HDAC-independent transcriptional repression by RBP1 is modulated by SUMO modification

By

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Abstract

The tumor suppressor gene *RB* regulates cell proliferation at the G1/S transition of the cell cycle. The retinoblastoma protein pRB associates with both HDAC-dependent and independent mechanisms to actively repress E2F-dependent genes required for entry into S phase. The retinoblastoma binding protein 1 RBP1 recruits the mSin3A/HDAC1 co-repressor complex to the pocket of pRB at growth arrest and accounts for the majority of the HDAC activity associated with pRB. However, transcriptional repression by RBP1 also involves HDAC-independent activities because repression is only partially relieved by the HDAC inhibitor Trichostatin A. This activity is mediated in part by residues 241 to 452 of RBP1 designated as the R1 domain. Hypermapping studies on the previously defined R1 domain of RBP1 revealed that amino acids 400 to 452 of RBP1 are sufficient to mediate HDAC-independent repression. Inspection of the minimal R1 region located two copies of the SUMO consensus motif ψ-K-X-E and subsequent experiments demonstrated that the R1 domain is post-translationally modified by SUMO on lysine 418 and 444. In addition, transcriptional repression by the R1 domain was abrogated by either mutagenesis of both SUMO acceptor lysines or in the presence of a SUMO specific protease implying that SUMO modification modulates HDAC-independent transcriptional repression by RBP1.

Résumé

Le gène suppresseur de tumeur RB contrôle la croissance des cellules lors de la phase G1/S du cycle cellulaire. Les déacetylases d'histones, de même que des mécanismes indépendants des HDAC, sont recrutés par la protéine pRB pour ainsi prévenir l'activité des facteurs de transcription E2F, qui contrôle l'expression de gènes requis pour l'exécution de la phase S. Le complexe corépresseur mSin3A/HDAC1 est associé à pRB grâce à la protéine RBP1 et cette interaction est nécessaire pour l'induction de l'arrêt cellulaire. Par contre, la répression de la transcription par RBP1 n'est que partiellement sensible à l'inhibiteur de HDAC Trichostatin A, suggérant un mécanisme de répression indépendant du remodelage des histones par déacétylation. Ce second domaine de répression nommé R1 est situé entre les acides aminés 241 et 452 de RBP1. Cette région circonscrit un autre domaine retrouvé dans un certain nombre de facteurs de transcription connus sous le nom de ARID. Une étude plus poussée sur R1 révèle que les résidus 400 à 452 sont suffisants pour permettre la répression indépendante des HDAC. De plus, une inspection attentive de cette région a permis de localiser deux sites putatifs conformant au consensus ψ-K-X-E qui permet la sumoylation. Diverses expériences ont ensuite permis de confirmer que le domaine de répression R1 est modifié par la protéine SUMO *in vivo* et que les lysines 418 et 444 sont les deux sites majeurs de sumoylation. La mutagénèse de ces deux sites ou l'expression d'une protéase capable de prévenir la sumoylation des protéines cause une perte de la répression par le domaine R1 et démontre un lien direct entre la sumoylation et le contrôle de la répression de la transcription par RBP1.

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List of Abbreviations

aa	amino acids
ALP	Aprotinin, Leupeptin, Pepstatin
ARID	A/T rich interacting domain
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCAA	Breast Cancer Associated Antigen
CBP	CREB binding protein
Cdk	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
C/EBP	CAATT enhancer binding protein
CHO	Chinese hamster ovary cells
ChIP	Chromatin immunoprecipitation
CMV	Cytomegalovirus
CpG	Cytosine followed by Guanine
CRD1	Cell cycle repression domain 1
CtBP	C terminal binding protein
CtIP	CtBP interacting protein
DBD	DNA binding domain
dl	deletion
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferase
Dri	Dead ringer
DTT	Dithiothreitol
E1A	Early region 1 A protein
E2F	Early region factor 2
eARID	extended ARID
EDTA	Ethylenediaminetetraacetic acid
EFα1	Enhancer factor 1
EMSA	Electric mobility shift assay
FBS	Fetal bovine serum
G1	gap phase 1
G2/M	gap phase 2/mitosis
GFP	Green fluorescent protein
GMP	GAP modifying protein
gr	gram
GST	Glutathione-S-transferase
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HEPES	N-(2-hydroxyethyl) piperazine-N-N-(2-ethanesulfonic acid)
HMT	Histone methyltransferase
HP-1	Heterochromatin protein 1
HPV	Human papilloma virus
HRP	Horse radish peroxidase
HSF	Heat Shock Factor

IP	immunoprecipitation
IPTG	Isopropyl B-D-thiogalactopyranoside
IxCxE	I stands for isoleucine, C for cysteine, E for glutamate and X any amino acid
K418R	lysine 418 of RBP1 substituted to arginine
K433R	lysine 433 of RBP1 substituted to arginine
K444R	lysine 444 of RBP1 substituted to arginine
K1204R	lysine 1204 of RBP1 substituted to arginine
kDa	kilodalton
LxCxE	as above, except L stands for leucine
MEF2	Myocyte enhancer factor 2
min	minute
mif2	Mitotic fidelity of chromosome transmission protein 2
mL	milliliters
MLP	adenovirus major late promoter
mRNA	messanger RNA
NAD	Nicotinamide adenine dinucleotide
NEM	N-ethyl maleimide
NLS	nuclear localization signal
NZFP	Novel Zinc-finger Protein
O/N	over night
PcG	Polycomb group proteins
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PIAS	Protein Inhibitor of Activated STAT
PML	Promyelocytic leukemia protein
PMSF	Phenylmethylsulfonyl fluoride
PSG	Penicillin, Steptomycin, glutamine
PVDF	polyvilyidene difluoride
RanBP2	Ran GAP binding protein 2
RbAp	Retinoblastoma associated protein
RBP1	Retinoblastoma binding protein 1
RBP1L1	Retinoblastoma binding protein 1 like 1
Rpm	revolutions per minute
RSV	Rous sarcoma virus
rt	room temperature
SAE	SUMO Activating Enzyme
SAP	Sin3 associated polypeptide
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrilamide gel electrophoresis
Sec	seconds
SENP	Sentrin specific protease
snRNP	small nuclear ribonucleoprotein
Sp3	Specificity protein 3
SV40	Simian virus 40
SuPr-1	SUMO protease 1

Suv39H1	Suppressor of variegation 39 H1
SWI/SNF	Switch/sucrose non-fermenting
TBP	TATA binding protein
TBS	Tris buffered saline
TGF	Transforming Growth Factor
TK	thymidine kinase
TKO	Triple knockout
Tris	Tris hydroxymethyl aminoethan3
TSA	Trichostatin A
Uba	Ubiquitin-like activating enzyme
Ubc	Ubiquitin-like conjugating enzyme
ug	microgram
uL	microliter
Ulp	Ubiquitin-like protease

Chapter 1: Introduction

1.1 The prototypical tumor suppressor pRb

A hallmark feature of tumors is their ability to grow uncontrollably by avoiding intricate cellular defense mechanisms such as apoptosis, DNA repair and cell cycle regulation. In fact, several gene products required for growth arrest are inactivated or lost in tumors. Studies on a rare form of eye cancer known as retinoblastoma led to the formulation of the "two-hit" hypothesis of tumor formation due to high incidence of this disease in certain families (Knudson, 1971). Several years later, the retinoblastoma susceptibility gene *RB* was the first tumor suppressor cloned and has been observed to be frequently mutated in other tumors such as osteosarcoma, breast and prostate carcinomas (Friend et *al.*, 1986). Interestingly, many viral oncoproteins such as the adenovirus early region 1 A protein E1A, have the ability to interact with the retinoblastoma protein pRB, implicating the latter in growth suppression (DeCaprio et *al.*, 1988; Bandara et *al.*, 1991; Nevins, 1992). Subsequent experiments have revealed that the *RB* gene family plays a critical role in cell cycle progression, growth arrest and differentiation by blocking the activity of the E2F family of transcription factors.

1.1.1 *RB gene family members*

The retinoblastoma gene product encodes a ubiquitously expressed nuclear phosphoprotein of 928 amino acids that is sometimes referred to as a pocket protein due to its structure. The other pRB related proteins isolated, p107 and p130, share 50% homology to each other and 30% to pRB but have similar structural features (Li et *al.*, 1991; Hannon et *al.*, 1993). All three members contain two domains termed A and B, that

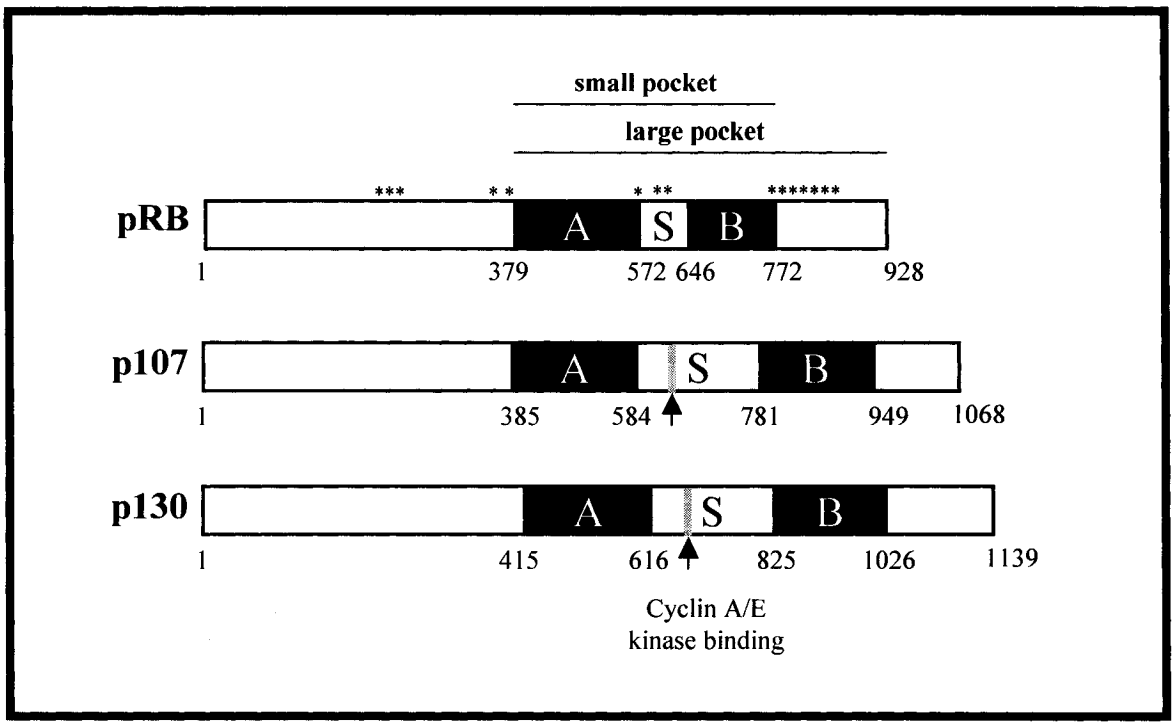


Figure 1.1. Schematic representation of the pRB family members. The small pocket of pRB is required for viral oncoprotein binding whereas the E2F family of transcription factors interact with the large pocket, which comprises the C-terminal region of pRB. The majority of the phosphorylation sites in pRB are located in the E2F binding region. In addition to the A and B domains that form the pocket, p107 and p130 also contain a larger spacer region, which harbors a binding region for cyclin A and E kinases that is absent in pRB. Adapted from Morris and Dyson, 2001.

together form the pocket region involved in many protein-protein interactions (Figure 1.1). In fact, the pocket of pRB mediates growth suppression and most mutations in pRB occur in this region (Mulligan and Jacks, 1998). Viral proteins such as E1A, the Simian virus 40 large T antigen and the human papilloma virus E7 protein all interact with the small pocket (amino acids 379-792) of pRB via a short peptide motif that contains the sequence LxCxE (Nevins, 1992). Several cellular proteins such as RBP1 and RBP2 have been isolated as pRB binding proteins due to the presence of this motif (Defeo-Jones et *al.,* 1991). Unlike viral proteins, the E2F transcription factors interact with the large pocket (amino acids 379-928) of pRB in an LxCxE independent fashion (Morris and Dyson, 2001). The interaction with pRB and E2F is controlled by phosphorylation as only hypophosphorylated pRB can bind to E2F (Chellapan et *al.*, 1991). In fact, the level of pRB phosphorylation varies depending on the cell cycle stage, where pRB is predominantly hypophosphorylated at the beginning of G_1 and hyperphosphorylated during S phase (Buchkovich et *al.*, 1989). There are several differences among the pocket proteins that are noteworthy. While the levels of pRB remain constant, p130 expression is high in G_0 and in early G_1 but decreases as cells enter S phase due to p130 degradation via the ubiquitin proteasome pathway (Smith et *al.*, 1998). The levels of p107 are low in early G_1 but increase as cells progress into S phase (Smith et *al.*, 1998; Classon and Harlow, 2002). The functional significance of these patterns of expression is reflected by the different interactions that occur with various members of E2F transcription factors at different stages of the cell cycle.

1.1.2 Pocket proteins and cell proliferation

The implication of pRB in cell proliferation was conclusively demonstrated by targeted disruption in mice. Embryos completely deficient for *RB* fail to develop and die around the 16th day of gestation with prominent defects in neurogenesis and haematopoiesis (Clarke et *al.*, 1992; Lee et *al.*, 1992). Furthermore, *RB*-/- mouse embryonic fibroblasts (MEF) display cell cycle defects that include a shortened G_1 phase and also a higher expression of E2F regulated genes (Herrera et *al.*, 1996). The lethal phenotype associated with *RB*-/- embryos appears to be caused by increased E2F1 activity as mutations inactivating E2F1 in these embryos increases their life span, suggesting that E2F can promote apoptosis (Tsai et *al.*, 1998). In contrast to pRB, mice lacking either p107 or p130 suffer no apparent developmental defects and are not prone to tumors (Cobrinik et *al.*, 1992; Lee et *al.*, 1996). In spite of this, overexpression of either p130 or p107 is capable of inducing growth arrest. Strong evidence implicating all three members of the pocket proteins in cell cycle control come from studies performed with *p107/p130/RB*-/- triple knockout (TKO) mouse embryonic fibroblasts. Following serum deprivation or contact inhibition, TKO MEFs fail to undergo G_1 arrest and were also more susceptible to transformation with the oncogene Ras, which is in sharp contrast to *RB*-/- MEFs or *p107; p130*-/- MEFs, (Sage et *al.*, 2000).

1.1.3 Cyclin-dependent kinases and pRB function

Cell division is a highly regulated process that consists of four distinct phases: G_1, S, G_2 and M. Entry into S phase is controlled in part by the activity of the pocket proteins and the E2F family of transcription factors. In addition, growth factors cause downstream

signals to activate sequentially a subset of protein kinases termed the cyclin-dependent kinases (CDK). This family of kinases regulates pRB function by directly affecting its level of phosphorylation. The activation of the kinases requires binding with a specific member of the cyclin family of proteins (Adams, 2001). The critical regulators of G1 progression in mammalian cells are the D and E type cyclins. Extracellular signals trigger a rapid rise in cyclin D levels in early G1 enabling the association and activation of the Cdk4/6 kinase (Sherr, 1993). This active cyclin D/Ckd4/6 complex phosphorylates pRB in specific residues in the C-terminal region (Bates et *al.*, 1994; Dyson, 1998). The increase in pRB phosphorylation causes a decrease in pRB/E2F complexes and disrupts LxCxE dependent interactions with cellular co-factors (Cooper and Shayman, 2001; Zhang et *al.*, 2000). This also coincides with an increase in cyclin E levels at the G1/S phase, which is in turn activated by E2F1 (Ohtani et *al.*, 1995). The developmental defects associated with *cyclin D1-/-* mice can be rescued by *cyclin E* knock-in reinforcing the notion that that the latter is a major downstream target of cyclin D1 (Geng et *al.*, 1999). Cyclin E then associates with Cdk2 and this active CDK complex will further cause an increase in pRB phosphorylation allowing more E2F release and subsequent entry into S phase (Lundberg and Weinberg, 1998). This point of the cell cycle is referred to as the restriction point because cells no longer require growth factors and are committed to completing the cycle. The activity of cyclin-dependent kinases is in turn regulated by a family of cyclin-dependent kinase inhibitors (CKI).

1.1.4 Cyclin-dependent kinase inhibitors

Mammalian cells express two different groups of CKIs that have been classified based on their ability to inhibit a specific set of CDKs. Growth inhibitory signals and DNA damage can induce the expression of CKIs such as p21 and the overexpression of all CKIs causes cells to withdraw from the cell cycle (Sherr and Roberts 1995; Morisaki et *al.*, 1999; Reynisdottir et *al.*, 1995). The $p21^{WAF1}$ (Cip/Kip) family of CKIs, which includes $p27^{Kip1}$, $p57^{Kip2}$ and $p21^{WAF1}$, can inhibit the activity of different cyclin-cdk complexes including cyclin D/cdk4-6 and cyclin E/cdk2 (Harper et *al.*, 1993; Xiong et *al.*, 1993). Unlike the Cip/Kip family, genes encoded by the *INK4a* locus regulate the activity of the cyclin D/cdk4-6 kinase complex. Members of this family of proteins includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ (Sherr and Roberts 1995; Hall et *al.*, 1995). p15 and p16 have both been demonstrated to associate directly with Cdk4 and Cdk6 thus preventing cyclin D binding and activation (Hall et *al.*, 1995). The observation that p16 is unable to cause growth arrest in the absence of pRB indicates that the INK4 family of CKIs are upstream effectors of the *RB* pathway (Lukas et *al.*, 1995).

1.2 E2F transcription factors

E2F1 was initially identified as a pRB binding protein that had previously been shown to be required for E1A mediated transactivation of the adenoviral E2A promoter (Helin et *al.*, 1992; La Thange et *al.*, 1987). The observation that E1A could displace pRB from E2F suggested that pRB participated in cell proliferation (Bandara et *al.*, 1991). In support of this, several groups have shown that overexpression of E2F1 bypasses growth arrest and allows cells to progress into S phase (Johnson et *al.*, 1993;

Qin et *al.*, 1994). The E2F transcription factors control the expression of an array of genes involved in DNA synthesis, DNA replication and cell cycle regulation.

1.2.1 E2F structure analysis

Mammalian cells express six different E2F transcription factors that share similar structural features (Figure 1.2). All members of the *E2F* gene family contain a DNA binding domain adjacent to a dimerization domain (Trimarchi and Lees, 2001). This domain mediates the interaction with the dimerization protein DP-1/2 family members and potentiates the DNA binding and transactivating properties of the E2F transcription factors (Girling et *al.*, 1993; Bandara et *al.*, 1993). The E2F family can be divided into two classes based on their transcriptional activity. E2F1/2 and E2F3 are primarily associated with transcriptional activation whereas E2F4/5 and E2F6 are associated with repression of E2F regulated genes. E2F2 and E2F3 are highly homologous to E2F1 with most of the homology lying in the DNA binding domain and the transactivation domain, the latter containing the pRB binding region (Ivey-Hoyle et *al.*, 1993; Lees et *al.*, 1993). In contrast to its mammalian counterparts, E2F6 is unable to bind pocket proteins as it lacks the C-terminal transactivation domain (Morkel et *al.*, 1997). Subsequent experiments have revealed that E2F6 represses E2F dependent transcription by recruiting the polycomb group protein B lymphoma Mo-MLV insertion region 1 Bmi-1, a repressor of the INK4A-ARF tumor suppressor locus (Trimarchi et *al.*, 2001).

E2F4 and E2F5 were both initially cloned due to their ability to interact with the other pocket proteins p107 and p130 and appear to have distinct properties that are not

shared by the activator E2Fs (Beijersbergen et *al.*, 1994; Hijmans, et *al.*, 1995; Vairo et *al.*, 1995). For instance, E2F4 and E2F5 lack nuclear localization signals and are targeted to the nucleus via interaction with pocket protein family members and, unlike the activator E2F transcription factors, expression of E2F4 or E2F5 fails to push quiescent cells into entering S phase (Muller et *al.*, 1997; Verona et *al.*, 1997). In addition, E2F4 and E2F5 levels remain constant during the cell cycle and are actually highest at G_0, whereas E2F1/2 and E2F3 are only present in actively cycling cells (Ikeda et *al.*, 1996).

1.2.2 Regulation of E2F activity

Expression of genes required for G_1/S progression such as *cyclin E* and *cyclin D* is tightly regulated by E2F1, along with genes that mediate DNA synthesis in S phase (Stevens and La Thange, 2003). E2F1 activity is directly inhibited by pRB, which binds directly the transactivation domain and prevents E2F1 from contacting the transcriptional machinery (Flemington et *al.*, 1993; Helin et *al.*, 1993). However, this model of repression cannot explain how the E2F sites in certain promoters act as negative regulatory elements (Weintraub et *al.*, 1992; Ohtani et *al.*, 1995). Consequently, several experiments demonstrated that pRB is tethered to DNA via E2F transcription factors and is able to repress transcription by recruiting various chromatin remodeling complexes (Luo et *al.*, 1998; Zhang et *al.*, 2000; Nielsen et *al.*, 2001; Lai et *al.*, 2001). Furthermore, while E2F1/2 and E2F3 only interact with pRB, E2F4 and E2F5 can interact with the three pocket proteins, and these interactions are regulated in a cell cycle dependent manner (Hijmans, et *al.*, 1995; Vairo et *al.*, 1995; Trimarchi and Lees, 2001).

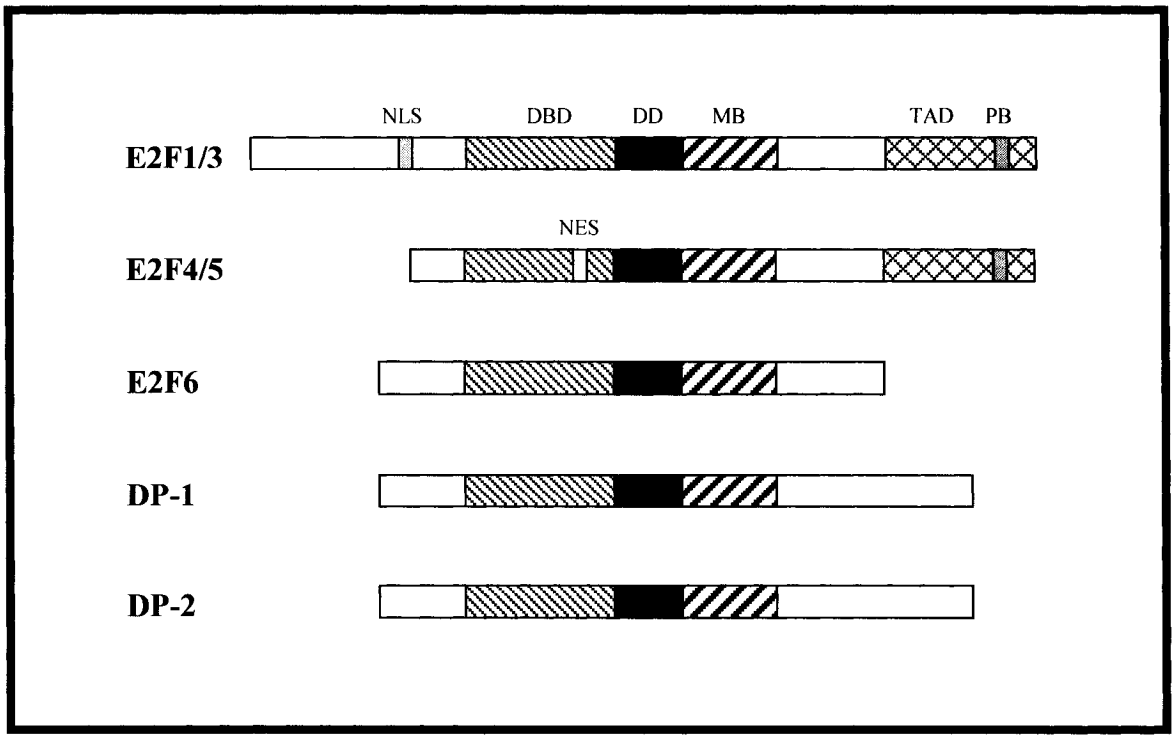


Figure 1.2. Structure of the E2F/DP family members. Schematic representation of the functional domains of E2F and DP proteins. E2F transcription factors and DP proteins all share homology in their DNA binding domain (DBD) and in the dimerization domain (DD). All E2Fs, except for E2F-6, have a transactivation domain (TAD) located at the C-terminus, which contains the pRb and pocket protein binding region (PB). The activator E2Fs (E2F-1/2/3) also possess an N-terminal cyclin A binding site and a nuclear localization signal (NLS) that is absent in E2F-4 and 5. Rather, the repressor E2Fs have a nuclear export signal present in their DNA binding domains. Adapted from Stevens and La Thange, 2003.

1.2.3 Pocket protein/E2F complexes and cell cycle regulation

Promoter occupancy by E2F complexes at different stages of the cell cycle has been extensively studied. Following serum starvation, analysis of E2F regulated genes such as *cyclin A*, *cdc2* and *e2f1* by chromatin immunoprecipitation (ChIP) indicates that p130/E2F4/5 are the predominant E2F complexes in quiescent cells (Takahashi et *al.*, 2000; Rayman et *al.*, 2002). This observation is consistent with the observation that *p107; p130-/-* MEFs have increased expression of E2F responsive genes in Go and G1, while *e2f4; e2f5-/-* MEFs fail to exit the cell cycle after expression of the cyclin-dependent kinase inhibitor p16 (Hurford et *al.*, 1997; Gaubatz et *al.*, 2000). In contrast to what is observed in human cells, p107/E2F4 complexes are also present at growth arrest in MEFs, suggesting that there are differences between the species (Rayman et *al.*, 2002). Furthermore, the recruitment of histone deacetylase 1 repressor HDAC1 to these E2F regulated promoters depends on the presence of either p107 or p130 but not pRB.

Surprisingly, pRB/E2F complexes *in vivo* were not detected on these E2F regulated promoters in quiescent cells or during the G1/S transition (Takahashi et *al.*, 2000; Rayman et *al.*, 2002). However, this finding could be due to the type of cell cycle withdrawal conditions used or the type of E2F promoter studied because pRB has been shown to occupy the *cyclin A* promoter of cells pushed into senescence by the cyclin-dependent kinase inhibitor p16 or the transforming growth factor TGFβ (Dahiya et *al.*, 2001). Alternatively, pRB may occupy E2F promoters transiently or in cells undergoing terminal differentiation where it recruits chromatin modifying enzymes to establish an irreversible cell cycle arrest (Nielsen et *al.*, 2001). Evidence for this hypothesis lies in the

observation that myoblasts deficient for pRB previously induced to differentiate into myotubes are still capable of re-entering the cell cycle following mitogenic stimulation (Novitch et *al.*, 1996). Moreover, pRB interacts with a large portion of the pool of HDAC1 in differentiated muscle cells and may contribute to turning off E2F regulated genes once the differentiation program is initiated (Puri et *al.*, 2001). Also, recent experiments performed in human fibroblasts indicate that pRB can be detected on E2F regulated promoters of genes such as *cyclin A* and proliferating cell nuclear antigen *pcna* only in senescent but not quiescent cells (Narita et *al.*, 2003). Interestingly, other studies indicate that pRB and E2F4 localize to discrete regions in the nucleus at growth arrest that correspond to origins of replication (Kennedy et *al.*, 2000; Lai et *al.*, 2001).

Following cell cycle re-entry, the levels of p130 begin to subside and pRB/p107 pocket proteins complex with E2F4, while E2F1/2 and E2F3 are sequestered by pRB early in G_1 (Morberg et *al.*, 1996). In late G_1, most of the pRB exists in its hyperphosphorylated form enabling E2F1/2 and E2F3 to activate genes required for progression into S phase. The importance of the activator E2Fs is demonstrated by the inability of *e2f1/2 and e2f3-/-* TKO cells to enter S phase (Wu et *al.*, 2001). Additionally, the lack of nuclear targeting by p130 causes E2F5 to localize in the cytoplasm, while E2F4/p107 complexes are exported to the cytoplasm via the nuclear export factor known as the chromosome region maintenance protein 1 CRM-1 (Gaubatz et *al.*, 2001).

1.3 Chromatin and Regulation of transcription

Expression of genes is a highly regulated process that is negatively or positively influenced by the structure of chromatin. Unpacking of the higher order structure of chromatin is required to allow DNA to be accessibility to the general transcription machinery. This can be achieved by several means such as nucleosomal rearrangement, disruption of interactions between nucleosomes and also by interruption of histone-DNA contacts (Gaston and Jayaraman, 2003). Dynamic changes in chromatin structure are mediated by ATP-dependent nucleosome remodeling complexes and histone modifying enzymes and both are implicated in the regulation of transcription (Narlikar et *al.*, 2002). Lysine residues in the N-terminal tail of histones can be modified by post-translational modifications such as phosphorylation, ubiquitination, methylation and acetylation. Acetylation of histones removes the positive charge on the lysine residue thereby disturbing the affinity for the phosphate backbone of DNA and this event is associated with transcriptional activation (Grant, 2001). Acetylation has a positive influence on transcription and methylation of histone tails creates a binding surface for proteins involved in the gene silencing (Lachner et *al.*, 2001, Bannister et *al.*, 2001). Evidence of cooperation between different types of histone modifying enzymes is apparent as methylation of specific residues can only occur after deacetylation of the latter (Rea et *al.* 2000). The interplay between histone modifications has lead to the proposal that a histone code is involved in recruiting different sets of proteins in order to achieve activation or repression of transcription (Strahl and Allis, 2000).

1.3.1 Active repression of E2F transcription by pRB

Over one hundred proteins have been demonstrated to bind pRB but the relevance of some of these interactions remains elusive (Morris and Dyson, 2001). Some protein partners suggest that pRB is involved in transcriptional activation, although this appears to be indirect in some instances (Puri et *al.*, 2001). On the other hand, several of these binding partners are chromatin modifying enzymes and have been implicated in pRB mediated transcriptional repression (Ferreira et *al.*, 2001). The retinoblastoma protein utilizes E2F/DP heterodimers to bind DNA and actively repress transcription. Furthermore, the pocket of pRB is capable of repressing transcription independently of E2F when recruited to promoters by a DNA binding domain (Weintraub et *al.*, 1995; Luo et *al.*, 1998). Transcriptional repressors can function by either binding to the basal transcription machinery, blocking the action of an activator, or facilitating the recruitment of a chromatin remodeling complex (Gaston and Jayaraman, 2003). The retinoblastoma protein appears to utilize all three mechanisms of repression, but the involvement of chromatin remodeling complexes has been the most extensively studied. Many histone modifying enzymes are recruited directly or indirectly to the pocket of pRB in an LxCxE dependent fashion and these interactions may be responsible for pRB-mediated exit from the cell cycle and silencing of E2F genes during terminal differentiation.

1.3.2 Chromatin-modifying enzymes and pRB

1.3.3 Histone Deacetylation

Histone deacetylases (HDAC) catalyze the removal of acetyl groups from histone tails to cause chromatin condensation and are often recruited by proteins involved in

transcriptional repression (Laherty et *al.*, 1997; Lai et *al.*, 2001; Fleisher et *al.*, 2003). There are three classes of HDACs based on their homology to their yeast counterparts. Class I HDACs are related to the yeast protein reduced potassium dependency 3 Rpd3, whereas the class II HDACs are related to the yeast protein histone deacetylase 1 Hda1 (Gray and Elkstrom, 2001). Although class I and II HDACs are structurally related, class II HDACs possess an N-terminal extension that is required for inhibition of the myocyte enhancer factor 2 MEF-2 activity, a protein in muscle differentiation initiation (Miska et *al.*, 1999). In contrast to class I HDACs, class II HDACs are exported to the cytoplasm during differentiation in a signal dependent manner where they associate with 14-3-3 proteins, thus allowing expression of muscle specific genes (Grozinger et *al.*, 2000). In mammalian cells, two class I HDAC co-repressor complexes have been isolated, the mSin3A-associated and the nucleosome remodeling and histone deacetylation (NURD) complexes (Zhang et *al.*, 1997; Xue et *al.*, 1998). Both contain HDAC1/2 but unlike mSin3A, the NURD complex has ATPase activity involved in nucleosomal remodeling. Although mSin3A lacks this kind of activity, it has been shown to co-purify with members of the SWI/SNF ATP-dependent remodeling complex (Sif et *al.*, 2001). The class III members of the HDAC family are related to the Sirtuin 2 Sir2 yeast HDAC. This family of HDACs is different from the others in that deacetylase activity is dependent on the cofactor NAD^+ and they are insensitive to the class I/II HDAC inhibitor Trichostatin A (Imai et *al.*, 2000).

The pocket of the retinoblastoma protein was initially shown to bind and inhibit transcriptional activators such as the lymphoid-specific factor E74-like factor 1 Elf-1 by

blocking its interaction with the basal transcription machinery when recruited to a promoter in an E2F-independent manner (Weintraub et *al.*, 1995). In addition, several reports indicated that active repression of E2F-dependent transcription by the pocket of pRB involved histone deacetylation because treatment with the HDAC inhibitor TSA severely impaired repression of E2F-dependent transcription (Luo et *al.*, 1998; Magnaghi-Jaulin et *al.*, 1998). Moreover, the pocket of pRB can repress transcription mediated by viral promoters such as SV40, Herpes simplex virus thymidine kinase (HSV TK) and adenovirus major late promoter (MLP) when brought to a synthetic reporter by a DNA binding domain (Luo et *al.*, 1998). Interestingly, pRB-mediated repression of the SV40 and TK promoters is insensitive to TSA, suggesting that the pocket of pRB contains both HDAC-dependent and independent repression mechanisms (Luo et *al.*, 1998; Lai et *al.*, 1999b). Subsequent experiments revealed that the pocket of pRB interacts with the class I histone deacetylases HDAC1/2 and HDAC3 in an LxCxE dependent fashion (Luo et *al.*, 1998; Magnaghi-Jaulin et *al.*, 1998; Lai et *al.*, 1999b).

Initial reports suggested that HDAC1 interacts directly with the pocket of pRB as deletion of a region containing the IxCxE motif abolished the interaction (Magnaghi-Jaulin et *al.*, 1998). However, some deletion mutants used to support this idea also lacked a large portion of the protein, which may have affected other binding regions. HDAC3 lacks an LxCxE motif so it may bind the pocket of pRB via an adaptor protein or through a different binding motif. Also, yeast two-hybrid experiments failed to detect direct HDAC1 or HDAC2 binding to pRB (Lai et *al.*, 1999b). Recent reports have shown that class I HDACs are recruited indirectly to the pocket of pRB via the LxCxE containing

pRB-binding protein RBP1 (Lai et *al.*, 1999b). In fact, removal of RBP1 by immunodepletion leads to a significant loss of the histone deacetylase activity associated with pRB (Lai et *al.*, 2001). Since the HDAC activity associated with pRB is not completely dependent on the presence of RBP1, other factors, such as the DNA methyltransferase 1 Dnmt1, may recruit HDAC1 to pRB (Robertson et *al.*, 2000).

1.3.4 Histone methyltransferases

Specific lysine residues of histones H3 and H4 can undergo methylation by histone methyltransferases (HMT) (Kouzarides, 2002). Suppressor of variegation 39 H1, SUV39H1, the first of the lysine histone methyltransferases isolated, methylates only lysine 9 of histone H3 and is involved in the formation of heterochromatin (Rea et *al.*, 2000). Successive reports then demonstrated that SUV39H1-mediated methylation of lysine 9 is required to create a binding surface for the heterochromatin-associated protein HP1 (Lachner et *al.*, 2001, Bannister et *al.*, 2001). Moreover, SUV39H1 can mediate transcriptional repression when recruited to a promoter and this activity is dependent on its catalytic domain (Firestein et *al.*, 2000). However, others have reported that SUV39H1 interacts with HDACs independently of the catalytic domain to repress transcription of a synthetic promoter (Vaute et *al.*, 2002).

pRB can associate with the SUV39H1 methyltransferase and participate in pRB-mediated transcriptional repression of E2F regulated genes (Nielsen et *al.*, 2001; Vandel et *al.*, 2000). In particular, SUV39H1 cooperates in pRB-mediated repression of the *cyclin E* gene in a methyltransferase dependent manner. Transcription of genes such as *cyclin E* and *cyclin A* is up regulated in mice lacking *suv39h1/2* (Nielsen et *al.*, 2001).

Furthermore, ChIP experiments on the *cyclin E* promoter using *RB-/-* MEFs conclusively demonstrated that HP1 promoter occupancy is abolished in the absence of pRB and this effect also correlated with a disruption in lysine 9 methylation (Nielsen et *al.*, 2001). Even though SUV39H1 lacks the LxCxE pRB-binding motif a peptide containing the LxCxE sequence can efficiently block the interaction between both proteins, suggesting that an adaptor protein is likely involved in bridging histone methyltransferase activity to the pocket of pRB (Vandel et *al*, 2000; Nielsen et *al.*, 2001). Interestingly, HP1 localization to heterochromatin is lost *in vivo* when cells are treated with the HDAC inhibitor TSA (Taddei et *al.*, 2001). Another recent report demonstrated that the E2F controlled promoters of the *cyclin A* and *pcna* genes have a higher amount of histone H3 lysine 9 methylation during senescence relative to quiescent cells (Narita et *al.*, 2003). Based on these findings, one could speculate that pRB recruits deacetylase activity to E2F-regulated genes to allow methylation by SUV39H1, thus establishing gene silencing by HP1. This event could then allow the recruitment of other proteins to maintain an irreversible cell cycle exit required during terminal differentiation.

1.3.5 DNA methylation

Another important biochemical feature of heterochromatin is DNA methylation. Mammalian cells express three DNA methyltransferases, Dnmt1, Dnmt3a and Dnmt3b (Burgers et *al.*, 2002). Dnmt1 is required for *de novo* methylation of DNA during development to maintain the established 5-methyl cytosine patterns of CpG dinucleotides and has been implicated in gene silencing (Li et *al.*, 1992; Okano et *al.*, 1999). The methyl mark on DNA is recognized specifically by methyl cytosine binding proteins (MeCP), which recruit histone deacetylases to repress transcription (Jones et *al.*, 1998).

Recently, Dnmt1 was shown to interact with HDACs independently of its catalytic domain and is recruited by the Dnmt1 associated protein co-repressor DMAP1 to replication foci (Rountree et *al.*, 2000). Dnmt1 was recently isolated as part of an E2F/pRB/HDAC1 containing complex (Robertson et *al.*, 2000). Interestingly, Dnmt1 interacts with the pocket of pRB in an LxCxE dependent fashion but lacks this structural motif. Mapping experiments reveal that the N-terminus of Dnmt1 is required for pRB binding, which has been shown to mediate HDAC binding (Robertson et *al.*, 2000; Rountree et *al.*, 2000). Also, Dnmt1 participates in the repression of E2F regulated genes only in the presence of pRB and this ability is not completely abolished upon removal of its catalytic domain. In addition, *de novo* methylation of the reporter constructs could not be detected, which suggests that DNA methylation may not be utilized by pRB for transcriptional repression (Robertson et *al.*, 2000). However, the observation that Dnmt1 and pRB have both been detected at replication foci could indicate that pRB targets Dnmt1 during growth arrest and participates in the control of replication. Interestingly, it was shown that the mouse Dnmt1 promoter contains E2F1 binding sites that can be repressed by pRB in both HDAC-dependent and independent manners (Kimura et *al.*, 2003). This effect implicates Dnmt1 in the pRB tumor suppression pathway, because DNA hypermethylation has been detected in different tumor cell lines (Baylin and Herman, 2000).

1.4 LxCxE interactions and growth suppression

The transforming ability of viral oncoproteins such as E1A and SV40 large T is dependent on their interaction with pRB via the LxCxE motif (Decaprio et *al.*, 1988;

Bandara et *al.*, 1991). These findings led to the identification of many pRB binding proteins that utilize the LxCxE motif to participate in pRB mediated growth arrest, including RBP1 (Defeo-Jones et *al.*, 1991; Lai et *al.*, 1999a). In addition to binding the A/B pocket, the E2F family of transcription factors interacts with the pocket independently of the LxCxE motif by contacting the C-terminal region of pRB (Lee et *al.*, 1998; Fattaey et *al.*, 1993b). Structure determination of the pRB pocket domain has allowed the identification of key residues involved in recognition of the LxCxE consensus sequence (Lee et *al.*, 1998). Consequently, mutation of the residues involved in forming the LxCxE binding cleft results in a pRB protein that retains the ability to bind E2F but is significantly impaired in binding cellular proteins such as HDAC2 and cyclin D1 (Chan et *al.*, 2001). Strikingly, these mutant pRB molecules can efficiently block E2F transactivation and cause cell cycle arrest at levels comparable to wild type pRB, indicating that growth arrest is independent of LxCxE interactions (Chan et *al.*, 2001; Dick et *al.*, 2001). However, these pRB pocket mutants exhibit a dramatic reduction in transcriptional repression when brought to a promoter independently of E2F (Chan et *al.*, 2001). The types of mutations introduced in the LxCxE binding pocket may have different consequences on the overall structure because LxCxE containing proteins such as HDAC1 still bind these pocket mutants (Dick et *al.*, 2001). Also, some pRB mutants unable to associate with the LxCxE motif only cause a transient growth arrest since this effect is not seen in colony formation assays (Dahiya et *al.*, 2000). Therefore, some proteins may utilize the LxCxE motif to stabilize the interaction with pRB, whereas others like RBP1, rely on the presence of this motif to elicit their growth repressive ability and target proteins required for pRB mediated transcriptional repression.

1.4.1 Retinoblastoma Binding Protein 1 RBP1

The *rbp1* gene encodes a ubiquitously expressed nuclear phosphoprotein of 1257 amino acids in length with a predicted molecular weight of 142.6kDa (Fattaey et *al.*, 1993a). Interestingly, western blot analysis indicates that RBP1 migrates on SDS-PAGE at about 180kDa and some have proposed that this retarded migration is due to the fact that RBP1 contains a high amount (39%) of charged residues (Fattaey et *al.*, 1993a; Otterson et *al.*, 1993; Lai et *al.*, 1999a). Four alternative splice variants have been isolated and all retain the ability to interact with pRB in an LxCxE dependent manner (Otterson et *al.*, 1993). The predominant steady-state mRNA transcript is isoform I, whereas isoform IV was isolated from a bone marrow cDNA library. Splicing occurs within an internal exon of 207bp that encodes potential casein kinase II and $p34^{cdc2}$ phosphorylation sites, and two of these sites are missing in isoforms II and III (Otterson et *al.*, 1993). *In vivo*, RBP1 interacts with hypophosphorylated pRB in an LxCxE dependent fashion as this interaction is abolished by the E7 viral protein or in cells constitutively expressing E1A and SV40 large T antigen (Fattaey et *al.*, 1993a; Lai et *al.*, 1999b).

1.4.2 RBP1 structural motifs

Conserved domain searches indicate that RBP1 contains a putative A/T rich interacting domain (ARID) domain between amino acids 313 to 409, an N-terminal tudor domain encoded by amino acids 57 to 114 and a chromatin organization (Chromo) domain located near the LxCxE sequence from amino acid 593 to 633 (Figure 1.3). How each domain contributes to the biological function of RBP1 remains to be determined.

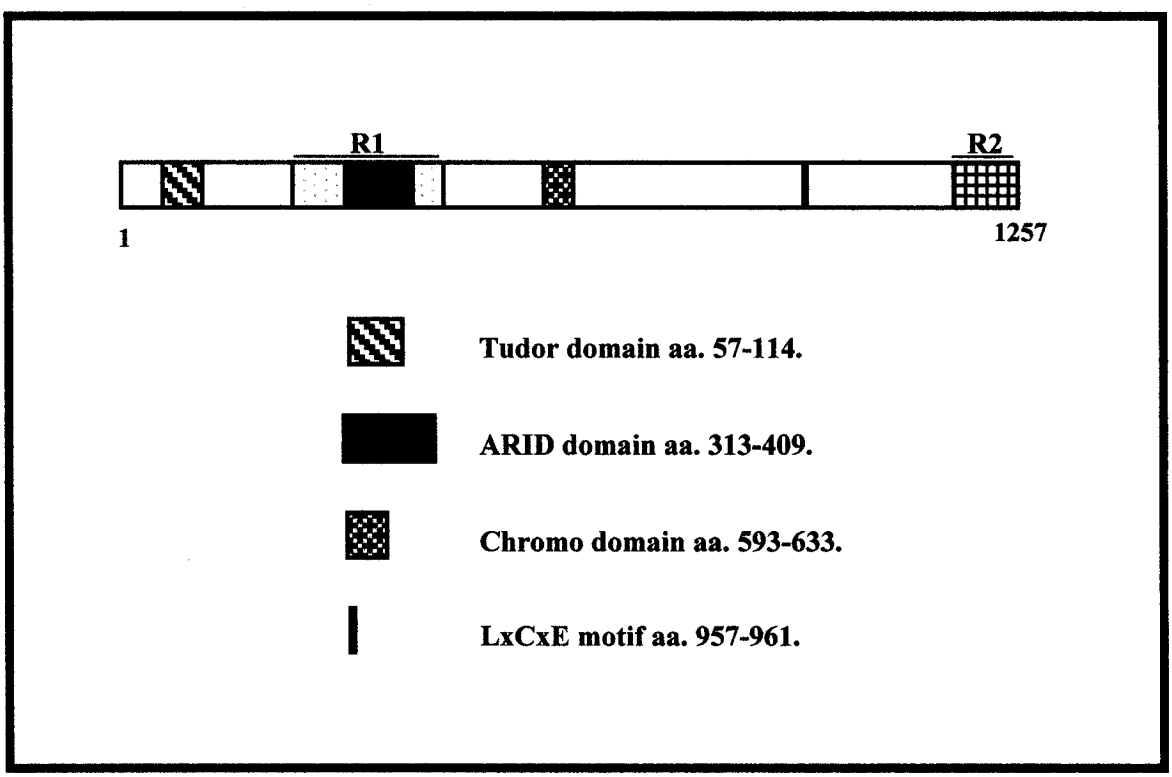


Figure 1.3. RBP1 structural motifs. The figure is made to scale and denotes the structural domains of RBP1 and their relative position. The location of the two repression domains are indicated above. R1 aa. 241-452, R2 aa. 1167-1257.

The ARID domain was initially described in the *Drosophila melanogaster* Dead-ringer protein Dri as a DNA binding motif that recognizes the consensus sequence TATTGAT (Gregory et *al.*, 1996). The mammalian genome contains thirteen unrelated genes that harbor the putative ARID domain that are involved cell proliferation, development and differentiation. In support of this contention, *jumonji-/-* mice die 15 days after gestation while mice lacking the murine *Mrf-2* gene, *desrt,* show reduced viability and abnormal development of reproductive organs (Takeushi et *al.*, 1995; Lahoud et *al.*, 2001). Jumonji is a nuclear protein involved in the negative regulation of cell growth by possibly reducing the levels of cyclin D1. On the other hand, the *desrt* gene product is a transcription factor involved in mouse organogenesis.

The minimal consensus sequence of the ARID domain spans 100 amino acids and 39 of these are perfectly conserved in terms of spacing and identity (Wilsker et *al.*, 2002). There are also five residues that are invariable and mutations of the latter result in impairment of DNA binding (Dallas et *al.*, 2000). Some proteins such as Dri have an additional 40 amino acids located either at the C-term or N-term of the minimal ARID and belong to the family of extended ARID domains (eARID) (Kortschak et *al.*, 2000). This additional region has been proposed to be involved in protein-protein interactions that may participate in stabilizing the interaction of Dri with DNA (Valentine et *al.*, 1998). Solution structure of the Dri ARID indicates that this novel type of DNA binding module binds DNA using a non-canonical helix-turn-helix motif (Iwahara et *al.*, 2002). Interestingly, not all ARID domains recognize a specific sequence, nor do they all have high affinity for A/T rich sites. The ARID containing SWI/SNF subunit p270 has no

preference for A/T rich sequences and a binding site selection experiment failed to detect any consensus sequence (Dallas et *al.*, 2000). In fact, it has been proposed that minimal ARID containing proteins bind to DNA less tightly relative to the eARID family members because of the lack of stabilizing contacts made by the additional α-helix (Iwahara et *al.*, 2002). RBP1 has been shown to bind with high affinity to calf thymus DNA but the relative contribution of the ARID in this interaction is unknown (Fattaey et *al.*, 1993a).

The chromo domain, which typically spans about 50 amino acids, was first described in the polycomb protein as a region with homology to a motif in HP1 (Paro et *al.*, 1991). HP1 is a heterochromatin binding protein that recognizes methylated lysine 9 via its chromo domain (Lachner et *al.*, 2001; Bannister et *al.*, 2001). The structure of the HP1 chromo domain bound to methyl lysine 9 indicates that three aromatic residues are involved in this interaction (Nielsen et *al.*, 2002). Interestingly, the chromo domain has also been reported to be an RNA binding module in some proteins. The *Drosophila* histone acetyltransferase MOF (Males-absent in on the first) binds RNA *in vitro* and this activity has been mapped to the chromo domain (Ahkhtar et *al.*, 2000). Sequence alignment of the MOF and HP1 chromo domains reveals that MOF lacks the aromatic cage involved in the recognition of methylated lysine residues (Nielsen et *al.*, 2002). The histone methyltransferase SUV39H1 also possesses a chromo domain that appears to be required for the interaction with histone deacetylases, indicating that in some instances chromo domains can also function as protein-protein interaction motifs (Vaute et *al.*, 2002). Analysis of the sequence of the chromo domain of RBP1 indicates that it may bind

methylated histones other than histone H3 or serve as a binding surface for proteins rather than RNA. However, studies on the function of the chromo domain of RBP1 have not been initiated. Tudor domains have been best characterized in the survival of motor neuron protein SMN as a motif that is involved in protein interactions (Buhler et *al.*, 1999). Reduced levels of SMN have been linked to spinal muscular atrophy and a point mutation within the SMN tudor domain impairs the interaction with the spliceosomal protein Sm. Furthermore, the integrity of the tudor domain of SMN is required for proper formation of U snRNPs *in vivo* (Buhler et *al.*, 1999; Selenko et *al.*, 2001). However, solution structure of the SMN tudor domain identified key residues involved in Sm protein binding and suggested that this module is likely to be involved in protein-protein interactions rather than direct binding to RNA (Selenko et *al.*, 2001). The function of the tudor domain in other proteins such as RBP1 has not been characterized. Recently, the p53 binding protein-1, 53 BP1, was shown to bind to chromatin via the tudor and Myb domains (Iwabuchi et *al.*, 2003). Interestingly, both these domains are required to bind directly to DNA *in vitro* suggesting that there could be synergism between tudor domains and nearby structural motifs.

1.4.3 RBP1 induces growth arrest

Our group has shown that RBP1 is present in p130/E2F and pRB/E2F complexes at growth arrest and that its overexpression reduces colony formation in an LxCxE dependent fashion (Lai et *al.*, 1999a). Endogenous RBP1 interacts with all three members of the pRB family of proteins in an LxCxE dependent manner as this interaction is abolished in cells expressing E1A and SV40 large T antigen (Lai et *al.*, 1999b). The

growth arrest mediated by the overexpression of RBP1 appears to be largely due to repression of E2F-dependent transcription. RBP1 represses an E2F driven promoter in a dose-dependent fashion and this effect is dependent on the presence of the LxCxE motif (Lai et *al.*, 1999a). Interestingly, RBP1 can also actively repress transcription when recruited to a promoter by a DNA binding domain independently of pRB. Mapping studies have located two independent transcriptional domains in RBP1 that may participate in active repression by the pocket of pRB (Lai et *al.*, 1999a; Lai et *al.*, 1999b).

1.4.4 HDAC-dependent and independent activities and RBP1

Two independent repression domains have been identified in RBP1 and are designated as R1 and R2. The R1 domain is located between amino acids 241 and 452 and encompasses the putative ARID DNA binding domain whereas the R2 domain is encoded by amino acids 1167 to 1257 (Lai et *al.*, 1999b). When fused to the Gal4 DNA binding domain, both repression domains can repress transcription from strong viral promoters containing Gal4 binding sites such as HSV TK, SV40 and adenovirus MLP. Transcriptional repression by the R2 domain of RBP1 is sensitive to the HDAC inhibitor TSA and binding studies demonstrated that R2 interacts only with HDAC1/2 and 3. Consistent with this contention, RBP1 represses E2F-dependent transcription in a manner that is sensitive to TSA, suggesting that RBP1 recruits both histone deacetylase dependent and independent repression functions to the pocket of pRB (Lai et *al.*, 1999b). In contrast to the R2 domain, repression by the R1 domain is insensitive to TSA and functions independently of histone deacetylases (Lai et *al.*, 1999b; Lai et *al.*, 2001).

Several studies indicate that RBP1 associates with an mSin3A corepressor complex containing mSin3A, RbAp46/48, HDAC1/2 and SAP30 with relatively high stochiometry (Zhang et *al.*, 1998b; Lai et *al.*, 2001; Fleisher et *al.*, 2003). RBP1 appears only to associate with the mSin3A complex as it cannot be detected in the ATP dependent NURD-HDAC chromatin remodeling complex (Zhang et *al.*, 1998a; Lai et *al.*, 2001). Our group has demonstrated that the R2 domain of RBP1 recruits the mSin3A complex by binding to the SAP30 polypeptide (Lai et *al.*, 2001). The association of HDAC activity with pRB is highly dependent on the presence of SAP30 and RBP1 confirming that RBP1 recruits HDAC1 and HDAC2 to the pocket of pRB. In addition, pRB-mSin3A-RBP1 and E2F4 have been shown to colocalize to discrete regions of the nucleus in human fibroblasts at growth arrest (Lai et *al.*, 2001). These studies also confirmed that both RBP1 and pRB only associate with class I HDACs and that the sites occupied at growth arrest are likely to correspond to origins of DNA replication (Kennedy et *al.*, 2000; Lai et *al.*, 2001). The mechanism of HDAC-independent repression by RBP1 has not been established.

1.5 Breast Carcinoma Associated Antigen BCAA

An IgG antibody from a breast cancer patient was recently used to identify novel tumor associated antigens from a cDNA library of breast cancer cells. Interestingly, the peptide sequence (KASIFLK), corresponding to the epitope, was identical to a sequence located in RBP1 suggesting that RBP1 may be overexpressed in tumors (Cao et *al.* 1999). However, the antigen recognized by the antibody was located in the cytoplasm, whereas RBP1 resides in the nucleus (Cao et *al.*, 1999; Lai et *al.*, 2001). Cloning of the tumor-

associated antigen revealed that it corresponds to a different protein, initially named breast cancer associated antigen BCAA (Cao et *al.*, 2001). Although BCAA shares only 37% amino acid sequence identity with RBP1, the highest homology is seen within the ARID, tudor and chromo domains (Cao et *al.*, 2001; Fleisher et *al.*, 2003). The most noticeable difference between both is the lack of an LxCxE motif in BCAA. Expression of BCAA in different tissues indicates that expression is restricted to testis in normal tissues but is high in breast, ovary, lung, colon and pancreatic cancer tissues (Cao et *al.*, 2001). The function of BCAA in tumor formation remains unclear but the high homology with RBP1 may indicate that it can block the transcriptional repression activities associated with RBP1 by competing for binding factors such as the mSin3A complex.

1.5.1 SAP180/BCAA associates with the mSin3A-HDAC complex

Recently, a novel mSin3A associated polypeptide, SAP180, was shown to co-purify with the mSin3A complex in an erythroleukemia cell line (Fleisher et *al.*, 2003). Mass spectrometry analysis indicated that SAP180 was in fact BCAA and subsequent experiments demonstrated that it associates with mSin3A components. In addition, BCAA, like RBP1, possesses transcriptional repression activity when tethered to a promoter (Fleisher et *al.*, 2003). The C-terminal region of BCAA is sufficient for binding to the mSin3A complex whereas the N-terminal region represses transcription independently of histone deacetylases (Fleisher et *al.*, 2003). The factor that is responsible in targeting the mSin3A complex to BCAA has not been characterized. Whether RBP1 and BCAA are members of the same mSin3A complexes has not been verified.

1.6 The Ubiquitin-like family of proteins

Post-translational modification of proteins by phosphorylation, acetylation or ubiquitination can have profound effects on their stability, sub-cellular localization and even influences protein-protein interactions. For instance, the small 76 amino acid polypeptide ubiquitin is covalently linked to lysine residues of proteins causing them to be degraded via the 26S proteasome (Schwartz and Hochstrasser, 2003). Over the years, many proteins have been discovered to possess structural similarity to ubiquitin and have now been classified as the ubiquitin-like family of proteins. Members of this family include the small ubiquitin-like modifier SUMO, Nedd8 (Neural precursor cell expressed, developmentally downregulated 8) and the autophagy proteins Apg8 and Apg12 (Seeler and Dejean, 2001). The ubiquitin-like protein SUMO is covalently linked to lysine residues of target proteins in a manner that is analogous to ubiquitination but the functional consequences that result from this modification are quite distinct from those mediated by ubiquitin.

1.6.1 SUMO-1 structure and function

The mammalian *sumo-1* gene encodes a 101 amino acid protein that is covalently attached to proteins and was initially referred as Sentrin-1/GMP-1 (gap modifying protein 1). Conjugation of SUMO to proteins occurs on specific lysine residues, where the ε-NH_2 side chain forms an isopeptide bond with a C-terminal glycine residue of SUMO-1. SUMO-1 is conjugated to acceptor lysine residues having the consensus sequence ψ-K-X-E, where ψ represents any hydrophobic amino acid, typically isoleucine, leucine or valine, and X represents any amino acid (Rodriguez et *al.*, 2001). Sequence comparison

demonstrates that SUMO-1 shares fifty percent homology with the yeast ubiquitin-like protein suppressor of mif two Smt3 and eighteen percent homology to ubiquitin at the primary sequence level (Matunis et *al.*, 1996). Smt3 is a yeast centromere binding protein that is capable of rescuing mutants of mif2, also a centromere binding protein. Although SUMO-1 shares little overall sequence similarity to ubiquitin, the tertiary structure of SUMO-1 resembles that of ubiquitin. Both contain the C-terminal diglycine residues required for isopeptide bond formation at the same position (Payer et *al.*, 1998). However, SUMO-1 possesses unique structural features, which exemplify its functional difference from ubiquitin. A highly charged N-terminal extension in SUMO-1, absent in ubiquitin, has been described as a potential protein-protein interaction module (Rangasamy and Wilson, 2001). Furthermore, the specific lysine residues critical for the formation of polyubiquitin chains are absent in SUMO-1, thus explaining the inability of SUMO-1 to form polymers (Payer et *al.*, 1998). SUMO-1 was initially discovered as a protein covalently linked to the nuclear import factor RanGAP-1 (Matunis et *al.*, 1996). This SUMO-modified form of RanGAP-1 has a high affinity for RanBP2, a component of the nuclear pore complex, and is the predominantly SUMO-1 modified protein in cells (Matunis et *al.*, 1996; Mahajan et *al.*, 1997; Rodriguez et *al.*, 2001). A growing number of SUMO substrates have now been identified and unlike ubiquitin conjugation, which is generally associated with protein degradation, SUMO modification is emerging as a very important event regulating crucial cellular processes such as nucleocytoplasmic trafficking, genomic stability and gene transcription.

1.6.2 SUMO-1 versus *SUMO-2/3*

Whereas yeast have evolved only one *SUMO* family member, mammalian cells express two additional proteins related to SUMO-1 whose functions are poorly understood. The SUMO-2 and 3 proteins are highly homologous to one another but only share fifty percent homology with SUMO-1, suggesting that they may have different functions from SUMO-1 (Saitoh et *al.*, 2000). In support of this idea, all three members have very different intracellular localization patterns. SUMO-1 predominantly localizes to the nuclear membrane and to discrete regions in the nucleus that correspond to nuclear bodies (Rodriguez et *al.*, 2001; Hong-Lin et *al.*, 2002). This result is consistent with the observation that SUMO-1 modification of proteins requires nuclear targeting, as SUMO-1 substrates such as p53, IκBα and HDAC4 fail to undergo modification with SUMO in the absence of their nuclear targeting sequence (Rodriguez et *al.*, 2001; Kirsh et *al.*, 2002). However, SUMO-2 and SUMO-3 are largely present in nuclear bodies and in the cytoplasm, respectively (Hong-Lin et *al.*, 2002). In addition, while virtually all of the SUMO-1 protein is conjugated in cells, SUMO-2 and SUMO-3 exist as free forms and their conjugation to proteins appears to occur only in response to protein damaging stimuli such as heat shock and oxidative stress *in vivo* (Saitoh et *al.*, 2000). SUMO-2 and SUMO-3 contain the SUMO specific motif, and both can form polymeric chains *in vitro* (Tatham et *al.*, 2001). Recently SUMO-2 and SUMO-3 chains have been observed *in vivo* and have been implicated in the formation of amyloid beta peptide, suggesting they may be involved in the onset of Alzheimer's disease (Li et *al.*, 2003). SUMO modification follows three major steps: 1) processing and activation of SUMO via E1

enzyme; 2) transfer of SUMO to the conjugating enzyme E2; and 3) Transfer of SUMO to substrate via an E3 ligase.

1.7 SUMO conjugation pathway

The first step in the SUMO pathway involves proteolytic processing of the C-terminal end of SUMO by ubiquitin-like proteases to expose the glycine residue involved in isopeptide bond formation. Several SUMO specific proteases have been described and will be discussed further. The mature SUMO moiety is then activated by adenylation on the exposed glycine residue by the heterodimeric E1 enzyme Uba2/Aos1 in an ATP-dependent fashion (Johnson et *al.*, 1997b). Following adenylation, the SUMO-AMP conjugate is linked to Uba2/Aos1 via a trans-esterification reaction involving the active site cysteine residue on the Uba2 subunit (Hay, 2001). The E1 activating enzyme was initially identified in yeast and later purified from human cells and named SAE1/SAE2, which stands for SUMO activating enzyme 1 and 2 (Desterro, et *al.*, 1999). Sequence alignment of the 70 kDa subunit of the SUMO E1 enzyme SAE2/Uba2 indicates homology to the C-terminus of Uba1, which encompasses the active site cysteine residue required for thioester bond formation with SUMO. The other subunit SAE1/Aos1 has a molecular weight of 38 kDa and has homology to the N-terminus of Uba1, which encodes the putative nucleotide-binding motif (Johnson et *al.*, 1997b). Unlike the ubiquitin E1 enzyme Uba1, which is a single polypeptide, *in vitro* experiments showed that SUMO-1 activation of Uba2 requires the presence of Aos1 (Desterro et *al.*, 1999).

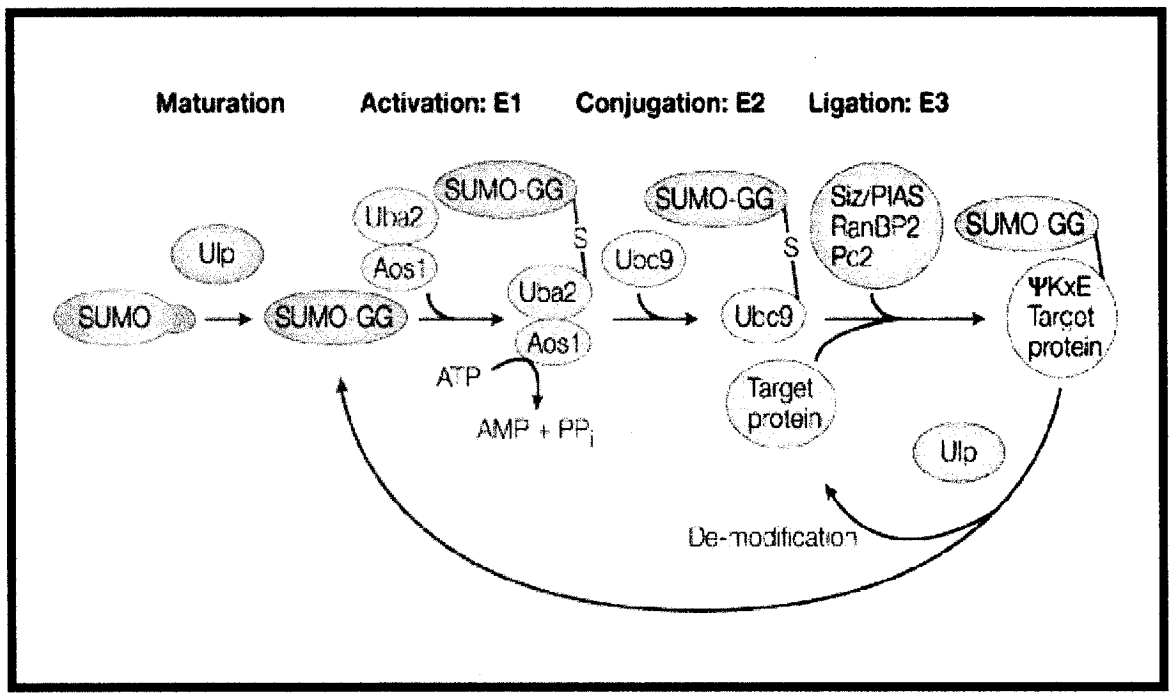


Figure 1.4 The SUMO Pathway. SUMO initially undergoes proteolytic processing by SUMO proteases (Ulp) to expose the C-terminal glycine residue required for attachment on the acceptor lysine. The SUMO moiety is then linked via a thioester bond to the SUMO E1 activating enzyme Uba2/Aos1 (SAE2/SAE1). The activated SUMO is then transferred to the conjugating E2 enzyme Ubc9, which then catalyzes the transfer of SUMO to the target protein. Several E3 ligases have been isolated and can increase the rate of substrate sumoylation by binding to Ubc9. SUMO modification is a reversible process and substrates undergo de-modification catalyzed by specific SUMO protease. Ulp: Ubiquitin-like protease. (Seeler and Dejean 2003).

The second step of the reaction involves the transfer of activated SUMO to the E2 conjugating enzyme Ubc9 and coincides with a thioester linkage formed between the C-terminal glycine of SUMO and cysteine 93 of Ubc9. This high-energy intermediate allows the formation of an isopeptide bond between the acceptor lysine ε-amino group and the SUMO C-terminal glycine (Tatham et *al.*, 2003). While the ubiquitin system utilizes several types of E2 conjugating enzymes, sumoylation only relies on Ubc9 as the E2 conjugating enzyme (Hershko et *al.*, 1998). Ubc9 was first described in yeast and is required for viability (Johnson et *al.*, 1997a). The mammalian ortholog of Ubc9 can complement a yeast strain harboring a temperature sensitive mutant Ubc9 suggesting that they are functionally related (Yasugi et *al.*, 1996).

Interestingly, *in vitro* experiments show that conjugation of SUMO to substrates such as p53 and PML can occur only in the presence of ATP, SUMO, Uba2/Aos1 and Ubc9, indicating that Ubc9 also acts as the E3 ligase (Desterro et *al.*, 1999; Rodriguez et *al.*, 2001). In contrast to the E3 ligases of the ubiquitin system, which provide substrate specificity, most SUMO modified proteins interact directly with Ubc9 and substrate recognition is achieved by direct recognition of the SUMO consensus sequence ψ-K-X-E, with the interaction relying heavily on the hydrophobic residue and the glutamate. Mutation of either residue to alanine abolishes substrate modification *in vitro* and *in vivo* (Rodriguez et *al.*, 2001; Bernier et *al.*, 2002). Recent structure-based studies on the Ubc9-RanGAP1 complex indicates that the hydrophobic residue does not fit into the active site via a lock-and-key system but is involved in hydrophobic van der Waals interactions (Bernier et *al.*, 2002). Not surprisingly, the enzymes involved in the SUMO pathway all

localize to the nucleus, further strengthening the idea that sumoylation is a nuclear process (Rodriguez et *al.*, 2001). More recently, immunogold labeling has demonstrated that Ubc9 can associate with the cytoplasmic filaments of the nuclear pore complex, a finding consistent with the modification of RanGAP-1 occurring on the cytoplasmic side of the nuclear pore complex (Zhang et *al.*, 2002). The discovery of SUMO E3 ligases has demonstrated that they are involved in substrate selectivity and are potentially responsible for enhancing the SUMO modified form of a subset of proteins. These findings are supported by the observation that some Ubc9 mutants, defective in SUMO modification of substrates such as p53 and IκBα, still retain the ability to modify RanGAP1 (Bernier et *al.*, 2002). Finally, sumoylation is a reversible process and several SUMO specific proteases have been characterized to date and all appear to have different substrate specificities.

1.8 SUMO E3 ligases

1.8.1 PIAS proteins

The protein inhibitor of activated STAT (PIAS) family of proteins were initially described as repressors of the transcription factor STAT1 (Liu et *al.*, 1998). The yeast equivalent Siz1p has been shown to be involved in SUMO modification of the septins (Takahashi et *al.*, 2001). This class of E3 enzymes is characterized by the presence of a RING-finger domain that is homologous to the class of RING-type ubiquitin ligases (Kahyo et *al.*, 2001). The SUMO specific E3 ligase activity of PIAS was initially shown to increase p53 sumoylation both *in vitro* and *in vivo* (Kahyo et *al.*, 2001). E3 activity relies on a cysteine residue within the RING-finger domain that is directly responsible for

binding to Ubc9, whereas SUMO binding occurs on a different location (Kahyo et *al.*, 2001; Kotaya et *al.*, 2002). In fact, it has now been proposed that E3 ligases increase the level of SUMO modification of their substrates by serving as adapters between Ubc9 and the substrate (Hochstrasser, 2001). Several transcription factors such as the GC box binding transcription factor Sp3 and PML are controlled by SUMO-1 modification (Ross et *al.*, 2002; Muller et *al.*, 1998). Interestingly, PIAS proteins appear to have selective E3 ligase activity towards transcription factors. Sumoylation of Sp3 negatively regulates its activity and this modification is enhanced by PIAS1 and not by another recently described E3 ligase RanBP2 thus implicating substrate specificity of E3 SUMO ligases (Sapetschnig et *al.*, 2002). SUMO-1 modification is becoming an important regulatory mechanism by which transcription factor activity is regulated and will be addressed later.

1.8.2 RanBP2 and nuclear import

Import of proteins into the nucleus requires the Ran GTPase activating protein 1 RanGAP-1 bound to the nuclear pore complex to drive the entry (Pichler and Melchior, 2002). A role for SUMO in nuclear import is supported by the observation that only SUMO-modified RanGAP-1 associates with the nuclear pore associated protein RanBP2 on the cytoplasmic side (Matunis et *al.*, 1997). Furthermore, RanBP2 possesses E3 ligase activity towards the PML-associated protein Sp100 and HDAC4, suggesting that it modulates their import into the nucleus (Pichler et *al.*, 2002b; Kirsh et *al.*, 2002). Although these results do not provide direct evidence to link SUMO with nuclear import, a yeast strain bearing a conditional mutation in Uba2 shows impaired nuclear import of a cNLS-GFP construct (Stade et *al.*, 2002). RanBP2 is structurally different from the PIAS

type E3 SUMO ligases due to the absence of the RING-finger motif and only appears to interact with substrates via Ubc9 (Pichler et *al.*, 2002b). There also appears to be substrate selectivity among the E3 ligases, as RanBP2 is not capable of catalyzing the SUMO modification of p53 *in vitro* (Pichler et *al.*, 2002b). Moreover, it is unclear to what extent SUMO modification is important for import of proteins. A point mutation in the E1B 55K adenoviral protein that abrogates SUMO-1 modification prevents nuclear accumulation, whereas a non-modifiable Sp100 is still capable of translocation into the nucleus upon overexpression (Endter et *al.*, 2001; Sternsdorf et *al.*, 1999). The coupling of SUMO modification with nuclear import may only be important for a subset of proteins that need to enter the nucleus at a critical time.

1.8.3 Pc2 group proteins

Recently, the human polycomb group protein Pc2 was identified as a novel type of E3 ligase. Polycomb proteins are involved in gene regulation and have also been shown to bind the co-repressor C-terminal binding protein CtBP, which is involved in repression of genes required for cell growth and differentiation (Sewalt et *al.*, 1999). Immunofluorescence studies demonstrated that both SUMO and Ubc9 colocalize to PcG bodies within the nucleus and Pc2 can be modified with SUMO. In addition, Pc2 is capable of increasing the level of CtBP sumoylation *in vitro* as well as increase the amount of CtBP and Ubc9 recruited to PcG bodies (Kagey et *al.*, 2003). These findings imply that, like PML bodies, PcG bodies may control the activity of SUMO modified proteins by sequestration.

1.9 SUMO specific proteases

SUMO specific proteases play a dual role in the SUMO modification pathway. They are involved in exposing the C-terminal glycine of SUMO and are also involved in maintaining the equilibrium of SUMO modified substrates. The family of ubiquitin-like SUMO proteases was first reported in yeast. Ulp1 is involved in cell cycle progression in yeast and catalyzes the removal of Smt3/SUMO from proteins (Li et *al.*, 1999). To date, nine different SUMO proteases have been functionally characterized in yeast, human and mouse (Dejean and Seeler, 2003). While they are classified as ubiquitin-like enzymes, they have no significant homology with their ubiquitin counterparts and are unable to cleave ubiquitin or Nedd8 from substrates. The major characteristic of SUMO proteases is a C-terminal cysteine residue that mimics a catalytic triad observed in the adenoviral protease domain (Li et *al.*, 1999). Interestingly, several of these enzymes can catalyze the removal of SUMO from similar substrates *in vitro* but fail to do so *in vivo* due to their diverse sub-cellular localization thereby controlling their substrate selectivity.

The mammalian SUMO protease SENP2 binds to the nucleoplasmic side of the nuclear pore complex and this interaction relies on the N-terminus of SENP2 (Hang et *al.*, 2002). When wild type SENP2 is overexpressed, only a modest amount of proteins can be desumoylated whereas removal of the N-terminus causes a dramatic loss of SUMO modified proteins, suggesting that interaction with the pore complex prevents substrate accessibility (Hang et *al.*, 2002). This is supported by the observation that SENP2 is capable of cleaving SUMO from RanGAP1 *in vitro* but not *in vivo*. Although the authors speculate that SENP2 activity against SUMO modified substrates is

controlled by association with the nuclear pore, it remains to be determined if the full length SENP2 can be dissociated from the complex to target other substrates.

Table 1.2. List of characterized SUMO specific proteases

Protease	Organism	Localization
Ulp2	Budding yeast	Nucleoplasm
Ulp1	Budding yeast	Nuclear pore complex
Ulp1	Fission yeast	Nuclear periphery
SUSP1	Human	Cytoplasm
SMT3IP	Human	Nucleolus
SENP1	Human	Nucleoplasm/nuclear bodies
SENP2	Human	Nuclear pore complex
Smt3ip2*	Mouse	Cytoplasm
SuPr-1*	Mouse	Nucleoplasm/nuclear bodies

Table 1. Shown are several SUMO proteases with known functions that have been described in eukaryotes. The sub-cellular localization of each is also indicated. * Both Smt3ip2 and SuPr-1 are splice variants of SENP2. Adapted from Seeler and Dejean, 2003.

Unlike SENP2, SENP1 is a human SUMO specific protease that localizes to the nucleoplasm and to discrete regions in the nucleus corresponding to nuclear bodies. *In vivo*, SENP1 is unable to remove SUMO-1 from RanGAP1 but does cause the loss of SUMO-modified PML demonstrating that localization of SUMO proteases controls their substrate selectivity (Gong et *al.*, 2000). However, desumoylation of PML by SENP1 did not correlate with a loss of PML bodies, thus questioning the biological relevance of this effect. SUSP1 is another human SUMO protease that localizes to the cytoplasm when

expressed in NIH3T3 and HeLa cells (Kim et *al.*, 2000). Although RanGAP1 is a cytoplasmic protein, SUSP1 is unable to process SUMO-RanGAP1 implying that it has other functions that remain to be determined.

Recently, the mouse SUMO protease SuPr-1 was isolated as an activator of c-Jun dependent transcription and sequence comparison revealed that it corresponds to a splice variant of SENP2/Smt3ip2. Strikingly, SuPr-1 actually lacks the N-terminal region of SENP2 that is necessary for targeting to the nuclear pore complex and localizes to nuclear domains corresponding to PML bodies (Best et *al.*, 2002). SuPr-1 also exhibited selectivity towards SUMO-PML and was unable to remove SUMO from RanGAP1, which is similar to SENP1. Furthermore, the transcriptional activity of Sp3 is directly stimulated by SuPr-1 mediated SUMO deconjugation (Ross et *al.*, 2002). Interestingly, the ability of SuPr-1 to activate c-Jun dependent transcription did not rely on its catalytic activity but rather on its interaction with PML. In fact, SuPr-1 fails to activate c-Jun transcription in PML deficient fibroblasts or in the presence of PML that cannot be modified by SUMO (Best et *al.*, 2002). This result suggests that SUMO proteases may also function by altering the localization of factors by bringing them to PML bodies or other nuclear domains independently of their sumoylation status.

1.10 SUMO and transcriptional control

The majority of SUMO conjugated proteins identified to date are transcription factors, implying a direct role of SUMO in transcriptional regulation. SUMO conjugation appears to control transcription factor activity by several mechanisms, including altering

subnuclear distribution, mediating protein-protein interactions and preventing ubiquitin-mediated degradation. Moreover, SUMO modification is commonly, but not exclusively, associated with negative effects on transcription. Transcriptional activity of several activators such as Sp3 and CAATT enhancer binding protein C/EBP is downregulated by SUMO modification of previously mapped inhibitory domains (Sapetschnig et *al.*, 2002; Kim et *al.*, 2002). In addition, inspection of the sequence of previously mapped transcriptional repression domains revealed that they contain consensus sites for sumoylation (Lai et *al.*, 1999; Yang et *al.*, 2002; Snowden et *al.*, 2000). Thus, SUMO modification could act as a rapid molecular switch to convert transcriptional activators into repressors during specific cellular processes.

1.10.1 SUMO and inhibition of transcription factor activity

SUMO modification of proteins occurs on specific lysine residues that have the ψ-K-X-E motifs. However, other post-translational modifications such as ubiquitination and acetylation occur on lysine residues (Freiman and Tijan, 2002). It has been shown that Sp3 is acetylated on the specific lysine residue that is also modified with SUMO (Braun et *al.*, 2001; Ross et *al.*, 2002). SUMO modification could block acetylation or vice versa to control the activity of transcription factors in response to various cell signaling pathways. In support of this idea, recent evidence indicates that acetylation of Sp3, following TSA treatment, converts it to a transcriptional activator (Amanamanchi et *al.*, 2003).

The inhibition of transcription factors by SUMO conjugation also appears to be controlled in part by sequestration within specific nuclear domains such as PML bodies. For example, SUMO-modified Sp3 localizes to discrete regions in the nucleus and is unable to activate transcription. However, overexpression of a SUMO specific protease relieves the inhibitory effect of SUMO on Sp3 transactivation and causes the redistribution of Sp3 such that it is diffusely present within the nucleus (Ross et *al.*, 2002). It has also been shown that the E3 ligase PIASy represses lymphoid enhancer binding factor LEF-1 activity by sequestration into nuclear bodies, but this effect is not dependent on the SUMO acceptor sites, indicating that SUMO modification of LEF-1 cannot account for the relocalization (Sachdev et *al.*, 2001). LEF-1 is a member of the HMG box family of transcription factors

1.10.2 SUMO and transcriptional repression

Increasing evidence indicates that transcriptional repressors are regulated by SUMO modification, but the exact contribution of SUMO in repression remains obscure. The CtBP co-repressor is sumoylated on a single lysine residue and mutation of the latter causes a decrease in transcriptional repression of the E-cadherin promoter (Lin et *al.*, 2003). Similarly, although the effect was not dramatic, a point mutant of HDAC4 that fails to undergo SUMO conjugation has impaired repression and deacetylase activity (Kirsh et *al.*, 2002). However, in both cases, this effect appears to be indirect, as sumoylation of both proteins mediates their nuclear import. Direct involvement of SUMO in transcriptional repression has now been established for several previously identified repression domains with unknown mechanisms of action.

The transcription factor C/EBPε possesses an inhibitory domain known as a synergy control motif that contains one copy of the SUMO consensus motif that is required for repression (Kim et *al.*, 2002). This domain is capable of repressing the strong activation domain of the yeast activator VP16 in *cis* and in *trans* but a point mutant in the putative SUMO site abolishes the repressive effects. In addition, repression is increased in a copy-dependent manner implying that SUMO is responsible for this activity (Kim et *al.*, 2002). The CRD1 repression domain of p300 and the R motif of the ETS-related transcription factor Elk-1 have been demonstrated recently to function in a SUMO-dependent manner (Girwood et *al.*, 2003; Yang et *al.*, 2003). In both cases, mutation of the lysine residues predicted to be SUMO modification sites leads to a loss of transcriptional repression. CRD1 actually contains two sites of sumoylation and both need to be mutated to achieve a complete relief of repression. Furthermore, this effect is not due to a block of other lysine modifications such as acetylation or ubiquitination as expression of a SUMO specific protease or a dominant negative form of the E2 enzyme Ubc9 leads to a dose-dependent relief of transcriptional repression (Girwood et *al.*, 2003; Yang et *al.*, 2003). Strikingly, p300 transcriptional repression and p21-mediated activation are unaffected when the CRD1 is substituted by a motif containing the PML and E1B-55K SUMO specific sites (Girwood et *al.*, 2003). This provocative result demonstrates that SUMO modulates directly the transcriptional repression mediated by p300. These repression domains appear to be mechanistically similar because the Elk-1 R motif can functionally replace the CRD1 and confer p21 inducibility in the context of p300 whereas Elk-1 is not responsive to p21 (Yang et *al.*, 2002).

The mechanisms by which SUMO exerts its repressive effects at the promoter level have not been characterized. When brought to a promoter by a DNA binding domain, SUMO-1 can effectively repress transcription and can functionally replace the R motif of Elk-1 (Ross et *al.*, 2002; Yang et *al.*, 2003). The CRD1 of p300 recruits HDAC6 in a SUMO-dependent fashion and suggests that in some instances, SUMO conjugation creates a binding surface for co-repressor complexes (Girwood et *al.*, 2003). Alternatively, the repressive effects may be explained by Ubc9, which has the ability to bind both SUMO or the SUMO consensus sequence located within the repression domains (Verger et *al.*, 2003). Nevertheless, several transcriptional repressors have repression domains that contain putative SUMO modification sites but the relative contribution of SUMO in the repression remains to be determined. Moreover, it suggests that SUMO specific proteases, in response to signaling pathways, could regulate transcription factor activity by directly controlling their level of sumoylation and implicates SUMO as a novel type of molecular switch.

1.11 Thesis proposal

The retinoblastoma protein causes growth arrest by blocking the activity of the family of E2F transcription factors, which are required for entry into S-phase (Helin et *al.*, 1992; Bandara et *al.*, 1991; Wu et *al.*, 2000). Early studies demonstrated that pRB binds directly to the transactivation domain of E2F thereby blocking its transcriptional properties (Flemington et *al.*, 1993; Helin et *al.*, 1993). However, this model of repression does not explain how mutations in the E2F sites of some promoters led to an increase in transcription (Weintraub et *al.*, 1992; Ohtani et *al.*, 1995). Members of the pocket proteins are targeted to promoters by E2F/DP dimers to actively repress E2F-dependent transcription by recruiting chromatin-modifying enzymes (Luo et *al.*, 1998, Lai et *al.*, 2001; Nielsen et *al.*, 2001). The retinoblastoma binding protein 1 RBP1 is responsible for targeting histone deacetylase activity to the pocket of pRB by associating with the mSin3A/HDAC1 co-repressor complex via SAP30 (Lai et *al.*, 2001). Interestingly, pRB-mediated repression of E2F-dependent transcription is only partially sensitive to the HDAC inhibitor TSA (Luo et *al.*, 1998; Lai et *al.*, 1999b). Consequently, RBP1 can also repress transcription in an HDAC-independent manner by an unknown mechanism when tethered to a promoter by a heterologous DNA binding domain (Lai et *al.*, 1999b).

Unlike ubiquitination, which is generally associated with protein degradation, post-translational modification with SUMO has been implicated in several cellular processes such as nuclear import and transcriptional regulation (Matunis et *al.*, 1997; Girwood et *al.*, 2003). In fact, the activity of several transcription factors is modulated by

SUMO conjugation. For instance, Sp3 transactivation is inhibited by SUMO conjugation by being sequestered into PML bodies (Ross et *al.*, 2002). A more direct role of SUMO in transcriptional repression comes from the observation that many previously identified repression domains function in a SUMO dependent manner (Yang et *al.*, 2003; Girwood et *al.*, 2003; Kim et *al.*, 2002). Modification with SUMO requires the consensus sequence ψ-K-X-E, where ψ represents a hydrophobic amino acid and X any amino acid (Rodriguez et *al.*, 2001). Many putative SUMO sites have been identified in RBP1, two of which are imbedded within the R1 domain of RBP1, which is responsible for the HDAC-independent repression. The goal of the present studies was to determine if these two potential sumoylation sites play any role in the regulation of repression by the R1 domain of RBP1.

Chapter 2: Materials and Methods

2.1 Cell Culture

H1299 human large cell lung carcinoma cells (ATCC CRL-5893) (Mitsudomi et *al*, 1992) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% PSG (Penicillin, Streptomycin and L-Glutamine). Chinese Hamster Ovary K1 cells (CHO-K1) (ATCC CCL-661) were grown in α Minimal Essential Medium (αMEM) also supplemented with 10% FBS and 1% PSG. Both cell types were cultured in incubators at 37°C containing 5% CO2.

2.2 Plasmid Constructs

Table 2.1 PCR Oligonucleotide primers.

Constructs	Forward Primer	Reverse Primer
pSG424 Gal4-R1 K418R	5'caccatgaaccaaaagtacgtgac gaaaaaaaagac 3'	5'gtctttttttcctcacgtacttttggttc atggtg 3'
pSG424 Gal4-R1 K444R	5'gcctttaacagaagtgcgcagtga acctgagg 3'	5'cctcaggttcactgcgcacttctgtta aaggc 3'
pSG424 Gal4-R1 K433R	5'ggaagaggctctccggttagatca agaaatg 3'	5'cagttcttgatctaaccggagagcct cttcc 3'
pSG424 Gal4-R2 K1204R	5'gaaaatattatatgtctttgagatctg aagttgcaacc 3'	5'ggttgcaacttcagatctcaaagaca tataatatttc 3'

Bacterial expression constructs pGEX2TK and pGEX2TK R1 have been described previously (Lai et *al*, 2001). Reporter constructs pG5-TK-Luc, pG5-MLP-luc, pRL-CMV can be obtained from Promega. The phRL-RSV construct was made by subcloning the RSV promoter from pRc-RSV (Invitrogen) upstream of the Renilla Luciferase gene replacing the TK promoter phRL-TK (Promega) vector by restriction enzyme digest using BglII and HindIII enzymes (Binda, 2003 unpublished). Mammalian

expression plasmids pcDNA3 Gal4-RBP1, pSG424 Gal4-R1, pSG424 Gal4-ARID, pSG424 Gal4-R2 and pcDNA3 Gal4-RBP1 deletion mutants have all been described elsewhere (Lai et *al,* 1999a,b). Gal4-R1 dl859c was generated by ligating the HindIII/Sca1 digested fragment from pSG424 Gal4-R1 into a modified pcDNA3 containing stop codons 3' of the multi-cloning site. The pSG424 Gal4-R1 C-term plasmid (SMRD), which corresponds to residues 400-452 of RBP1, was generated by subcloning the Sca1/Xba1 blunt filled fragment in-frame with the Gal4 DNA binding domain of pSG424, which expresses Gal4 fusion proteins under the SV40 promoter. All Gal4-R1 point mutations (K418R, K444R and K433R), either alone or in combination, were generated by PCR mediated site directed mutagenesis using the specific primers described above (see table). The amplified products were sequenced using specific primers by automated dideoxy sequencing. The pSG424 Gal4-R2 K1204R mutant was also generated using the method described previously. The Gal4-R1 and Gal4-R1 point mutants expressed under the control of CMV promoter were constructed by subcloning the HindIII/Xba1 fragment from pSG424 into pcDNA3, which also contains a T7 promoter. The pcDNA3 Gal4-RBP1 K418R/K444R double mutant was generated using the enzyme Bsu361 and replacing the fragment of RBP1 with the one from the pSG424 Gal4-R1 K418/444R Bsu361 restriction enzyme digest. The vectors pcDNA3 HA-SUMO-1, 2 and 3 were kind gifts from Ronald T. Hay (Desterro et *al.*, 1997; Tatham et *al.*, 2001). The mammalian expression plasmid pEBB-HA containing the SuPr-1 murine cDNA and SuPr-1 C466S mutant, expresses proteins under the EFα1 promoter and were provided by Leonard Zon (Best et *al*, 2002). The pcDNA3 HA-SuPr-1 and HA-SuPr-1

C466S plasmids were produced by subcloning the cDNA of SuPr-1 from the pEBB backbone into pCAN-HA by BamH1 and Not1 restriction enzyme digest.

2.3 Antibodies

The mouse monoclonal antibody specific for the HA tag, HA-11, is commercially available from Babco. The monoclonal antibody RK5C1 (anti-Gal4) raised against the Gal4 DNA binding domain was purchased from Santa Cruz Biotechnology. The anti-GMP (SUMO-1) mouse monoclonal antibody was obtained from Zymed Laboratories.

2.4 Nuclear extract preparation

Thirty-six 150mm plates of H1299 cells were grown to 95% confluence. Cells were washed twice with 1X PBS, scraped off in ice cold 1X PBS and collected by centrifugation at 3000 rpm in a Sorval RT7 for 5min at 4°. Cells were resuspended in 2.5 pellet volumes of Cytoplasmic Lysis Buffer (10mM HEPES pH7.9, 1.5mM $MgCl_2$, 10mM KCl and 0.5mM DTT) complemented with 1X ALP (Aprotinin 2ug/mL, Leupeptine 5ug/mL and Pepstatin 0.4ug/mL) protease inhibitor cocktail and 1mM PMSF by gently pipetting up and down and allowed to lyse on ice for 10min. Nuclei were then pelleted by centrifugation at 2000 rpm using the Sorval RT7 at 4° for 5min. The supernatant was discarded and the pellet, which consisted mostly of nuclear components, was gently washed with 2.5 volumes of initial pellet volume of Cytoplasmic Lysis Buffer to remove any residual cytoplasmic fraction from the nuclear pellet. Following centrifugation, the nuclear pellet was resuspended in 3.5mL of 75mM NaCl Affinity Chromatography Buffer (AC buffer) (50mM HEPES pH 7.9, 1mM EDTA, 1mM DTT,

1mM PMSF and 20% Glycerol) and lysed by three 10sec bursts of sonication. The lysate was cleared by centrifugation at 15 000 rpm for 30min at 4°.

2.5 GST protein purification

BL21 DE3 bacteria (Invitrogen) were transformed with pGEX2TK or pGEX2TK R1 onto 2YT plates containing 100ug/mL ampicillin (Bishop) and were grown for 16hrs at 37°. Single colonies from each were grown in 4mL of 2YT bacterial culture media with 100ug/mL ampicillin O/N for a maximum of 16hrs at 37°. The following morning, each 4mL culture was transferred to 400mL of pre-warmed media containing 100ug/mL ampicillin (Bishop) and was grown for 3hrs at 30° (OD590 1.2). The cultures were then induced with 0.2mM IPTG (Bishop) to induce the expression of the GST fusions at 30° for another 2 hours. Bacteria were harvested by centrifugation at 3000 rpm in a Sorval centrifuge for 10min at 4°. The bacterial pellets from each 400mL culture were resuspended in 20mL of ice cold GST Lysis Buffer (1X PBS, 1mM EDTA, 1% Triton-X 100, 2mM DTT) complemented with 1X ALP (Aprotinin 2ug/mL, Leupeptine 5ug/mL and Pepstatin 0.4ug/mL), and 1mM PMSF protease inhibitors. Each bacterial pellet was lysed with three 10sec bursts of sonication. Lysates were then cleared by centrifugation at 15 000 rpm for 20min at 4°. The supernatants were collected and then incubated with 400uL of an ice cold 1:1 slurry of Glutathione 4B Sepharose beads (Amersham Pharmacia Biotech) in 1X PBS for 1hr at 4° using end-over-end rotation. The beads were pelleted at 2000 rpm for 2 min at 4° in a Sorval RT7 and washed with 2mL of GST Lysis Buffer five times for 10min each wash end-over-end at 4°. GST fusions were then eluted four times from the beads using 600uL of 75mM NaCl AC Buffer containing 10mM reduced glutathione (Sigma) pH 8.5 and incubating at room temperature for 5min each

time followed by centrifugation at 2000 rpm. Pooled eluates were spin-dialysed and concentrated using Centricon spin colums (Millipore) in 75mM AC buffer to remove glutathione. Concentrations of the GST fusion proteins were determined by standard Bradford assay (Biorad). The purity of each was verified by separating the proteins on a 12% polyacrilamide gel followed by Coomassie brilliant blue staining.

2.6 Affinity chromatography

For each sample, two hundred microliters (bed volume) of Affigel-15 resin (Biorad) was washed with three volumes of cold ddH2O by repeat suspension and centrifugation at 2000 rpm at 4°. GST alone or GST-R1 protein was coupled to the resin at a protein/resin concentration of 4mg/mL in a final volume of 400uL of 75mM AC buffer. Coupling was performed at 4° for 4hrs by end over end rotation and the efficiency of coupling was verified by Bradford assay on aliquots of the supernatant. The resin/protein slurry was then incubated with 75mM NaCl AC Buffer containing 100mM ethanolamine (Sigma) for 1hr at 4° to block free amino groups, followed by incubation with 75mM NaCl AC Buffer containing 1mg/mL BSA for another 30min. The resin was then washed with 1mL of 1M NaCl AC Buffer for 10min at 4° and equilibrated using three 1ml washes of 75mM NaCl AC Buffer. Five column volumes of H1299 cell nuclear extract was added to the matrix and incubated for 1hr at 4° with gentle shaking end over end. As controls, the nuclear extract was also applied to the matrix alone and some of the resin/protein slurry was incubated with 75mM NaCl AC Buffer only. The flow through was collected and the columns were then washed three times with 75mM NaCl AC Buffer. Bound proteins were then eluted in a step wise fashion using two times 200uL of each of the following buffers: 75mM NaCl AC Buffer with 1% Triton-X 100; 300mM

NaCl AC Buffer; 1M NaCl AC Buffer; and finally 1% SDS sample buffer. Twenty percent of each fraction collected was separated by SDS-PAGE on 6% or 12% polyacrilamide gels and eluted proteins were detected by silver staining.

2.7 Silver stain and mass spectrometry analysis

All steps were carried out at room temperature. Gels were prepared for silver staining by fixing O/N in Fixing Solution (50% methanol, 10% acetic acid). The following day, gels were rinsed once in a solution of 20% ethanol for 10min followed by a 10min wash in ddH2O. The gels were then reduced in a solution of sodium thiosulfate (0.2g/L) for 1min and then washed twice with ddH2O for 30sec each time. Gels were incubated in silver nitrate (2g/L) for 30min. The gels were then rinsed for 30sec in Developing Solution (Na_2CO_3 30g/L, 1.4mL of a 37% formaldehyde solution/L, sodium thiosulfate 10mg/mL) and incubated in Developing Solution until the desired intensity was reached. The Developing Solution was removed and replaced with a 1% acetic acid solution to stop the reaction for 20min. Bands of interest were excised with clean scalpels and transferred to clean eppendorf tubes and stored at 4°. Bands were sent out to the McGill Sheldon Biotechnology Center or the Centre Proteomic de l'Universite Laval for sequencing by mass spectrometry.

2.8 Transcriptional reporter assays

H1299 cells were seeded at a density of 100 000 cells/well in 6 well plates and were transfected 18hrs later with 350ng of pG5-TK-luc reporter, 350ng of pSG424 Gal4 fusion and 10ng of pRL-CMV co-reporter using DMRIE-C reagent (Invitrogen). Cells were assayed for Luciferase activity 30hrs post-transfection using the Dual Luciferase

Assay kit (Promega) following the manufacturers protocol with a Luminometer (EG&G Berthold). Each sample was performed in duplicate. Firefly Luciferase activity was divided by Renilla Luciferase activity to normalize for transfection efficiency and the activity of each was compared to cells transfected with Gal4 alone, which was arbitrarily assigned a value of 100%. In the experiment involving the SuPr-1 SUMO protease, CHO-K1 cells were seeded at a density of 200 000 cells/well of 6 well plates and transfected 18hrs later with 200ng of pG5-TK-luc reporter, 200ng of Gal4 fusion construct (SV40 or CMV promoter), 5ng of phRL-RSV and either 0, 10 or 100ng of an HA-SuPr-1 expression plasmid (CMV or EFα1 promoter) or pEBB HA-SuPr-1 C466S mutant (EFα1) using Lipofectamine reagent (Invitrogen). The amount of DNA was brought to 505ng for all samples using pEBB (EFα1) or pCANHA (CMV) empty vector when necessary. Cells were harvested 27hrs post-transfection and Luciferase activity was monitored as described previously.

2.9 SUMOylation assay and Immunoprecipitation

H1299 cells were seeded in 60mm plates at a density of 300 000 cells/plate 24 hrs before transfection. Prior to transfection, cells were washed with serum free DMEM and infected with 20ul of vaccinia virus in a 500uL volume and incubated at 37°C for 1hr. Cells were transfected with 1.5ug of pcDNA3 Gal4-R1 or Gal4-R1 point mutants and 1.5ug of pcDNA3 HA-SUMO-1, HA-SUMO-2 or HA-SUMO-3 using DMRIE-C reagent (Invitrogen). The vector pcDNA3 Gal4 alone was included as a negative control. Cells were harvested 24hrs post-transfection using 1X PBS containing 1mM EDTA. Floating and adherent cells were washed once with 1XPBS and pelleted by centrifugation at 3000 rpm for 5 min at 4°. Cells were lysed in 3 pellet volumes of RIPA buffer (50mM Tris-

HCl pH 8.0, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1mM PMSF, 1mM Na_3VO_4, 1X ALP (Aprotinin 2ug/mL, Leupeptine 5ug/mL and Pepstatin 0.4ug/mL) and 25mM N-ethyl-maleimide (NEM) protease inhibitors. Cell lysis was performed on ice for 20 min followed by centrifugation at 13 000 rpm at 4° for 15 min to pellet debris. The cleared extracts were quantified and brought to a volume suitable for immunoprecipitation, typically 300 to 400uL using RIPA complete buffer. Extracts were incubated with 1ug of RK5C1 antibody (Santa Cruz) for 30 minutes at 4° with gentle agitation end-over-end. After 30 minutes, 20ul of a 1:1 slurry of Protein A Sepharose 4 fast flow beads (Amersham Pharmacia Biotech) in RIPA Buffer was added to each and incubation was continued at 4° for another 1hr 30. Samples were then washed five times in RIPA buffer containing 25mM NEM by inverting the tubes twenty times for each wash. Beads were then boiled in 30uL of 2X sample buffer for 5 min. Experiments involving the SuPr-1 SUMO specific protease had the following modifications; 1ug of pcDNA3 Gal4-R1, pcDNA3 HA-SUMO-1 and pcDNA3 HA-SuPr-1 were transfected with DMRIE-C reagent (Invitrogen). Cells were harvested and lysed as above, followed by immunoprecipitation following similar conditions.

2.10 Western blot Analysis

Extracts were quantified using the Bradford reagent (Biorad) and proteins were separated by SDS-PAGE on polyacrilamide gels. Proteins were then transferred to Immobilon-P PVDF membranes (Millipore) previously activated with methanol using a semi-dry transfer apparatus in 1X Semi-dry Transfer Buffer (48mM Tris-HCl, 39mM Glycine, 0.037% SDS, 20% methanol). Membranes were then blocked O/N at 4° or at room temperature for 1 hr in blocking solution, 1X TBS-T (25mM Tris-HCl pH 7.2,

137mM NaCl, 2.7mM KCl and 0.1% Tween-20 (v/v)) containing 5% milk. Prior to addition of primary antibody, membranes were rinsed three times for 10 sec with 1X TBS-T. Primary antibodies were diluted in 1X TBS-T containing 0.02% sodium azide according to the recommended working dilution for RK5C1 (1:100), anti-SUMO-1 (1:500) and HA-11 (1:1000) and typically incubated for 1hr to 1hr 30 at room temperature. Membranes were rinsed quickly three times in 1X TBS-T, followed by five washes of five minutes each. The membranes were then incubated with goat anti-mouse Horse Radish Peroxidase (HRP) conjugated secondary antibody (Jackson Immuno Research Labs) at a dilution of 1 in 10 000 in blocking solution (1X TBS-T, 5% milk) at room temperature for 1hr. The membranes were washed in the same fashion as after the primary antibody using 1X TBS-T. Following the last wash, membranes were incubated with enhanced luminol reagent (NEN Life Science) according to the manufacturer's protocol to allow visualization of proteins upon exposure of the membranes to photographic film (Kodak).

Chapter 3: Experimental Results

3.1 Isolation of a potential RBP1 R1 domain interacting protein

Transcriptional repression by the Retinoblastoma Binding Protein 1 RBP1 involves both HDAC dependent and independent mechanisms (Lai et *al*, 1999b). HDAC dependent repression is mediated by the R2 domain of RBP1, which associates with the mSin3A/HDAC co-repressor complex via the SAP30 polypeptide (Lai et *al*, 2001). However, the mechanism by which R1 represses transcription when brought to a synthetic promoter by the Gal4 DNA binding domain remains unsolved. Affinity chromatography using the R1 domain was utilized to isolate potential binding partners that may mediate the HDAC independent mode of repression by RBP1. Briefly, H1299 cell nuclear extract was incubated with GST or GST-R1/Affigel 15 affinity columns prepared as described in Materials and Methods. The GST/Affigel affinity resin was included as a control to monitor for non-specific interactions. Bound proteins were eluted in a stepwise fashion using buffers of increasing salt and detergent concentrations and visualized by silver staining.

Fractions collected from the affinity chromatography are depicted in the silver stained SDS-PAGE gels in Figure 3.1. The gel in panel A shows 40uL (20%) from each fraction that was collected after binding (Figure 3.1A, lanes 1, 3 and 5) and represents the flow through. Lanes 2 and 4 in Figure 3.1A correspond to GST and GST-R1 coupled resins that were incubated with the Affinity Chromatography Buffer (AC Buffer) only and serve as controls for proteins that may leach from the columns. Forty microliters of each elution is represented in Figures 3.1B to E. The AC Buffer containing 1% Triton-X 100, which contains a low salt concentration, eluted many proteins that bound non-

specifically to the matrix, as these species were also present in the GST and GST-R1 lanes (Figure 3.1B compare lanes 1, 3 and 5). However, there was a specific band that appeared only in the GST-R1 lane that was not present in the GST lane (Figure 3.1B compare lane 5 to 3 indicated by arrow). The AC Buffers containing 300mM and 1M NaCl also eluted many non-specific proteins as shown in Figure 3.1 panel C and D in lanes 1, 3 and 5. The SDS sample buffer revealed no specific band in the GST-R1 lane as the bands that appeared were in fact proteins that had leached off the resin because they were present in the lane where GST-R1 was not incubated with nuclear extract (Figure 3.1E compare lane 4 and 5). The 60kDa band above the GST-R1 in lane 5 likely corresponds to BSA, which was used during the preparation of the columns.

The 1% Triton-X 100 AC Buffer, which breaks hydrophobic interactions, yielded a single band of approximately 20kDa in size that was specific to the R1 domain. This band is also seen in fraction 2 of this elution but at a lower overall intensity (data not shown). The intensity of the band is similar to that of the non-specific binding proteins and suggests that the interaction with R1 is weak or that the protein may not be abundant. The band was excised and sent for mass spectrometry analysis to determine its identity. Unfortunately, no identification was achieved due to insufficient amounts of protein in the sample. Several attempts of this kind were conducted but we were unsuccessful in identifying R1 binding species. It became a concern that use of bacterially synthesized R1 protein may not be the best approach as such material was post-translationally un-modified and such modifications may be of importance in complex formation.

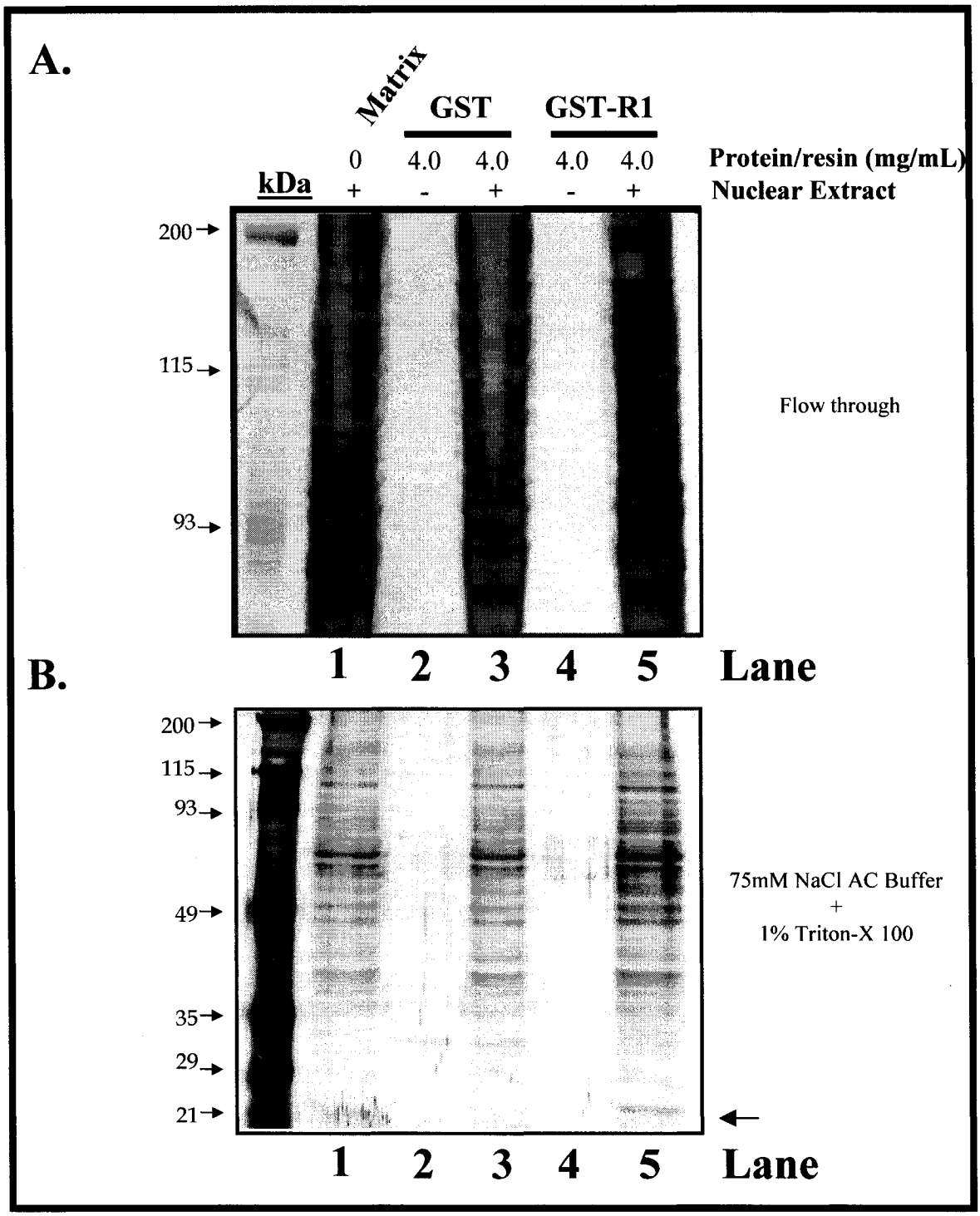


Fig. 3.1. Isolation of a potential R1 binding protein using affinity chromatography. Panel A. *Flow through elutions collected after incubation with H1299 cell nuclear extract.* Forty microliters of each fraction (20%) was separated on a 6% polyacrylamide gel by SDS-PAGE and proteins were visualized by silver staining. Panel B. *Elution with the AC Buffer containing 1% Triton-X 100 reveals a 20 kDa band that interacts specifically with the R1 domain.* Twenty percent of each fraction was separated by SDS-PAGE on a 12% polyacrylamide gel followed by silver staining. Note the specific band from the GST-R1 lane that is not present in the GST alone lane.

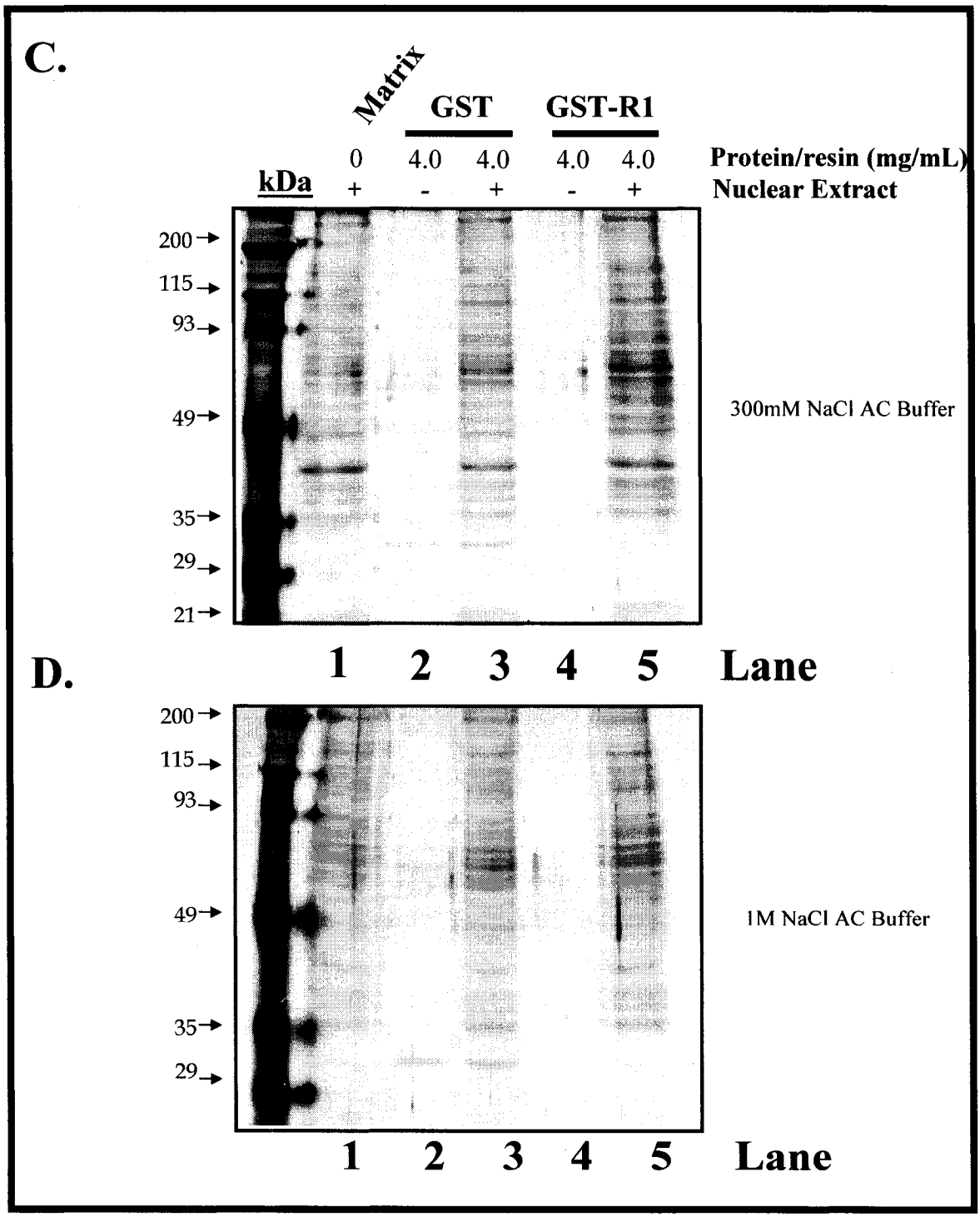


Fig. 3.1. continued. Panels C and D. *Elution with buffers of increasing salt concentration reveals proteins that bind non-specifically to the R1 domain.* Forty microliters from each fraction was separated as in B. and visualized again by silver staining. Proteins bound to the GST-R1 resin were also bound to the GST/resin and the resin alone indicating non-specific interactions.

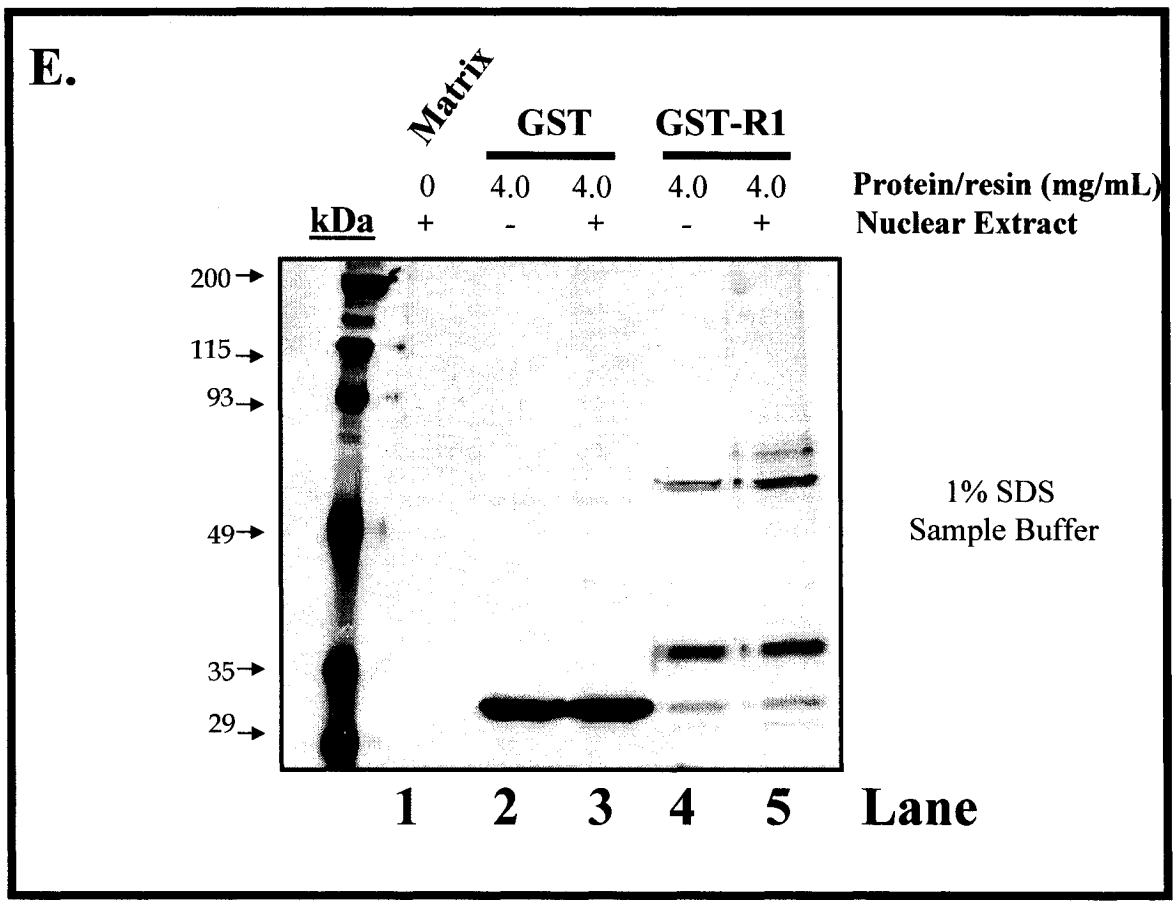


Fig. 3.1. continued. Panel E. *Elution with SDS sample buffer yields proteins that leached from the resin.* Twenty percent of fractions from various elutions were separated as mentioned on a 12% polyacrylamide gel and silver stained as before.

3.2 Mapping of the minimal region sufficient for R1 repression

Before studying the type of modification that may potentially govern repression by R1, further mapping studies were undertaken on the R1 domain. Alanine-scanning mutagenesis has been successfully used to identify critical residues in repression motifs of transcription factors such as Elk-1 (Yang et al, 2002). However, this type of experiment can be tedious with large repression domains. Previous work by our group demonstrated that the R1 domain is a strong repression motif that corresponds to amino acids 241 to 452 of RBP1, which encompasses the putative DNA binding ARID domain (Lai et *al*, 1999a,b). Preliminary mapping studies on the R1 domain indicated that amino acids 241 to 303 of RBP1 were not involved in the repression by R1. However, removal of this region was shown to allow the R1 domain to function as an activator of transcription (Lai, unpublished). To investigate the requirement of the ARID domain for repression by R1 and to identify a minimal region required for repression, several deletion constructs of Gal4-R1 were generated and their ability to repress transcription was tested using the pG5-TK-luc reporter construct. This reporter expresses firefly luciferase under the control of the herpes simplex virus thymidine kinase (HSV TK) promoter. A cassette containing five copies of the Gal4 binding site is also located upstream of the promoter.

H1299 cells were transfected with the pG5-TK-luc reporter and various Gal4-R1 deletion constructs and the repression activity of each was compared to Gal4 alone using Luciferase assays. The repression activity obtained for each construct is shown in Figure 3.2 Panel A. Relative to Gal4 alone, Gal4-R1 repressed transcription by 72.8%. Removal

of the N-terminus of R1 (Gal4-R1 ARID) had no significant effect, although the repression was slightly higher. However, when the last 53 amino acids of Gal4-R1 were deleted (Gal4-R1 dl859c), a significant amount of the repression associated with the R1 domain is lost (2.4 fold relief). Furthermore, this C-terminal portion of R1 was seen to act as a strong repression motif when fused to Gal4 (Gal4-R1 C-term). The impaired ability of the Gal4-R1 dl859c construct in transcriptional repression was not due to a lower level of expression relative to the other deletion constructs (Figure 3.2B compare lanes 1 to 5). Similar results were also obtained in CHO-K1 cells (data not shown). A summary of the R1 deletion constructs along with their repression activity has been presented in Figure 3.3A. The Gal4-R1 dl859c construct, which includes the ARID domain, retained some ability to repress transcription relative to Gal4. Nevertheless, the data indicated that the ARID domain does not participate in R1 mediated repression and that amino acids 400 to 452 are sufficient and necessary for transcriptional repression by R1.

3.3 The R1 domain contains putative sites for SUMO modification

The activity of many transcription factors is controlled by post-translational modifications such as acetylation, ubiquitination or sumoylation, which all take place on lysine residues (Freiman and Tijan, 2003). The function of the R1 domain of RBP1 may be controlled by such modifications. Modification with SUMO-1 requires the consensus sequence ψ-K-X-E, where ψ represents the hydrophobic amino acids valine, leucine or isoleucine and X signifies any amino acid (Rodriguez et *al*, 2001).

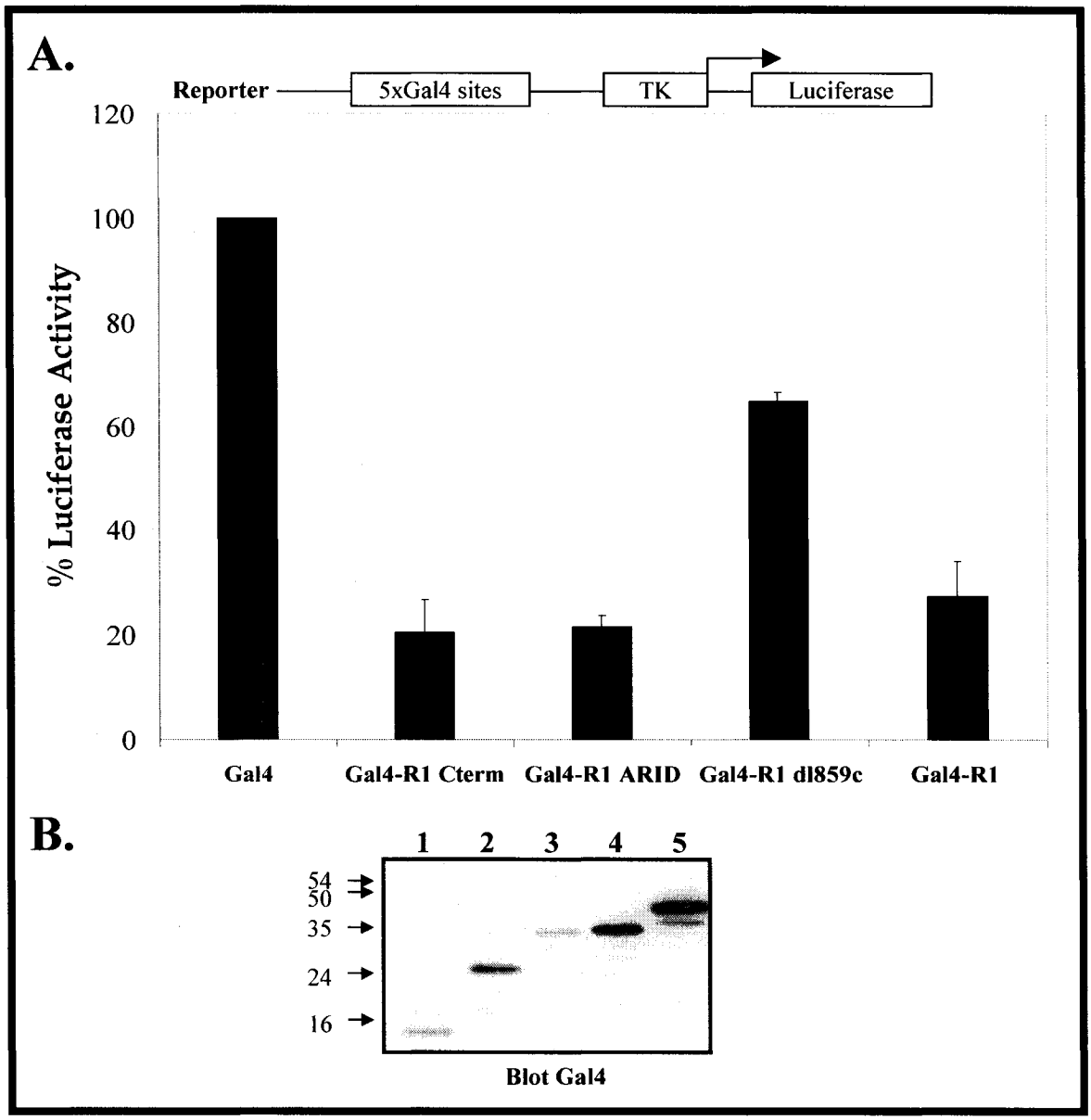


Fig. 3.2. Transcriptional repression by the R1 domain of RBP1 maps to amino acids 400 to 452. Panel A. *Transcriptional repression activity of various Gal4-R1 deletion constructs.* H1299 cells were transfected with the pG5-TK-luc reporter plasmid along with Gal4-R1 deletion mutants. The pRL-CMV-luc Renilla Luciferase co-reporter was also included to normalize transfection efficiencies. Cells were assayed for luciferase activity 30 hrs post-transfection using the Dual Luciferase Assay system following the manufacturer's protocol. The luciferase activity of each was compared relative to Gal4 alone, which was set at 100. Values shown are the mean of 2 independent experiments with each sample performed in duplicate. Panel B. *Expression of the Gal4-R1 deletion mutants.* H1299 cells were transfected with plasmids encoding Gal4-R1 deletion mutants and their expression was confirmed by western blot. Cells were harvested 24hrs post-transfection and 40ug of whole cell extract was separated by SDS-PAGE on a 15% polyacrylamide gel and the proteins were detected using the RK5C1 (Gal4) mouse monoclonal antibody. Sizes of the mutants were close to their expected molecular weights. Lane 1-Gal4 DNA binding domain, Lane 2-Gal4-R1 C-term, Lane 3-Gal4-ARID, Lane 4-Gal4-R1 dl859c and Lane 5-Gal4-R1.

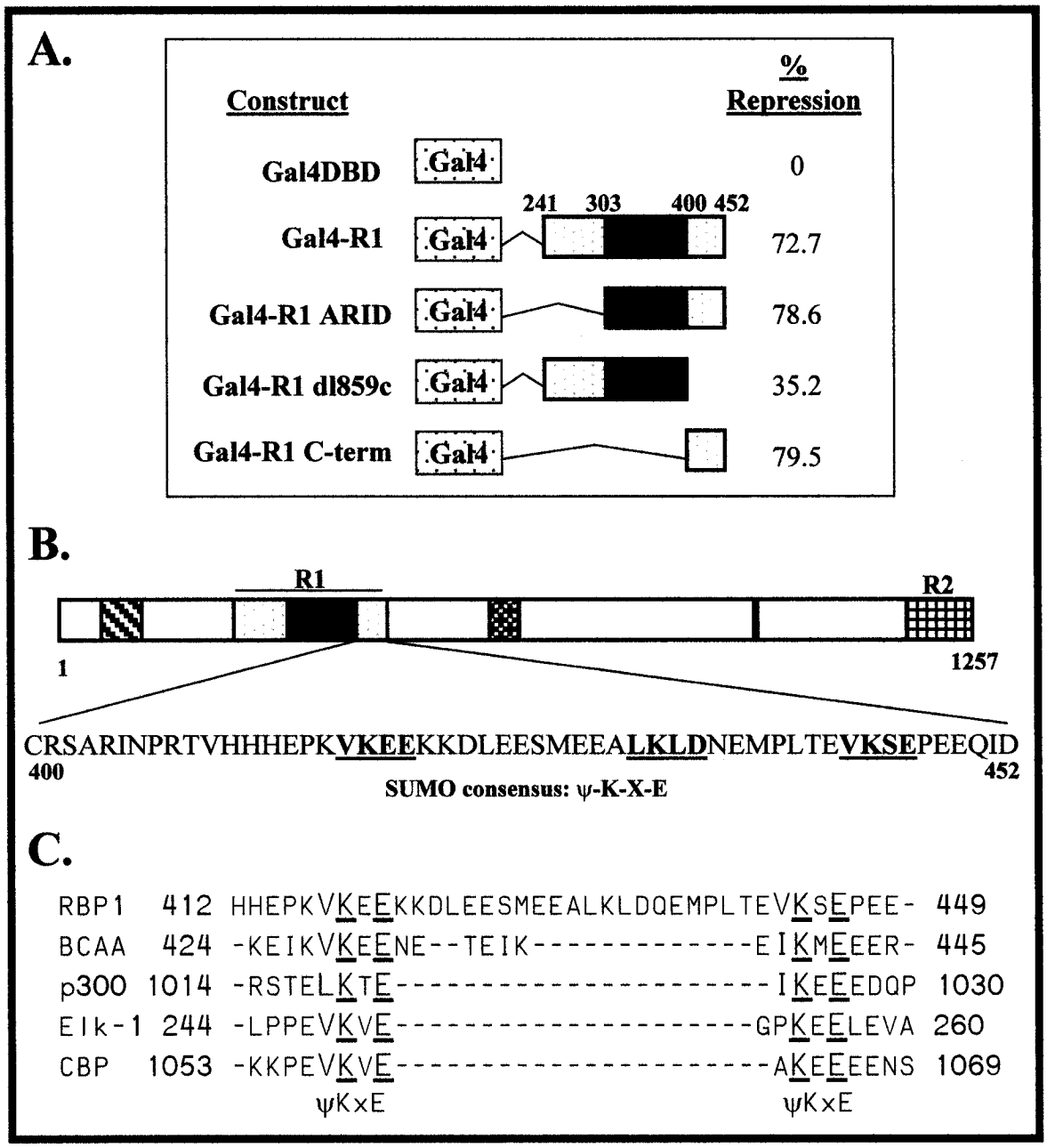


Fig. 3.3. The R1 domain of RBP1 contains three putative sites of sumoylation. Panel A. *Schematic representation of the Gal4-R1 fusion constructs used to map the minimal region required for R1 repression.* Shown is the percent repression of each relative to Gal4 alone and the amino acids from RBP1 encoded by each fusion. Panel B. *Diagram of RBP1 showing the position of the R1 domain relative to the other known structural motifs.* The sequence shown represents the minimal region sufficient for R1 repression and the putative sumoylation sites are highlighted in bold and underlined. Panel C. *Alignment of the C-terminal region of R1 from RBP1 with the homologous region of BCAA and other mapped repression motifs found in several transcription factors.* These repression domains are characterized by at least one N-terminal SUMO consensus and a stretch of acidic residues located at the C-terminal end. Note the presence of a spacer region that separates the SUMO consensus sites of the R1 domain, which is absent in the other repression motifs. Sequence alignment was performed using T-Coffee version 1.41 found on the ExPASy proteomics tools web site.

Inspection of the sequences in the minimal region sufficient for R1 repression revealed that three lysine residues, K418, K433 and K444, all lie within the consensus site for modification by SUMO-1 (Figure 3.3B). Although the sequence surrounding K433 contains a D instead of E, this lysine can still be considered a potential site of sumoylation (Ron T. Hay, personal communication). In addition, an alignment performed with residues from this region of R1 indicated that it had homology with other sumoylated repression motifs (Figure 3.3C).

PCR mediated site directed mutagenesis was used to convert the lysine residues to arginines in Gal4-R1 (aa. 241-452) to determine whether or not they participate in repression by R1. The repression activity of the Gal4-R1 (aa. 241-452) point mutants, either alone or in combination, was assessed using the pG5-TK-Luc reporter in H1299 cells by monitoring Luciferase activity and the results are depicted in Figure 3.4A. The Gal4-R1 K418R and K444R point mutants caused a partial relief of repression relative to wild type Gal4-R1, and this relief was not simply due to a lower level expression (Figure 3.4B). However, the Gal4-R1 K418/444R double mutant caused a relief of repression of about 2.5 fold, which was similar to that seen with the Gal4-R1 dl859c mutant (Compare Figure 3.2A with 3.4A). In addition, when all three lysines were converted to arginines, the repression activity of this mutant form was similar to that observed with the double K to R mutant, indicating that K433 is dispensable for R1 repression. Similar results were obtained using the pG5-MLP-Luc reporter, which is controlled by the adenovirus major late promoter (data not shown).

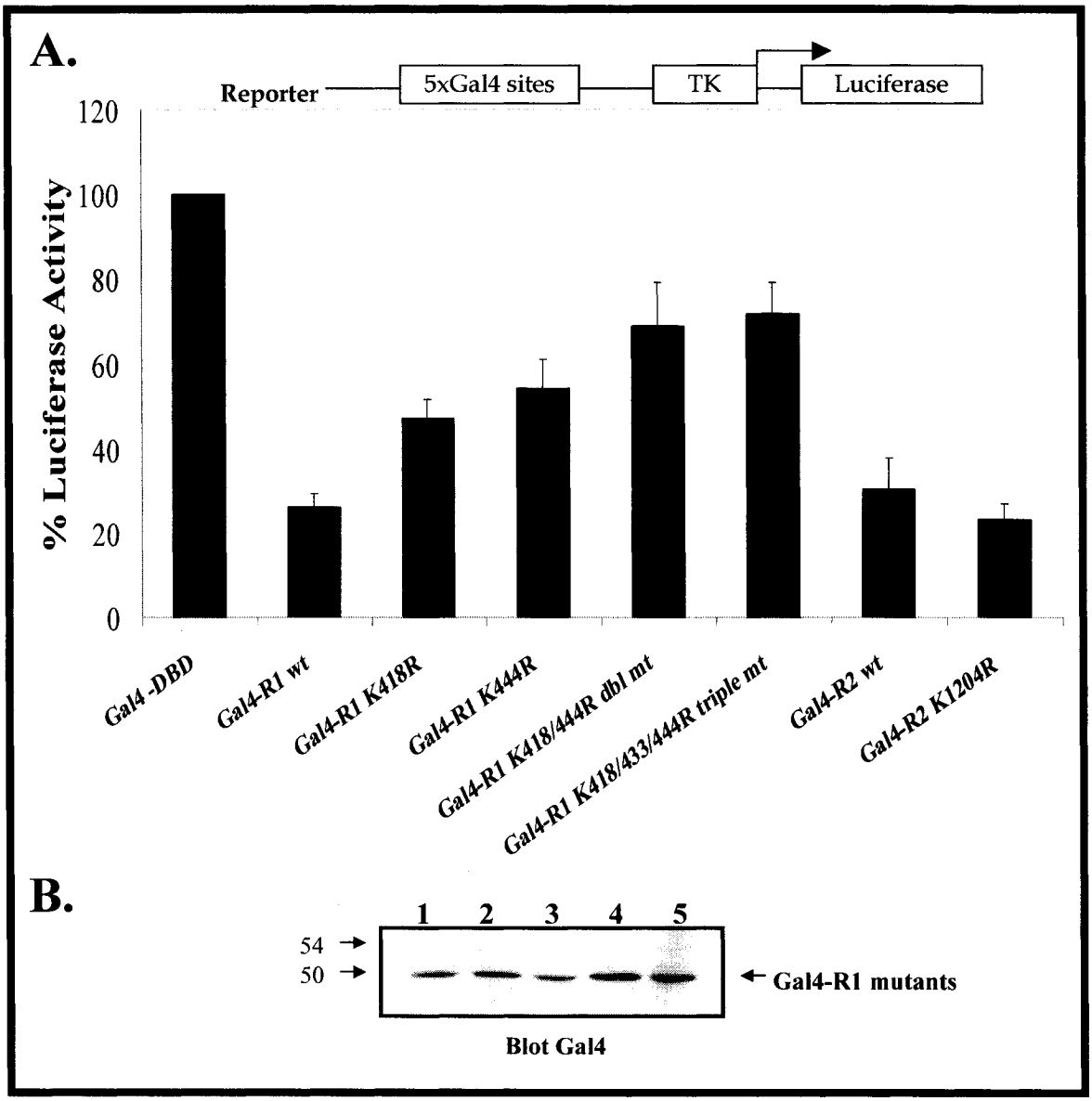


Fig. 3.4. Mutations in the putative SUMO specific sites abolishes transcriptional repression by the R1 domain of RBP1. Panel A. *Transcriptional repression activity of the Gal4-R1 and Gal4-R2 point mutants.* H1299 cells were transfected with the pG5-TK-luc reporter plasmid along with the pSG424 Gal4-R1 K to R point mutants and the pRL-CMV-luc co-reporter, included to allow normalization of transfection efficiency. Cells were harvested and assayed for Luciferase activity as before and the activity of each was compared to that of Gal4 alone, which was arbitrarily assigned a value of 100%. Values shown are the mean of at least three independent experiments of samples were performed in duplicate. Panel B. *Expression of the Gal4-R1 point mutants.* H1299 cells were transfected with 2ug of plasmids expressing Gal4-R1 point mutants and harvested 24hrs post-transfection. Forty micrograms of whole cell extract was separated by SDS-PAGE and the expression level of each was monitored by probing with the Gal4 antibody RK5C1. Lane 1-Gal4-R1 wt, Lane 2-Gal4-R1 K418R, Lane 3-Gal4-R1 K444R, Lane 4-Gal4-R1 K418/444R dbl mt and Lane 5-Gal4-R1 K418/433/444R triple mt.

Our group has shown previously that the RBP1 R2 repression domain functions by associating with HDAC complexes (Lai et *al.*, 1999b; Lai et *al.*, 2001). Analysis of the amino acid sequence of the R2 domain revealed the presence of a lysine residue within a SUMO consensus motif. Thus, a mutant in which K1204 of R2 was converted to an arginine was produced by PCR and the repression activity of this mutant. Interestingly, the Gal4-R2 K1204R mutant did not relieve repression by wild type Gal4-R2 but actually increased slightly, although this increase was minimal (Figure 3.4A). Thus, sumoylation does not appear to play a role in R2 mediated repression. Nevertheless, in the case of R1, K418 and K444, which are putative sumoylation sites, appear to be required for transcriptional repression.

3.4 The R1 domain of RBP1 is modified by SUMO

Mutagenesis of two lysine residues predicted to be SUMO modification sites in the R1 domain of RBP1 revealed that they are required for transcriptional repression. However, substitution of lysine to arginine can prevent other types of modifications to occur on R1. In fact, the transcription factor Sp3 as been documented to be acetylated at the same lysine residue that is modified by SUMO-1 (Braun et *al*, 2001). To demonstrate that the R1 domain of RBP1 is modified by SUMO-1, HA-SUMO-1 was overexpressed in H1299 cells along with Gal4-R1 (aa. 241-452). Prior to transfection, cells were infected with Vaccinia virus, which drives the expression of proteins by activating the T7 promoter due to rapid production of T7 RNA polymerase in the cytosol (Fuerst et *al.*, 1986). Since pcDNA3 contains a T7 promoter, this treatment thus allows more SUMO-1 to be available as a large portion of the pool of free SUMO-1 is attached to the nuclear import factor RanGAP1 (Muller et *al*, 2001). Extracts were prepared in the presence of

25mM N-ethyl maleimide (NEM), a cysteine protease inhibitor that blocks the activity of desumoylating enzymes, and they were immunoprecipitated using a Gal4 specific antibody. Western blot analysis using an anti-SUMO-1 antibody revealed the presence of two sumoylated species of 60kDa and 110kDa respectively that were immunoprecipitated specifically by using anti-Gal4 antibodies from samples containing Gal4-R1 but not Gal4 alone (Figure 3.5A compare IP lane 6 with 8). Similarly, western blotting with a Gal4 specific antibody confirmed that the two higher migrating species are SUMO modified forms of Gal4-R1 and not SUMO conjugated proteins that interact with R1 (Figure 3.5B). The 90kDa SUMO conjugated protein that was detected in cell lysates in the absence of exogenous SUMO-1 likely corresponds to RanGAP1 (Figure 3.5A WCE lanes 1 and 3).

A similar experiment was performed with the other SUMO family members SUMO-2 and SUMO-3, which are typically conjugated to proteins only in response to protein damaging stimuli. As shown in Figure 3.6A, western blot analysis using anti-HA yielded two SUMO modified forms of Gal4-R1 when either HA-SUMO-2 or 3 were overexpressed, as these forms are recognized by the anti-Gal4 antibody (Figure 3.6B). The presence of major and minor SUMO modified forms of Gal4-R1 suggested that there are only two sites of sumoylation. The major form was consistent with the expected size of Gal4-R1 modified with one SUMO, an effect that typically adds 15kDa to the overall size of the modified protein. However, the minor form appeared larger than the expected size of Gal4-R1 with two covalently attached SUMO molecules, indicating that other modifications may be involved. Nonetheless, the R1 domain of RBP1 was clearly post-translationally modified in the presence of exogenous SUMO at two sites, involving all

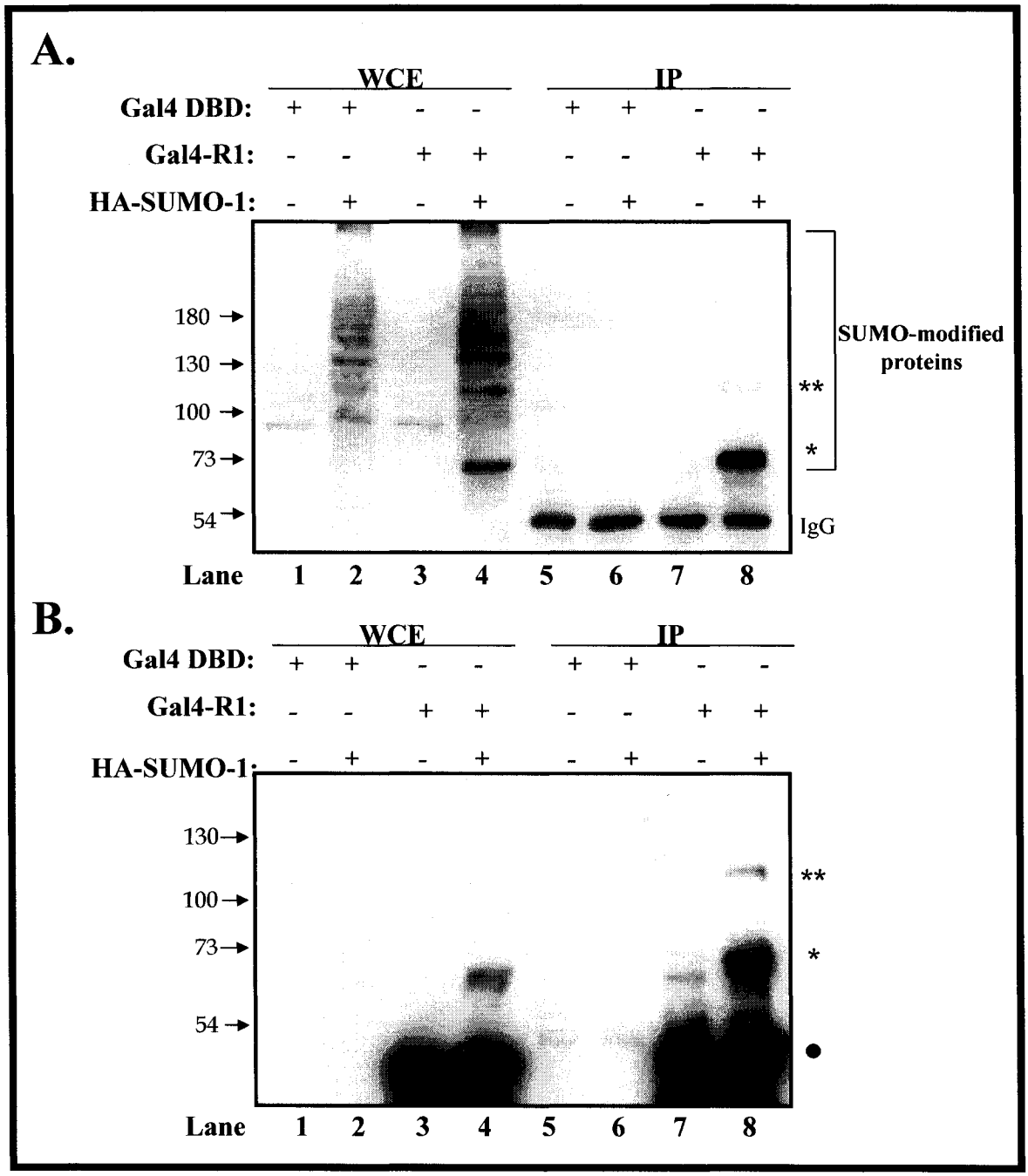


Fig. 3.5. The R1 domain of RBP1 is sumoylated *in vivo*. Panels A and B. H1299 cells were transfected with pcDNA3 Gal4-R1 along with HA-SUMO-1 or empty vector using DMRIE-C reagent. The Gal4 DNA binding domain was included as a negative control. Prior to transfection, cells were infected with Vaccinia virus for 1 hour. Cells were harvested 24hrs post-transfection and lysed in RIPA buffer containing 25mM NEM isopeptidase inhibitor. Extracts were immunoprecipitated with Protein-A Sepharose and 1ug of RK5C1 (anti-Gal4) antibody. Bound proteins were boiled in 2X sample buffer and separated on 10% polyacrylamide gels using SDS-PAGE followed by western blotting with either anti-SUMO-1 (Panel A) or RK5C1 (Panel B). Asterisks indicate the SUMO-modified forms of Gal4-R1 whereas the closed circle is the native Gal4-R1. Note that Gal4 alone was run of these gels.

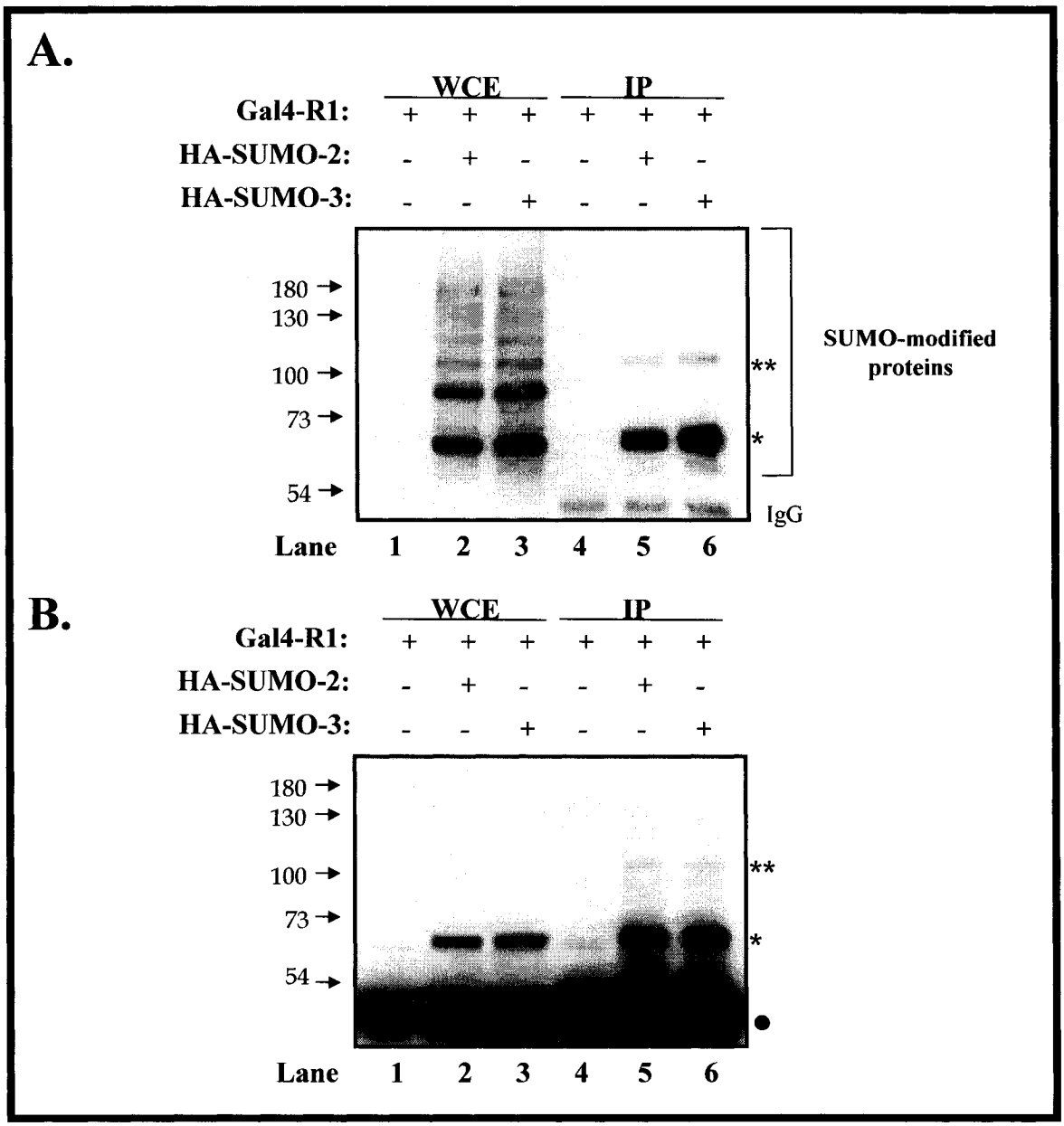


Fig. 3.6. The SMRD of RBP1 is also modified by SUMO-2 and SUMO-3 *in vivo*. Panels A and B. H1299 cells were transfected as in Figure 3.5 with plasmids encoding HA-SUMO-2 or 3 instead of HA-SUMO-1. Cells were harvested and lysed as mentioned previously and extracts were immunoprecipitated with a Gal4 specific antibody following similar conditions. Proteins were separated on 10% polyacrilamide gels using SDS-PAGE and the SUMO modified forms of Gal4-R1 were visualized by western blotting with the HA (Panel A) or RK5C1 (Panel B) antibodies. Asterisks indicate the SUMO modified forms whereas the closed circle indicates the un-modified form.

three SUMO family members. For the purpose of this discussion, the R1 domain of RBP1 will now be referred to as SMRD, which stands for Sumo Modified Repression Domain.

3.5 The major SUMO acceptor sites in the R1 domain are K418 and K444

It has recently been shown that the Cell Cycle Repression Domain 1 CRD1 of p300 represses transcription in a SUMO dependent manner by recruiting HDAC6 (Girwood et *al*, 2003). Transcriptional repression by the SMRD of RBP1 requires two lysine residues that are potential sites of sumoylation. To study the possible involvement of SUMO modification in SMRD mediated repression, H1299 cells were infected with Vaccinia virus followed by transfection with the Gal4-R1 K418R or K444R point mutant along with HA-SUMO-1. Cell extracts were prepared as before and immunoprecipitated using a Gal4 specific antibody. Western blot analysis using anti-SUMO-1 demonstrated that the minor SUMO modified form of 110kDa observed with wild type Gal4-R1 is lost with the Gal4-R1 K418R or K444R point mutants (Figure 3.7A, left Panel, lanes 6 and 8). The 50kDa band observed in the all lanes of Figure 3.7A corresponded to the antibody heavy chain and was not a SUMO conjugated protein interacting with the SMRD (Figure 3.7A, left panel, lanes 5 to 8). The results shown in Figure 3.7B, left panel, confirmed that the SUMO modified species observed are consistent with Gal4-R1 with one SUMO molecule covalently attached. Furthermore, no SUMO modified species of Gal4-R1 were detected when both K418 and K444 were mutated as shown by immunoblotting for SUMO-1 or Gal4, thus indicating that these are the only available SUMO acceptor sites in the SMRD (Figure 3.7A and B, right Panels). These results also demonstrated that K433, which is not required for repression by the SMRD, is not a SUMO modification

site. Thus, the major sites of sumoylation in the SMRD of RBP1 are K418 and K444, suggesting that SUMO modification mediates transcriptional repression.

3.6 Repression by the SMRD of RBP1 is dependent on SUMO-1 modification

In order to demonstrate that SUMO modification is required for SMRD mediated repression, it was hypothesized that expression of a SUMO-1 specific protease could prevent Gal4-R1 conjugation with exogenous SUMO-1. To this end, HA-SUMO-1 and wild type Gal4-R1 were co-expressed in H1299 cells along with the SUMO-1 specific protease HA-SuPr-1, an isoform of SENP2 (Best et *al*, 2002). Cells were infected with vaccinia virus prior to transfection as before to drive the expression of each construct and extracts were prepared as in previous studies. Western blot analysis showed that the ladder of SUMO modified proteins observed when HA-SUMO-1 was added disappeared when HA-SuPr-1 was overexpressed (Figure 3.8A compare WCE lane 2 with 3). In addition, the two SUMO modified forms of Gal4-R1 immunoprecipitated with the Gal4 antibody, upon HA-SUMO-1 overexpression, were lost in the presence of the SUMO-1 protease (Figure 3.8 A and B compare IP lane 5 to 6). The loss of protein sumoylation associated with addition of the SUMO-1 protease was dependent on its catalytic activity and did not occur due to squelching of HA-SUMO-1, as the level of free HA-SUMO-1 was higher in samples where it was present (Figure 3.8C compare lanes 2 and 3, indicated by arrows). Unexpectedly, Figure 3.8B indicated that the 55kDa band, present in the WCE lane and not in the IP lane, likely corresponds to the SUMO-1 protease and not a higher migrating form of Gal4-R1. Hence, the SUMO-1 specific protease SuPr-1 was capable of catalyzing the removal of SUMO-1 from the SMRD of RBP1.

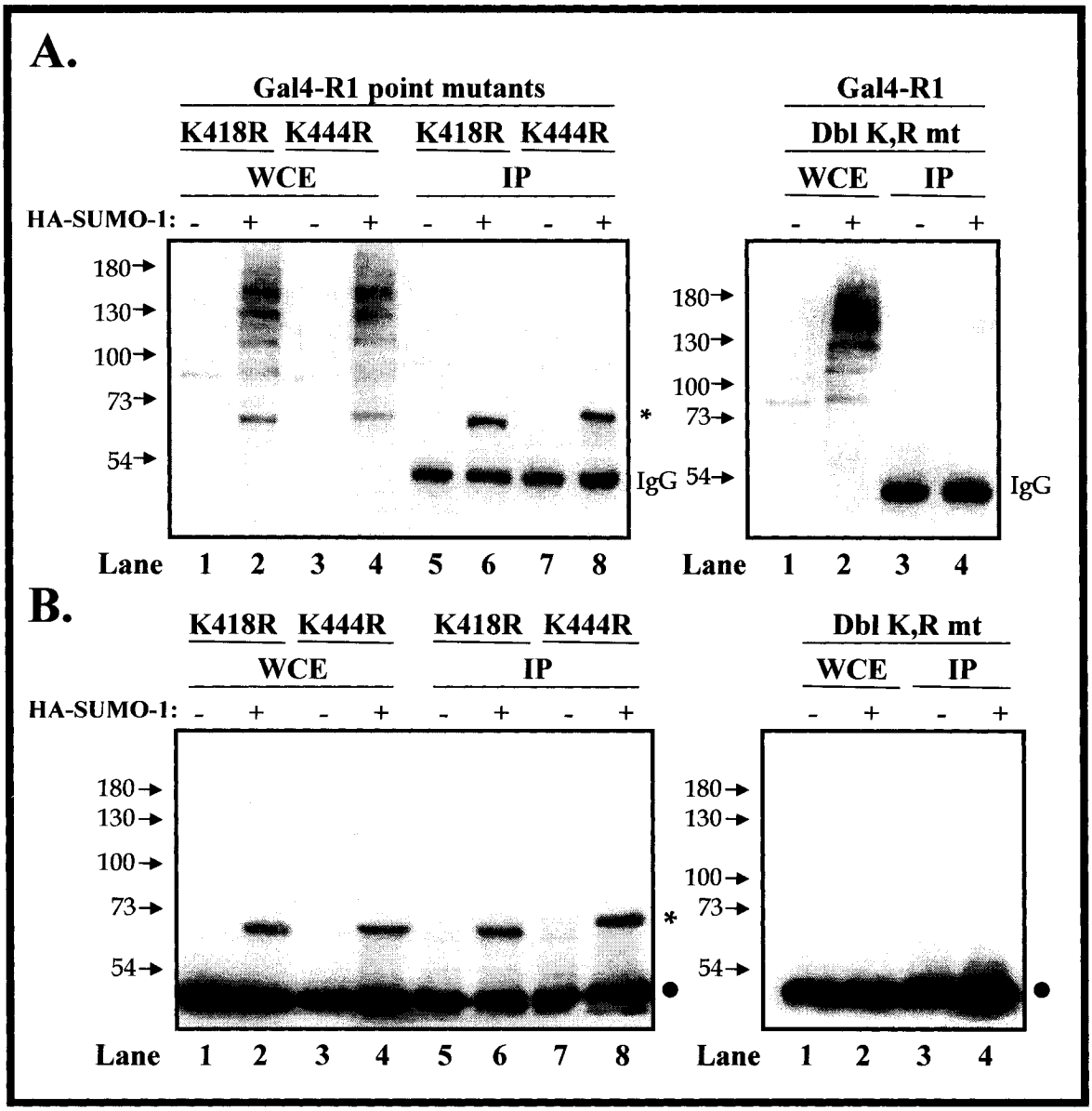


Fig. 3.7. Lysine 418 and 444 are the major SUMO modified sites in the SMRD of RBP1. Panels A and B. H1299 cells were transfected with plasmids expressing Gal4-R1 point mutants along with HA-SUMO-1 or empty vector as described in Materials and Methods. Cells were lysed and extracts immunoprecipitated as described in Figures 3.5 and 3.6 using the anti-Gal4 antibody. Samples were analyzed with either the anti-SUMO-1 antibody (Panel A, left and right) or with the mouse monoclonal anti-Gal4 antibody RK5C1 (Panel B right and left). Asterisks indicate the SUMO-modified Gal4-R1 point mutants whereas the closed circle corresponds to its unmodified form.

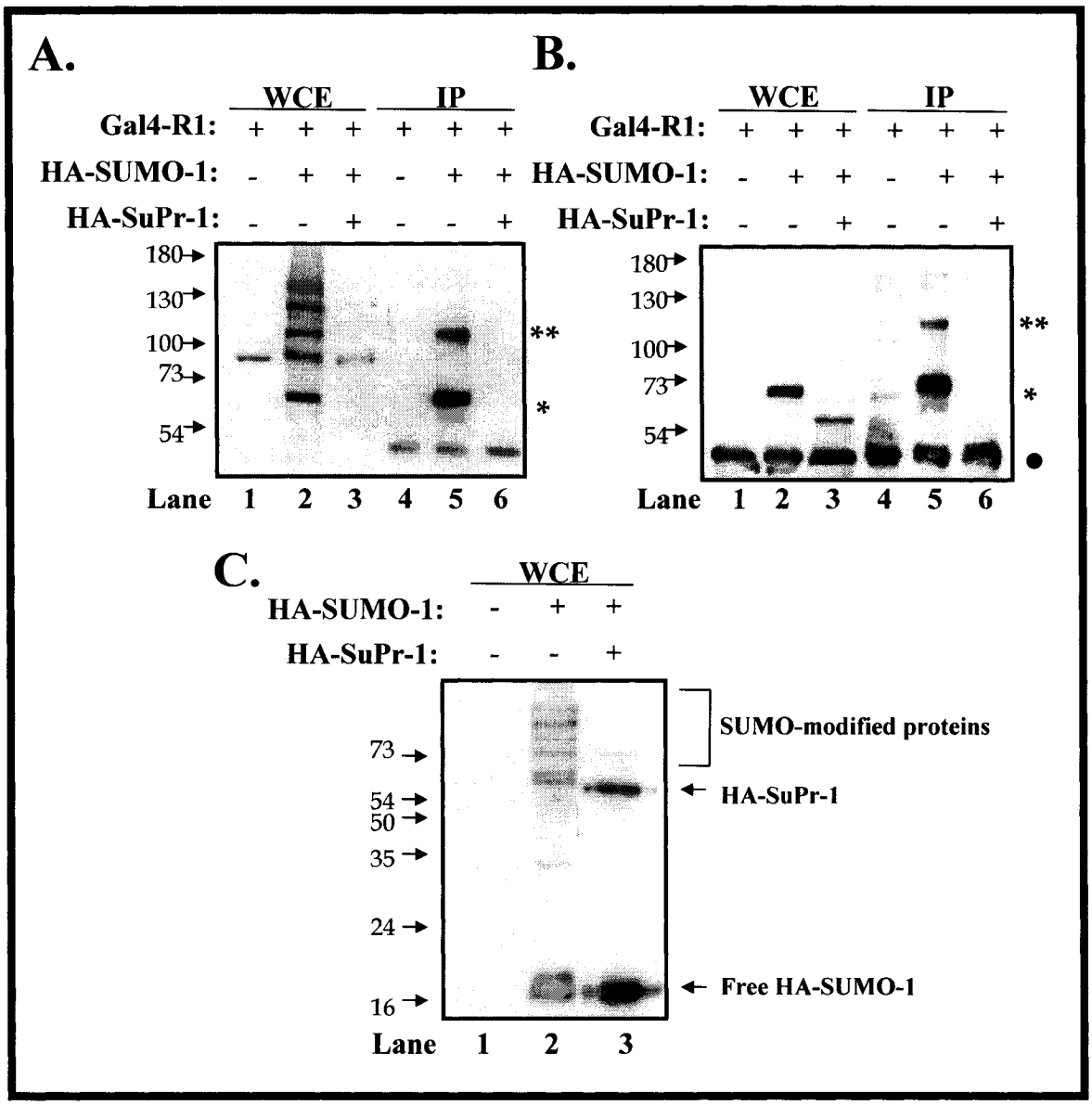


Fig. 3.8. The SUMO specific protease SuPr-1 hydrolyzes SUMO-1 from Gal4-R1. Panels A, B and C. H1299 cells, previously infected with vaccinia virus, were transfected with equal amounts of Gal4-R1, HA-SUMO-1 and HA-SuPr-1 expression vectors. Cells were harvested 24 hrs post-transfection and lysed with RIPA Buffer complemented with 25mM NEM. Extracts were immunoprecipitated as described previously using the anti-Gal4 antibody RK5C1 and proteins were subjected to SDS-PAGE. SUMO modified forms of Gal4-R1 were visualized by western blotting with an anti-SUMO-1 (Panel A) or anti-Gal4 (Panel B) antibody. Panel C. *Expression level of the SUMO specific protease SuPr-1.* Twelve and a half micrograms of whole cell extract was separated on a 15% polyacrylamide gel by SDS-PAGE and the proteins visualized by immunoblotting with an HA specific antibody. Arrows indicate that HA tagged SuPr-1, which migrates at 55 kDa and the free HA-SUMO-1 that has a molecular weight of 17 kDa.

Since the SMRD of RBP1 can be desumoylated by SuPr-1, overexpression of the latter could potentially relieve transcriptional repression by the SMRD. The ability of the SMRD to repress transcription in the presence of increasing amounts of HA-SuPr-1 was tested in CHO-K1 cells using the pG5-TK-Luc reporter. The phRL-RSV-Luc plasmid was included to normalize transfection efficiency. The results from this experiment are shown in Figure 3.9A. The Luciferase activity of each sample was compared to that observed with Gal4 alone in the presence of 0, 10 and 100ng of DNA expressing HA-SuPr-1, all set at 100%, as the protease seemed to increase the activity of the Luciferase reporter. Repression by Gal4-R1, which encodes amino acids 241-451 of RBP1, was relieved in a dose-dependent manner in the presence of HA-SuPr-1 with a fold relief of about 2.5. Furthermore, repression by the Gal4-SMRD construct, which is the region required for SMRD mediated repression, was also affected by the presence of the SUMO-1 protease. Interestingly, the fold relief observed with the Gal4-SMRD construct was about 4 fold, which was higher than that seen with the Gal4-R1 construct. However, increasing amounts of HA-SuPr-1 failed to stimulate the activity of the Gal4-R1 K418/444R double mutant, indicating that the SUMO-1 acceptor sites are required for SuPr-1 mediated relief of repression. Expression of SuPr-1 under the control of either EFα1 or CMV relieved SMRD repression but the fold relief was higher with the CMV promoter, which is stronger than EFα1 in CHO-K1 cells (Figure 3.9C compare lane 2 with 4).

The SUMO-1 specific protease SuPr-1 was found to be capable of stimulating the transcriptional activity of c-Jun independently of its catalytic activity (Best et *al*, 2002).

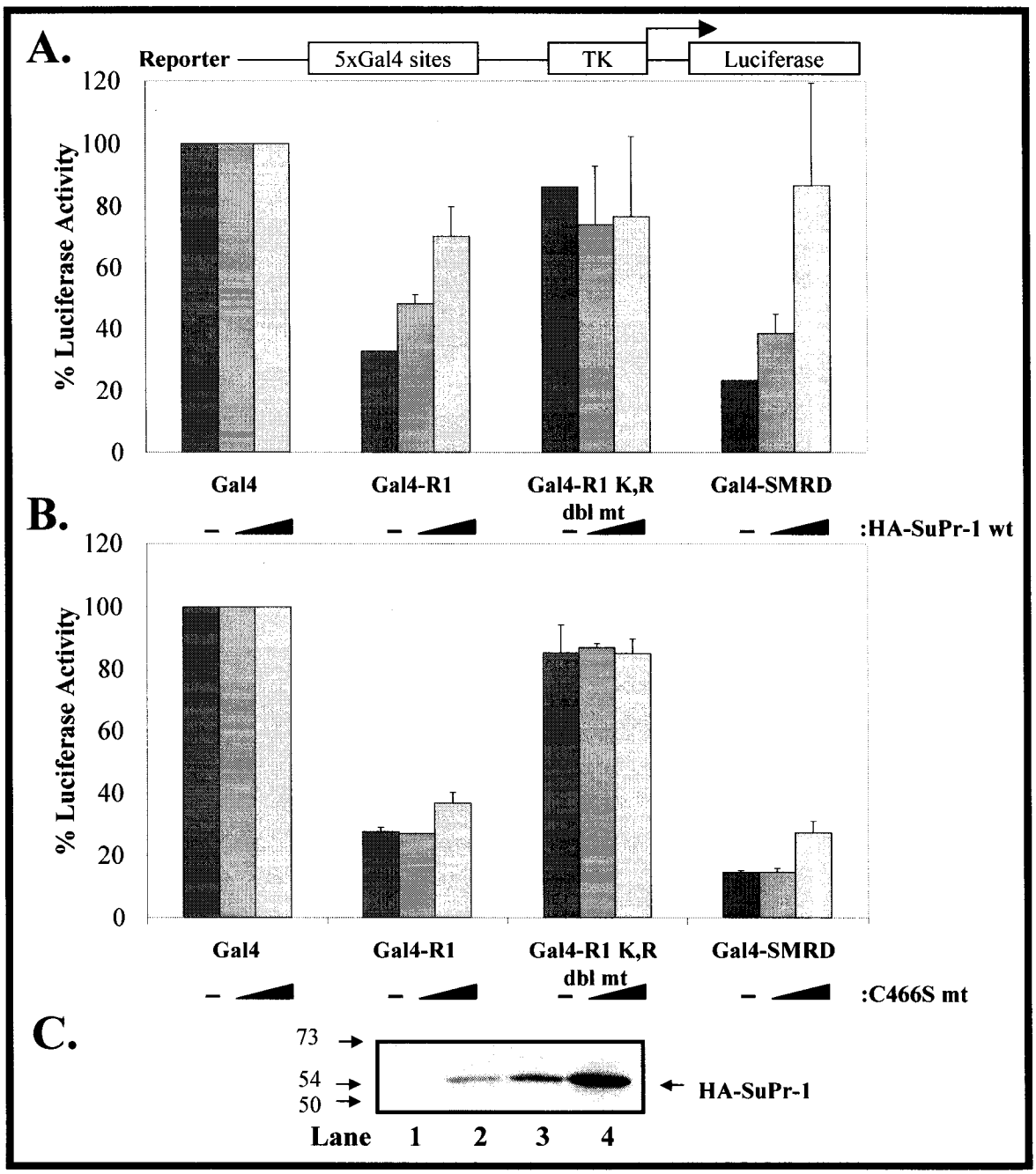
Therefore, a similar experiment was performed as described in Figure 3.9A using a catalytically inactive form of HA-SuPr-1 and its ability to relieve the repression mediated by the SMRD of RBP1 was tested. Again, the activity of each was compared to Gal4 containing 0, 10 and 100ng of DNA expressing the catalytically inactive SuPr-1 because it still retains the ability to induce the promoter construct. Figure 3.9B demonstrates that increasing amounts of the C466S HA-SuPr-1 point mutant was incapable of relieving transcriptional repression by either Gal4-R1 (aa. 241-452) or Gal4-SMRD (aa. 400-452). Hence, these results imply that transcriptional repression by the SMRD of RBP1 is dependent on conjugation with SUMO-1.

3.7 SUMO-1 modification differentially regulates RBP1 transcriptional activity

The SUMO acceptor lysines K418 and K444 are both required for transcriptional repression by the SMRD upon recruitment to a synthetic promoter by the Gal4 DNA binding domain. To study the repression of the SMRD in the context of full length RBP1, K418R and K444R mutations were both introduced to full length Gal4 tagged RBP1 and the effect of SuPr-1 on transcriptional repression was determined using Luciferase assays. CHO-K1 cells were transfected with full length Gal4-RBP1 or the Gal4-RBP1 K418/444R double mutant and increasing amounts of the HA-SuPr-1 expression vector. Repression activity was assessed using the pG5-TK-Luc reporter as described previously, and the pRL-RSV-Luc co-reporter was included to normalize transfection efficiency. In the presence of the SUMO-1 protease SuPr-1, transcriptional repression by wild type Gal4-RBP1 increased in a dose-dependent manner by about 2 fold with 100ng of DNA expressing HA-SuPr-1 (Figure 3.10). Furthermore, this apparent increase in repression activity was not dependent on the SMRD of RBP1, as repression by the Gal4-RBP1

K418/444R double mutant was enhanced in a similar fashion by SuPr-1. Thus, addition of a SUMO-1 protease increased transcriptional repression by full length Gal4-RBP1 independently of the SMRD.

Since the R2 domain is highly active in the Gal4-RBP1 K418/444R double mutant, R2 mediated transcriptional repression was analyzed in the presence of increasing amounts of the SUMO-1 protease SuPr-1. Figure 3.10 illustrates that SuPr-1 causes an increase (1.5 fold) in Gal4-R2 repression, although the repression in the absence of protease was already very high (88% repression). The Gal4-R2 K1204R construct harboring a mutation in the putative SUMO modification site has a higher ability to repress transcription compared to wild type Gal4-R2, which is consistent with what was seen previously. Addition of SuPr-1 did not significantly increase the repression by the Gal4-R2 K1204R mutant (1.2 fold increase), although the reporter may have been maximally repressed at this point. These results suggested that SUMO-1 modification negatively regulates repression by R2. Interestingly, the increase in Gal4-RBP1 transcriptional repression caused by the SUMO-1 protease relied on the presence of R2. This effect stemmed from the fact that transcriptional repression was relieved in a dose-dependent manner by SuPr-1 when R2 was deleted from Gal4-RBP1, which is consistent with the SMRD requiring modification by SUMO-1 for transcriptional repression (Figure 3.10 last lane). These results implied that sumoylation is involved in RBP1 mediated transcriptional repression and that SUMO-1 modification may have different consequences on the HDAC-dependent and independent modes of repression by RBP1.


A.
Reporter
5xGal4 sites
TK
Luciferase
% Luciferase Activity
120
100
80
60
40
20
0
Gal4
Gal4-R1
Gal4-R1 K,R dbl mt
Gal4-SMRD
:HA-SuPr-1 wt
B.
% Luciferase Activity
120
100
80
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20
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Gal4
Gal4-R1
Gal4-R1 K,R dbl mt
Gal4-SMRD
:C466S mt
C.
73
54
50
HA-SuPr-1
Lane 1 2 3 4

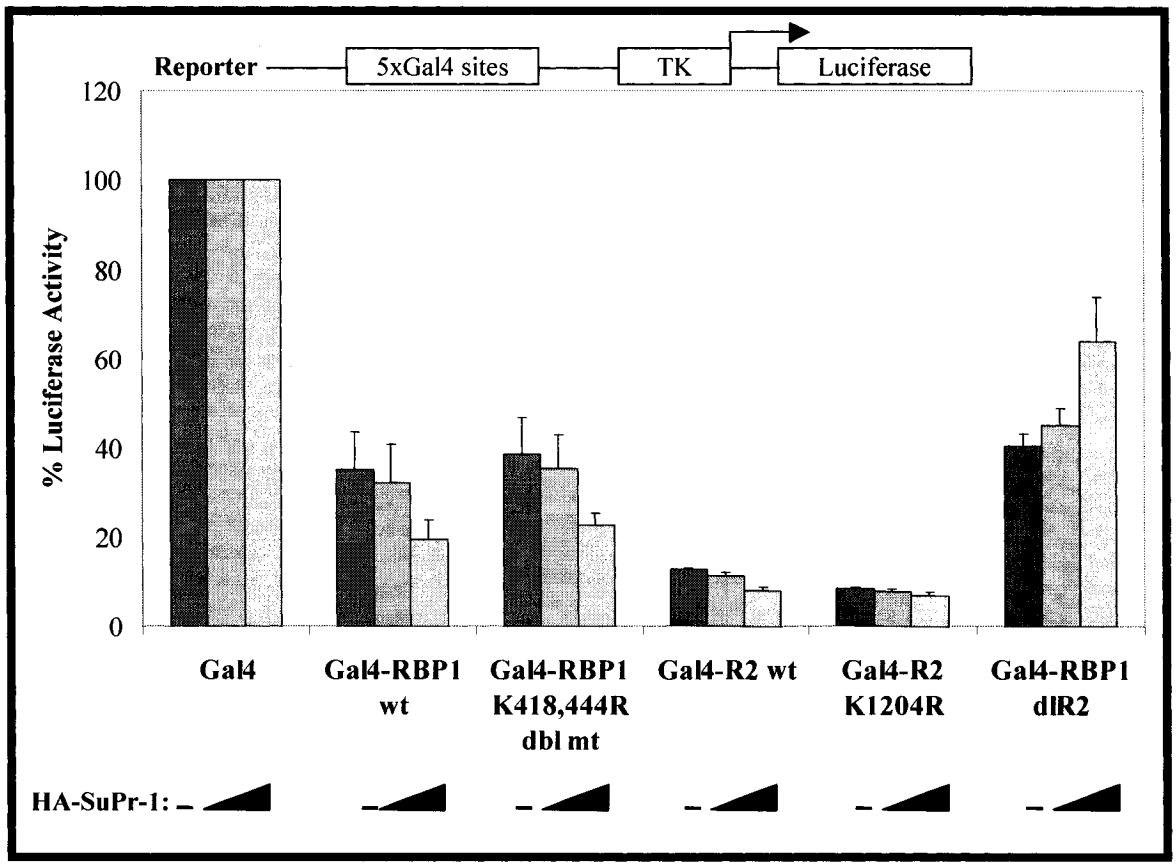

Fig. 3.10. RBP1 transcriptional activity is modulated by sumoylation. CHO-K1 cells were transfected as in Figure 3.9 with increasing amounts of a plasmid expressing HA-SuPr-1 under the control of EFα1 promoter and the indicated Gal4-fusion constructs. The phRL-RSV plasmid was used to normalize transfection efficiency. Cells were harvested 27 hrs post-transfection as before and the Luciferase activity of each sample was measured with the Dual Luciferase Assay System. The normalized Luciferase activity of each sample was compared to Gal4 alone with 0, 10ng or 100ng of the HA-SuPr-1 vector, which were arbitrarily set at 100%. The graph shown is the average of two independent experiments in which samples were performed in duplicate.

Chapter 4: Discussion

Several attempts were made to identify the HDAC-independent mode of repression by RBP1. Preliminary studies on the mechanism of R1 repression indicated that the ARID domain of RBP1 could activate transcription by associating with HAT activity (Lai, unpublished). In contrast, mapping studies performed on the R1 domain demonstrated that the ARID domain actually represses rather than activates transcription of the G5-TK-Luc reporter, which is consistent with the fact that the C-terminal 53 amino acids of R1 are largely responsible for the repression (Figure 3.2A). Interestingly, the reporter used to demonstrate that residues 303 to 452 of RBP1 activate transcription was G5-E1B-CAT, suggesting that the activation by the ARID domain might be promoter specific. Further experiments should be performed to clarify the role, if any, of the ARID domain in the activation of transcription.

The results presented in Figure 3.2A show that repression by R1 is independent of the ARID and maps to amino acids 400 to 452 of RBP1. However, residues 241 to 400 of R1 still retain some residual repression activity relative to Gal4. Although it remains unknown for RBP1, the ARID domain is predicted to bind DNA with a potential preference for A/T rich regions (Wilsker et *al.*, 2002). Therefore, the ARID domain may have some non-specific affinity for the TATA element and thus artificially competes with TBP for promoter occupancy, resulting in a slight decrease in the rate of formation of the pre-initiation complex. However, a point mutation in Tyr 389 of the ARID, one of the five conserved residues among the ARID domains important for DNA binding, failed to eliminate the repression associated with the Gal4-ARID dl859c construct (data not shown).

The activity of many transcriptional repressors is influenced by sumoylation. In fact, many previously characterized repression domains such as the R motif in Elk-1, the SC motif in the C/EBP family of transcription factors and the CRD1 domain of p300 all function in a SUMO-dependent manner (Girwood et *al.*, 2003; Kim et *al.*, 2002; Yang et *al.*, 2003). The minimal region required for R1 repression contains two copies of the consensus sequence required for modification with SUMO and both these residues were shown to be sumoylated *in vivo* by exogenous SUMO (Figure 3.3B and 3.5). Mutagenesis studies revealed a strict correlation between conjugation with SUMO and transcriptional repression by the SMRD of RBP1. In addition, both SUMO acceptor lysines were shown to be required for the full repression activity of the SMRD, suggesting that they function independently from one another (Figure 3.4A). Interestingly, this situation is analogous to the CRD1 repression domain of p300, which relies on two SUMO sites to interact with HDAC6 (Girwood et *al.*, 2003).

These results imply that the SMRD of RBP1 belongs to the novel class of domains that rely on SUMO conjugation to exert their repressive effects. An alignment performed with these repression domains shows that they all contain an N-terminal SUMO site and some also contain an additional SUMO site on the C-terminal side (Figure 3.3C). Interestingly, RBP1 has a large spacer region between the two sites that is not present in most of the other SUMO regulated repression domains suggesting that the two SUMO sites need to be positioned accordingly for function. Domain swap experiments could be performed to determine if the SMRD of RBP1 can functionally replace the Elk-1 R motif or the CRD1 of p300 to block their transactivation potential. In

addition, hypermapping experiments should be initiated to determine if only the sequence encompassing the SUMO sites is sufficient to elicit the repressive effects of the SMRD of RBP1.

Lysine residues can undergo a plethora of post-translational modifications that includes SUMO conjugation (Freiman and Tijan, 2003). The SUMO pathway can be inhibited by several means to show that SUMO modification is necessary for SMRD activity. Transcriptional repression by the SMRD of RBP1 was relieved in a dose-dependent manner in the presence of a SUMO-1 specific protease whereas a catalytically inactive form of the protease failed to impair the activity of the SMRD (Figure 3.9A and B). These results strongly support the idea that mutation of both K418 and K444 within the SMRD leads to a loss of repression due to a lack of SUMO modification. However, the SuPr-1 C466S mutant was expressed under the control of the EFα1 promoter, which is weaker in CHO-K1 cells relative to the CMV promoter (Figure 3.9C). Unfortunately, the catalytic mutant SuPr-1 had not been cloned downstream of the CMV promoter at the time these studies were done. Therefore, it will be important to study the repression by the SMRD with increasing amounts of the CMV driven SuPr-1 catalytically inactive point mutant to eliminate the possibility that the effect seen is due to higher expression. On the other hand, inhibition of the SUMO E2 ligase Ubc9 could also be used to show that the SMRD of RBP1 represses transcription in a SUMO-dependent manner. This determination can be achieved by RNA interference mediated against Ubc9 or by expression of a dominant-negative mutant Ubc9 that lacks the active site cysteine at

position 93 required for protein conjugation with SUMO (Yang et *al.*, 2003; Girwood et *al.*, 2003).

Sumoylation may not be the only post-translational modification that is involved in transcriptional repression by the SMRD of RBP1 because the migration of the double SUMO modified SMRD was higher than that expected (Figure 3.5 and 3.6). Other modifications such as phosphorylation or acetylation may regulate the sumoylation of the SMRD of RBP1. Recently, it was observed that SUMO modification of the heat shock factor HSF-1 only occurs if a nearby serine residue is phosphorylated suggesting that there may be an interplay between SUMO and other post-translational modifications (Hietankangas et *al.*, 2003). Although lysine 433, which is positioned between the two SUMO acceptor sites of the SMRD, had no apparent effect on transcriptional repression in the context of the double K to R mutant, it would be worthwhile to study this mutation alone to determine if it is involved in controlling the SUMO modification of the nearby residues. Whether or not lysine 418 and 444 can also be ubiquitinated has not been investigated to date.

In addition, examination of the primary sequence of RBP1 reveals that there are other potential sumoylation sites other than those in the SMRD. In fact, some of these are close to the LxCxE motif, the chromo domain and there is one site in the putative Tudor domain. One known SUMO substrate that is heavily sumoylated is the PML protein, which contains three sites of sumoylation and these regulate the formation of PML bodies (Muller et *al.*, 1998; Best et *al.*, 2003). The abundance of SUMO consensus sites makes it

attractive to speculate that the slow migration pattern of RBP1 is due to modification with many SUMO molecules and that such modifications regulate important protein-protein interactions necessary during growth arrest. Our group has demonstrated that RBP1 localizes to discrete regions of the nucleus at growth arrest (Lai et *al.*, 2001). Therefore, A GFP tagged RBP1 could be used to determine if SUMO modification, such as in the SMRD, is involved in sub nuclear partitioning. Although the SMRD alone can be modified by exogenous SUMO, it will be imperative to show that this modification also occurs in full length RBP1 and that the endogenous RBP1 can be modified by SUMO.

The SMRD of RBP1 functions as a strong repression motif when brought to a synthetic promoter by a heterologous DNA binding domain (Lai et *al.*, 1999a,b). However, transcriptional repression by the SMRD must also be studied in the context of full length, non-Gal4 tagged RBP1. Surprisingly, expression of a SUMO protease led to an increase in Gal4-RBP1 transcriptional repression and this effect was independent of the SMRD (Figure 3.10). These results suggest that SMRD repression in the context of full length RBP1 would need to be studied in the absence of the R2 domain, which seems to have a dominant effect over the SMRD because deletion of R2 causes the SuPr-1 protease to relieve repression by the SMRD. To address this, the repression by the Gal4-RBP1 double K418, 444R mutant could be monitored in the presence of TSA, which could inhibit the HDAC-dependent repression mediated by the R2 domain of RBP1.

Conversely, the repression of E2F-dependent transcription by pRB and/or RBP1 is only partially sensitive to HDAC inhibitors suggesting that the SMRD is responsible

for this activity (Luo et *al.*, 1998; Lai et *al.*, 1999b). Although RBP1 requires the LxCxE motif to repress E2F-mediated transcription, whether both the R2 and the SMRD are required for the repression of E2F transcription remains to be tested. Also, it has not been established if the SMRD participates in the growth suppressive effects mediated by RBP1. Nonetheless, the results imply that the R2 domain, or members of the mSin3/HDAC1 complex, may actually be negatively regulated by sumoylation (Figure 3.10). Further experiments will need to establish the consequences, if any, of *in vivo* sumoylation on the functions of the R2 domain. Consequently, the data suggest that the RBP1 transcriptional activity may be differentially regulated by SUMO proteases, which could potentially be recruited to RBP1 at different stages of the cell cycle or in response to growth stimulatory signals.

The identification of potential binding proteins could aid in determining the mechanism of transcriptional repression by the SMRD. Affinity chromatography using GST-R1 identified a potential binding partner of approximately 20kDa in size but its identity could not be established by mass spectrometry analysis due to insufficient amounts recovered (Figure 3.1A). This could be explained by several ways. First, the GST-R1 bait may not have been present in adequate levels to compete out the protein or complex bound to the endogenous R1 domain of RBP1 from the nuclear extract. In contrast to the R2 domain of RBP1, which can associate with SAP30 *in vitro*, the R1 domain may not fold properly in bacteria and thus be non-functional. Alternatively, the lack of SUMO modification may explain why R1 could not stably associate with its binding partner. Since SUMO modification is a reversible process, the activity of SUMO

proteases must be inhibited in cell extracts to preserve the SUMO moiety on substrates. Therefore, a similar chromatography experiment could be performed where the nuclear extracts are prepared with NEM to block the activity of endogenous desumoylating enzymes. Also, because the levels of free SUMO-1 in the cell are low, the purified R1 domain could be sumoylated *in vitro* using recombinant Uba2/Aos1, Ubc9, SUMO-1 and ATP prior to incubation with nuclear proteins. SUV39H1 is recruited to the pocket of pRB to repress E2F-dependent transcription (Vandel et *al.*, 2000; Nielsen et *al.*, 2001). Interestingly, while this interaction is LxCxE-dependent, SUV39H1 lacks such a motif suggesting that another protein links it to pRB. Co-immunoprecipitation studies could be performed with the SUMO modified SMRD to determine if RBP1 serves as the adaptor for histone methylase activity associated with pRB. However, the negative effects of the SMRD on transcription may be mediated directly by the SUMO molecule or by the indirect recruitment of Ubc9 to the promoter.

The exact contribution of SUMO in transcriptional repression, once recruited to a promoter, remains elusive. For instance, the Elk-1 R motif, which contains only one copy of the SUMO consensus, blocks the activity of the adjacent transactivation domain (Yang et *al.*, 2003). SUMO conjugation may cause a conformational change in the activation domain that prevents interaction with the basal transcription machinery. RanGAP1 apparently also undergoes a SUMO-dependent conformational change that allows interaction with the nuclear pore because the binding site of RanBP2 does not overlap with the region that contains the SUMO site (Matunis et *al.*, 1998). SUMO modification of the SMRD may allow binding to the basal transcription factors such as TBP thus

preventing formation of the pre-initiation complex. The minimal region required for SMRD repression has a high degree of homology to the region of NZFP that mediates interaction with TBP (Kim et *al.*, 2003). In addition, the region in BCAA that corresponds to the SMRD is capable of repressing a Gal4 controlled reporter construct that is only driven by the TATA box element (Binda, unpublished). Chromatin immunoprecipitation could be performed to determine if the SMRD prevents TBP occupancy or recruitment of TBP-associated factors to the G5-TK-luc reporter. On the other hand, the SMRD may control the DNA binding ability of the adjacent ARID domain of RBP1. Stimulation of p53 DNA binding activity is controlled by acetylation of a lysine residue located near the DNA binding domain while SUMO modification can increase the *in vitro* DNA binding ability of HSF1 and HSF2 (Gu et *al.*, 1997; Goodson et *al.*, 2001; Hong et *al.*, 2001). Experiments involving E2F transcription should help determine if the SMRD truly functions as a repression domain in the context of full length RBP1.

The RBP1 homolog, BCAA, is highly expressed in tumor cells (Cao et *al.*, 1999; Cao et *al.*, 2001; Fleisher et *al.*, 2003). Our group had initially proposed that BCAA may promote tumorigenesis by acting as a dominant negative of RBP1 by squelching the factors responsible for the growth suppressive properties. However, the finding that BCAA and RBP1 associate with the mSin3A complex suggests that they may perform similar functions (Fleisher et *al.*, 2003). In fact, initial results have demonstrated that BCAA and RBP1 synergize to repress E2F-dependent transcription (Binda, unpublished). Key experiments will ultimately show if the R1 of BCAA also represses in a SUMO

dependent manner but the likelihood is high as it contains two copies of the putative SUMO sites that align with RBP1.

Finally, the data presented show that transcriptional repression by the R1 domain of RBP1 functions in a SUMO dependent manner and thus has been renamed Sumo Modified Repression Domain. This motif contains two SUMO acceptor lysines and both are required for transcriptional repression. The SMRD of RBP1 belongs to the growing class of repression motifs that are sumoylated and suggests that RBP1 transcriptional activity can be regulated by post-translational modification by SUMO. How SUMO conjugation causes SMRD-mediated repression at the promoter level remains unknown.

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