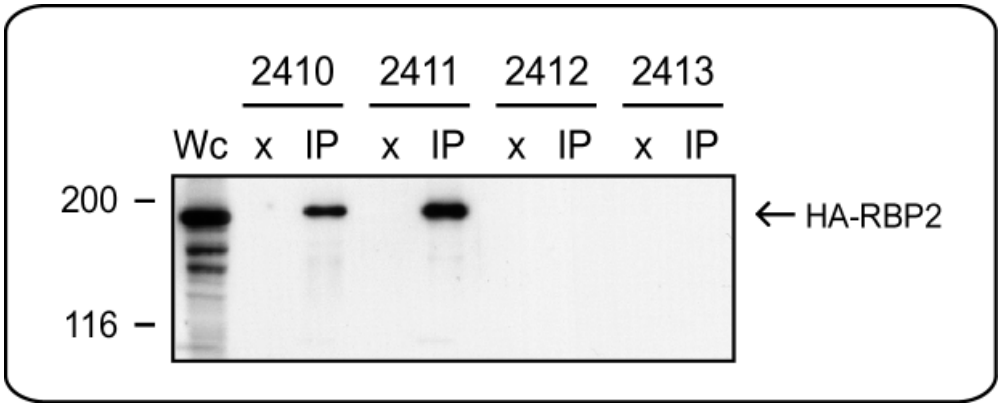


Fig. 17 Immunoprecipitation with α-RBP2 antibodies. HA-RBP2 transfected H1299 cell extracts were lysed, diluted 5-fold and split to 1ml aliquots. Half the aliquots were immunoprecipitated with α-RBP2 antibodies (IP lanes). The other aliquots were used as controls for non-specific immunoprecipitation (X lanes). Precipitates were separated by SDS-PAGE on a 6% polyacrylamide gel, transferred to PVDF membranes and blotted with the α-HA.11 mouse monoclonal antibody (BabCo). Wc: whole cell extract used as a control for HA-RBP2 expression and position. 2410-2411: α-RBP2 1416-1447 antibodies. 2412-2413: α-RBP2 C60 antibodies. Sizes are expressed in kDa.

Once functional antibodies were obtained, the rabbits were exsanguinated and whole serum was collected. Two rabbits were immunized per antigen; however antibody purification was done on only one representative serum for each antigen. Although the antibody raised against the C60 fragment of RBP2 was not responsive in the initial characterization attempts, it was hypothesized that affinity purification might yield better results. Antibody purification was done as described in Experimental Materials and Methods.

The dialyzed antibodies were quantified and submitted to SDS-PAGE. Figure 18 shows that the antibodies were purified successfully with little trace of exogenous protein contamination.

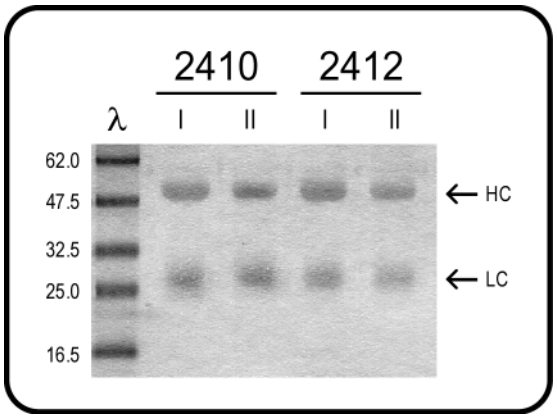


Fig. 18 Purification of α-RBP2 antibodies. Antibodies obtained from New Zealand white rabbits were affinity purified using column-bound GST-RBP2 fusion proteins. The purified antibodies were quantified and 10μg was loaded in a 10% polyacrylamide gel. Following SDS-PAGE, the gel was stained by coomassie blue. λ: Protein weight marker. 2410: α-RBP 1416-1447 antibody. 2412: α-RBP2 C60 antibody. HC: Heavy chain. LC: light chain. I and II correspond to the high pH and low pH elution fractions respectively. Sizes are expressed in kDa.

Lane 1 contains a broad-range protein size marker (New England Biolabs). Lane 2 and 3 contain the high pH and low pH elution fraction of antibody 2410 respectively. Both the heavy and light chains migrated to their predicted size of approximately 50 kDa and 25 kDa, respectively. After antibody purification, immunoblotting and immuno-precipitation testing was redone as described above. Unfortunately, antibody purification did not alter the previous pattern of activity. Both fractions of α-RBP2 2410 produced a strong signal with minimal background in western blotting at dilutions of up to 1:2000 and could also specifically immunoprecipitate HA-RBP2. No positive result could be obtained from either fraction of α-RBP2 2412 (data not shown).

In a final characterization step, α-RBP2 2410 was used to visualize HA-RBP2 *in vivo* by immunofluorescence. H1299 cells were grown in 4-well tissue culture microscope slides and transfected with HA-RBP2 by lipofection. Immunofluorescence was carried out as described in Experimental Materials and Methods. Typically obtained results can be seen in figure 19.

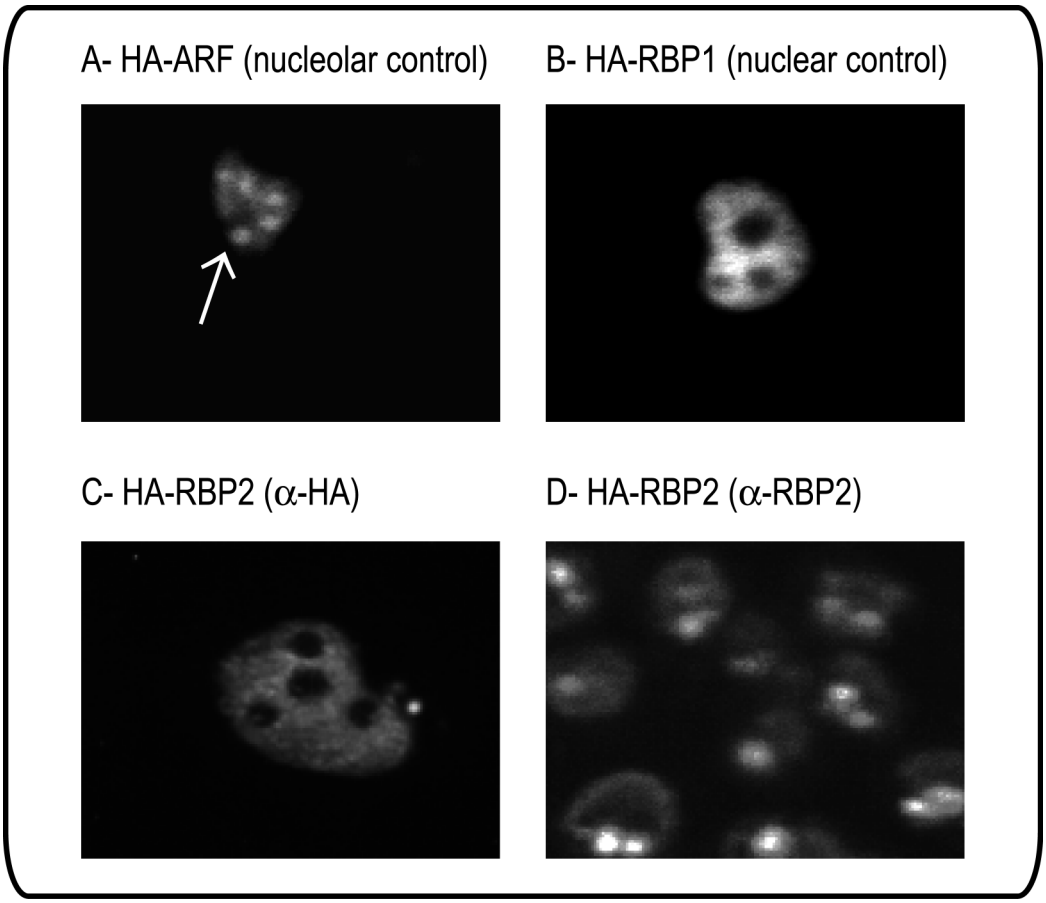


Fig. 19 RBP2 Immunofluorescence. H1299 cells were grown in chambered tissue culture microscope slides and transfected by lipofection with HA-tagged constructs as indicated. Immunofluorescence was carried out using monoclonal mouse α-HA or affinity purified rabbit polyclonal α-RBP2 2410 primary antibodies. Alexa-conjugated species-specific secondary antibodies were used as fluorophores. Panels were transfected as indicated and probed with α-HA unless otherwise noted. All antibodies were used at 1:1000. Slides were prepared as described in Experimental Materials and Methods and visualized using a laser confocal microscope.

Cell fractionation experiments had previously reported RBP2 to be localized to the nucleus (Fattaey *et al.*, 1993; Kim *et al.*, 1994). HA-ARF and HA-RBP1 were used as controls for nucleolar and nuclear localization respectively. As expected, ARF localized to nucleolar regions (the compact circular regions of higher intensity as pointed by the arrow in panel A) while HA-RBP1 demonstrated strong nuclear localization with nucleolar exclusion, as shown by the dark nucleolar regions in panel B. Panel C confirms that RBP2 is localized exclusively to the nucleus. Panel D illustrates that the rabbit polyclonal α-RBP2 antibody cannot be used in immunofluorescence because of the high amount of non-specific background signal it produces. Trials were done at various dilutions but all were negative and produced the same non-specific noise (data not shown).

Chapter 4: Discussion and future work

RBP2 is a transcriptional repressor that plays a role in E2F-dependent transcription regulation. Using the G_5TkCAT reporter system, the repression activity of RBP2 was found to require the C-terminus but could be recovered if the NtR, a 17-kDa fragment at the extreme N-terminus, was expressed alone. These data suggest a model where the C-terminal domain might regulate the activity of NtR such that, upon deletion of the C-terminus, RBP2 adopts a conformation that either disrupts or masks the NtR repression domain. In this model, the C-terminus would not be required to possess intrinsic repression activity but would be necessary to allow NtR-mediated repression in the context of the full-length protein.

The NtR fragment contains two domains of interest, jmjN and a truncated ARID lacking the last 42 amino acids. It is provocative to note that the 42 amino acids deleted from the ARID domain contain the helix-turn-helix DNA binding domain conserved across all ARID containing proteins (Iwahara *et al.*, 1999) as well as the putative tyrosine phosphorylation site. It will be informative to generate GAL4-RBP2 deletion mutants within the NtR to map the repression domain with greater accuracy and determine if the truncated ARID or the jmjN domain correspond to the minimal repression domain of RBP2.

A growing body of evidence reveals that transcription in higher eukaryotes occurs on a chromatin template and repressors must, more often than not, modify chromatin structure either directly or through the recruitment of appropriate protein complexes. Further work is required to clarify the mechanism by which RBP2 represses transcription.

The discovery of specific inhibitors of histone deacetylases such as trichostatin A (TSA) as well as TSA-responsive reporters (Brehm *et al.*, 1998; Lai *et al.*, 1999) could be very informative tools in this process. Transcriptional assays using the constructed panel of deletion mutants and TSA sensitive reporters, such as the G_5E1B-CAT (which expresses the CAT gene under control of the Ad E1B promoter flanked by GAL4 binding sites), should give indications concerning the possible involvement of histone deacetylases in RBP2-mediated repression. This approach should be done in parallel with *in vitro* deacetylase assays, where RBP2 is immunoprecipitated under mild conditions. The immunoprecipitate is then incubated with a broad range of radiolabeled acetylated substrates and radioactivity release is measured to determine if any deacetylation activity is associated with RBP2.

Preliminary transcription assays on TSA sensitive reporters indicated that RBP2 represses in a TSA-insensitive fashion (Côté and Lai, data not shown). Furthermore, preliminary RBP2 *in vitro* deacetylase assays indicated that RBP2 does not recruit deacetylase activity (Albert Lai, personal communication). These results were not included in this thesis as time constraints prevented confirmation of the preliminary data with full and appropriate controls. Furthermore, the NtR domain shows homology with the R1 domain of RBP1, which has been shown to repress in an HDAC-independent manner (Lai *et al.*, 1999). Although still unconfirmed, the above-mentioned facts indicate that the mechanism by which RBP2 represses transcription does not involve the recruitment of histone deacetylases.

This does not rule out the possibility completely, however, as newly discovered histone deacetylases are not all TSA sensitive (Carmen *et al.*, 1999). In addition, the substrate for the initial deacetylase assays, histone H4, might not be the physiological target of any deacetylase activity potentially recruited by RBP2, thus the need for a broader range of substrates.

RBP2 possesses at least 4 protein-protein interacting domains and two possible DNA binding domains. The multi-domain structure of RBP2 alone would suggest its possible involvement as a bridge between DNA and as yet unknown proteins or complexes. RBP2 could potentially coordinate the activities of many regulatory complexes by providing a docking surface for multiple proteins while tethered to DNA.

Determining the interacting partners of RBP2 would significantly increase our understanding of this multifunctional protein. This could be achieved by several means. Constructing a yeast two-hybrid screen using specific domains of RBP2, such as the ARID or PHD domains, as bait would be an obvious method to identify domain-specific interacting proteins. However, such a screen would only detect directly interacting partners.

Mobility shift and supershift assays could also be performed with the newly characterized RBP2 antibodies to identify RBP2-containing E2F complexes. Using this technique on cells arrested at specific stages of the cell cycle could also provide data on the cell cycle expression and complex formation profile of RBP2.

Finally, with advances in protein identification by mass spectroscopy, it could be possible to perform RBP2 IPs on *in vivo* labeled cell extracts to determine a pattern of interacting proteins with the subsequent goal of isolating specific bands for protein identification. This technique has successfully been used by other members of our group (Boivin *et al.*, 1999) but might prove to be challenging in the case of RBP2.

Several groups have reported difficulties when performing *in vivo* interaction studies with RBP2, possibly because of very low endogenous RBP2 expression levels (Fattaey *et al.*, 1993; Kim *et al.*, 1994). The construction of an adenovirus vector expressing RBP2 instead of the normal viral genome could circumvent this problem because cells could be infected at very high efficiency with the recombinant adenovector to increase RBP2 protein levels.

It had previously been reported that overexpression of RBP2 in transcriptional assays could partially reverse Rb-mediated repression of E2F-dependent transcription (Kim *et al.*, 1994). The results shown in figure 12 did not reproduce this observation. However, two noteworthy facts must be taken into account. The assays were performed in *RB* $^{-/-}$ cell lines and RBP2 was always coexpressed with exogenous Rb. The published results did not show if RBP2 could repress transcription when expressed alone. Furthermore, the reporter plasmid used in those assays was the pATF-E2F-CAT, which contains other regulatory sequences besides E2F binding sites (Weintraub *et al.*, 1992) and could possibly skew the assay results.

The reporter plasmid used in the assays performed in the course of this project contained only a minimal TATA box regulated by E2F binding elements to produce unbiased results demonstrating that RBP2 can repress E2F-dependent transcription through a mechanism where the presence of Rb is not compulsory. The cell line used in the E2F transcription assays is deficient in functional Rb yet RBP2, when expressed alone, could still repress E2F-dependent transcription. These cells do however contain functional p107 and p130, which could act to recruit RBP2 to E2F promoters.

In conclusion, RBP2 functions as a transcriptional repressor with growth suppressive capabilities. The role of RBP2 in RB family complexes remains uncertain, as is its possible involvement with other complexes that regulate gene transcription and cell proliferation. Further work is required to identify RBP2-interacting proteins and the mechanism through which RBP2 regulates transcription and cell proliferation.

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