EUR. J. ONCOL.; Vol. 20, n. 2, pp. 100-108, 2015

Acetylation of retinoblastoma-like protein-2 (Rbl2/p130): an additional check on cell proliferation?

Muhammad Saeed
Department of Biosciences, COMSATS Institute of Information Technology (CIIT), Islamabad, Pakistan

Summary. Pocket proteins are important regulators of cell cycle progression. The family includes three members, namely: retinoblastoma protein (pRb/p105), the founding member of the family, retinoblastoma-like protein-1 (Rbl1/p107) and retinoblastoma-like protein-2 (Rbl2/p130). Pocket proteins exert their role by binding with members of the E2F family of transcription proteins, thus making them unavailable for the gene transcription required for entry into the S-phase of the cell cycle. The functional activity of pocket proteins is regulated by phosphorylation. Once phosphorylated, the Rb-E2F complex is disrupted. The abrogation of Rb functions leads to unchecked G1-S phase transition and uncontrolled cell division. The ultimate impact is tumor formation. Recently, we have demonstrated that Rbl2/p130 is also subject to posttranslational acetylation. The exact role of Rbl2/p130 acetylation in cell cycle control and tumor genesis remains elusive. This review discusses acetylation being a prerequisite for phosphorylation and investigates a possible role of Rbl2/p130 acetylation in regulating the G1-S phase of the cell cycle.

Key words: acetylation, retinoblastoma, Rbl2/p130

«Acetilazione della proteina-2 retinoblastoma simile (Rbl2/p130): un ulteriore controllo della proliferazione cellulare?»

Riassunto. La proteine 'pocket' del retinoblastoma, sono importanti regolatori della progressione del ciclo cellulare. Questa famiglia include 3 membri: proteina del retinoblastoma (pRb/p105), membro fondatore della famiglia, la proteina-1 retinoblastoma simile (Rbl1/p107) e la proteina retinoblastoma silmile (Rbl2/p130). Le proteine pocket esercitano il loro ruolo legandosi ai componenti della famiglia E2F delle proteine di trascrizione, rendendole non disponibili per la trascrizione genetica richiesta per entrare nellafase S del ciclo cellulare. La funzionalità delle proteine pocket è regolata dalla fosforilazione. Una volta fosforilate, il complesso Rb-E2F viene distrutto. L'abrogazione della funzionalità di Rb, comporta una non corretta transizione della fase G1-S e una non controllata divisione cellulare. Il risultato finale è quindi la formazione del tumore. Recentemente, abbiamo dimostrato che Rbl2/p130 è anche soggetto ad acetilazione post translazionale. Il ruolo esatto della acetilazione di Rbl2/p130 nel controllo del ciclo cellulare e nella genesi del tumore rimane elusiva. In questo articolo discutiamo l'acetilazione come prerequisito per la fosforilazione e indaghiamo il possibile ruolo della acetilazione di Rbl2/p130 nella regolazione della fase G1-S del ciclo cellulare.

Parole chiave: acetilazione, retinoblastoma, Rbl2/p130

The retinoblastoma protein family

A human pediatric retinal tumor (retinoblastoma) occurs sporadically or inheritably in children. A biallelic mutation in the retinoblastoma (Rb) gene was found to occur in the neoplasm (1, 2). Identification and subsequent cloning of the Rb gene opened a new avenue in cancer research and soon the Rb gene was reported to be mutated in many other forms of human cancers (2, 3).

Shortly after the discovery of the tumor suppressor Rb gene, two other proteins sharing the characteristic structural and functional properties of the retinoblastoma protein (pRb) were identified (1-4). These proteins were named Rbl1/p107 and Rbl2/p130 and all three members of the family were collectively referred to as "pocket proteins". The term "pocket proteins" derives from the conserved pocket domain (1, 4, 5) present in all three members of the Rb family through which they bind to viral onco-proteins (1, 3, 6) and cellular factors such as the E2F family of transcription factors (1, 6, 7).

The members of the pocket protein family show homology in their domain structure. An A-domain is separated from a B-domain by a spacer region (Figure 1). Rbl1/p107 and Rbl2/p130 are more related to each other than to the founding member of the family (4,5). All three members are localized in the nucleus specifically during G_0/G_1, whereas in the nucleolus they are found in the S-phase (4,6). Claudio et al. (2002) reviewed some features of the Rb family that are similar to other housekeeping genes, such as the lack of a canonical TATA or CAAT box found in the promoters of most differentially expressed genes, the presence of a GC-rich region immediately surrounding the main transcription initiation site, the presence of multiple consensus sequences for binding the Sp1 transcription factor, and the presence of multiple transcription start sites (4).

Members of the Rb family exert their functions as negative regulators of cell cycle progression (1, 7–11) through interaction with members of the E2F family of transcription factors, thereby arresting the transcription at their regulated promoters. Members of the E2F transcription factors can act as transcriptional activators (E2F1, E2F2, E2F3a) as well as repressors (E2F3b, E2F4-E2F8) (1, 2, 6, 7).

Although the members of the Rb family are described as tumor suppressor proteins, they have physiological roles in development, differentiation, apoptosis, and angiogenesis (1, 4-7, 12, 13). Genome-wide searches, employing chromatin immunoprecipitation (ChIP) and microarray techniques for E2F targets, have identified many genes that are indirectly regulated by the Rb family of proteins. These include genes involved in DNA repair, mitochondrial biogenesis and metabolism (14, 15). Rb family proteins control G_1/S transition by two mechanisms: first, the members of the Rb protein family bind to the E2F family of transcription factors and stop the transcription of genes required for S-phase entrance (1, 4-7, 12, 16); second, they recruit chromatin remodeling factors to keep chromatin in a repressed state (1, 2, 9, 12, 15, 17, 18). Members of the Rb family show genetic redundancy and functional compensation in regulating different cellular processes, which makes it difficult to study the

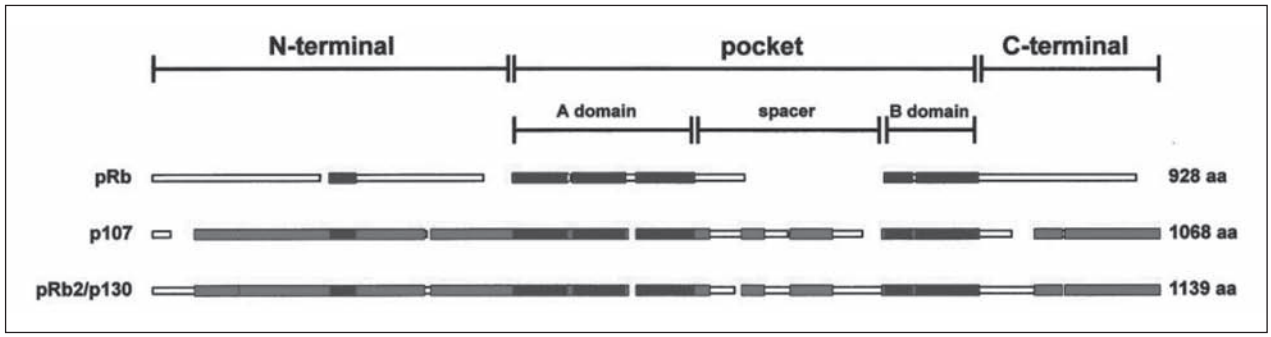

Figure 1. Schematic representation of structural homology among members of the pocket protein family. Blue boxes represent the regions of homology among all three members, whereas red boxes indicate regions shared only between Rbl1/p107 and Rbl2/p130. Adapted from Paggi and Giordano, 2001 (5).

effects of deficiency on the part of one member of the pocket protein family (1-4, 19).

Rb family members are post-translationally regulated proteins. Phosphorylation of Rb family members leads to dissociation of Rb/E2F complexes and inactivation of Rb proteins. Rb family proteins remain in this inactive state until the passage through mitosis, after which they are re-engaged through the action of a phosphatase (20). Phosphorylation of Rb family proteins by cyclin/CDK complexes takes place in a cell cycle dependent manner (1,3). The retinoblastoma protein (pRb) has also been reported to be acetylated during differentiation (21, 22). The exact functions of pRb acetylation in the cell cycle remain unclear.

Despite the similarities between pRb, Rbl1/p107, and Rbl2/p130, it has been shown -in single-, double-, and triple-knockout mouse models as well as in tissue culture studies - that individual family member do also have unique functions in cell cycle regulation and differentiation (1, 3-7). Members of the Rb family show different expression profiles during different stages of the cell cycle (3,6). pRb is expressed steadily through the cell cycle. Rbl2/p130 is highly expressed in quiescent and differentiated cells, and its levels drop when quiescent cells are stimulated to enter the cell cycle. On the other hand, Rbl1/p107 is expressed at lower levels in quiescent cells and its levels rise when cells enter the S-phase (Figure 2).

Members of the Rb family show preferences in binding to E2F transcription factors. pRb predominantly interacts with E2F1-4 whereas Rbl1/p107 and Rbl2/p130 primarily interact with E2F4 and E2F5 (1, 3, 7, 18) (Figure 3). These interactions also take place at distinct time points during the cell cycle. pRb binds to E2F in both quiescent and cycling cells, whereas Rbl2/p130 binds to E2Fs only during G_1. However, Rbl1/p107 interacts with E2Fs during the S-phase of the cell cycle (3).

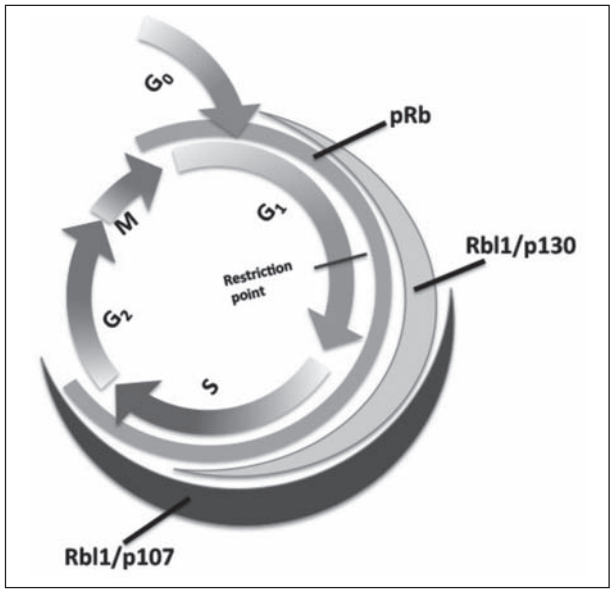

Figure 2. Expression patterns of pocket proteins. pRb (blue) is expressed steadily during different stages of the cell cycle. Rbl2/p130 (orange) expression is high in arrested cells, whereas Rbl1/p107 (purple) expression is high during the S- phase of the cell cycle.

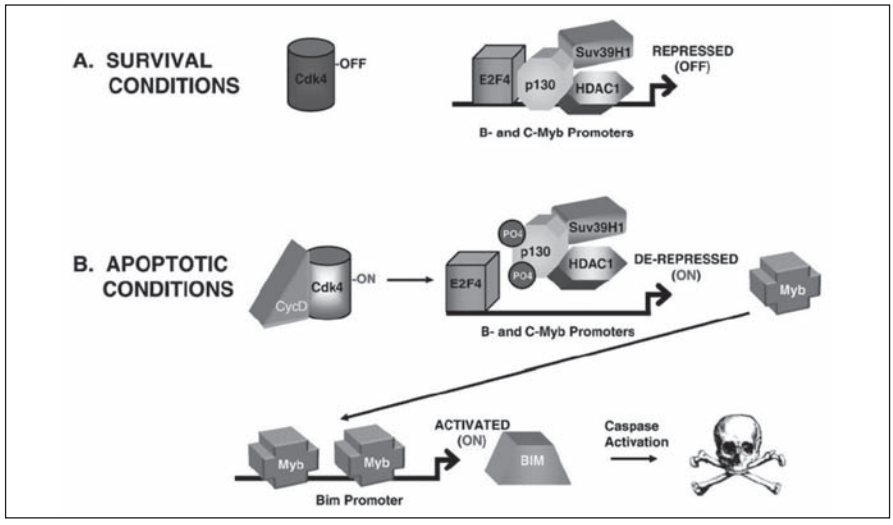

Figure 3. Scheme depicting the molecular pathway of neuron cell death. (A) Under survival conditions, Rbl2/p130 forms complexes with E2F4 and recruits chromatin modifiers to repress transcription. (B) Under apoptotic conditions, hyper-phosphorylation of Rbl2/p130 leads to de-repression of transcription and elevated levels of b- and c-myb, which then induce the transcription of BH3-only protein Bim. Bim promotes caspase activation and apoptosis. Adapted from Greene et al., 2007 (9).

Again, Rbl1/p107 and Rbl2/p130 (but not pRb) bind and inhibit cyclin E/CDK2 and cyclin A/CDK2 kinases (1, 3, 23). This activity is mechanistically similar to that of the CIP/KIP family of CDK inhibitors (CKI) (1, 23).

Retinoblastoma-like protein-2 (Rbl2/p130)

Cytogenetically, p130 maps to human chromosomal area 16q12.2 (2, 4, 5, 24), an area repeatedly altered in human cancers (2, 5). The Rbl2 gene consists of 22 exons, which are spread over 50 kb of genomic DNA; the mRNA is 4.6 kb in length (4, 24). Human Rbl2/p130 has 1139 amino acids with a molecular weight of 130,000. Mouse Rbl2/p130 has 1135 amino acids and is ~91% homologous to human Rbl2/p130. The Rbl2/p130 promoter shows the characteristic structural organization of "housekeeping" and growth control-related genes; a typical TATA or CAAT box is missing, but several GC-rich zones and potential binding sites for numerous transcription factors are present (24).

Rbl2/p130 shows structural similarity with other members of the retinoblastoma family, the greatest homology occurring in the bipartite pocket region of the protein (6). The spacer region in Rbl2/p130 and Rbl1/p107, which separates the A and B subdomains of the pocket, is longer than the spacer region in pRb (4, 6). This region enables them to form stable complexes with cyclin A-CDK2 and cyclin E- CDK2 (4, 6, 23) leading to the inhibition of CDK2-associated kinase activity (16, 25). It has been reported that Rbl2/p130 is required to inhibit CDK2 and thus to prevent S-phase entry in mitogen deprived p27-/-fibroblasts (1). Through this inhibition, Rbl2/p130 also regulates proteolytic degradation of p27 through cyclin E-CDK2 dependent phosphorylation (26). In addition to the spacer region, Rbl2/p130 and Rbl1/p107 share an extended region of homology in the amino terminal, which is missing in pRb (6); this region is identified as a CDK inhibitory domain (16).

Rbl2/p130 is a nuclear phosphoprotein like other members of the pocket protein family. A mutation in the C-terminus of the protein was reported to lead to an altered localization of the protein (27). Two independent nuclear localization signals (NLS) can be identified in the C-terminus of Rbl2/p130, whereas an additional nuclear localization signal was mapped in the unique loop region (935KRKRR939) of Rbl2/p130 (28). Rbl2/p130 is capable of shuttling between nucleus and cytoplasm, while an N-terminal leucine-rich region was identified for cytoplasmic localization of Rbl2/p130 (28).

Rbl2/p130 has been shown to block the cell cycle, whereas pRb overexpression sometimes fails to do so. The human cervix carcinoma cell line, C33A, is inhibited by Rbl2/p130 overexpression, but not by pRb (29). Similarly, the T98G human glioblastoma cell line is sensitive to the growth-responsive effects of Rbl2/p130, which was resistant to the growth-suppressive effects of pRb and p107 (30). Moreover, in HONE-1 (a nasopharyngeal carcinoma derived cell line), a drastic reduction in Rbl2/p130 expression levels has been reported, while expression levels of other Rb family members remained consistently elevated (30). On the other hand, Saos-2 human osteosarcoma cells were growth-arrested in G_0/G_1 of the cell cycle by all three members of the retinoblastoma family (4, 30). In the light of these observations, it has been proposed that the growth-suppressive properties of retinoblastoma family proteins are cell type specific. Due to the presence of E2F4-p130 complexes in abundance in quiescent cells, some authors have proposed E2F4-p130 complexes as a marker of the G0 phase of the cell cycle (4, 31).

The phenotypes of the knockout of Rbl2/p130 are dependent on the genetic background in which they are analyzed. $p130^{-/-}$ mice from a pure C57BL/6J or mixed 129/Sv:C57BL/6J genetic background develop normally and exhibit no obvious adult phenotype (3, 6, 19), whereas on a Balb/cJ background they show an embryonic lethal phenotype (19, 32). Mice lacking Rbl2/p130 and Rbl1/p107 are born with shortened limbs and rib bones and they die shortly after birth, due to an apparent failure to respire (6, 19). This suggests that Rbl1/p107 and Rbl2/p130 have essential but overlapping functions during development and differentiation. Inactivation of pRb in $p130^{-/-}$ mice leads to *retinoblastoma*, whereas mice deficient in both pRb and Rbl2/p130 show embryonic lethality (6, 19).

Rbl2/p130 has been shown to play a role in regulation of neuron survival and death (9, 33). Inappro-

priate activation of CDK4 in neurons leads to the hyper-phosphorylation of Rbl2/p130 and dissociation of Rbl2/p130 and associated chromatin modifiers Suv39H1 and HDAC-1 from transcription factor E2F4 (23). This results in de-repression and transcription of genes encoding for b- and c-myb. Elevated levels of b- and c-myb in neurons induce transcription of the pro-apoptotic BH3-only protein Bim (Figure 4). Bim then interacts with apoptotic machinery, leading to caspase activation and apoptotic death (9).

Is Rbl2/p130 a tumor suppressor?

pRb, the leading member of the pocket protein family, is a bona fide tumor suppressor which complies with all the requirements of a tumor suppressor protein. Several reports show it to be deleted or mutationally inactivated in several malignancies (2, 5, 10); moreover, exogenous expression of pRb in cancer cells has also reverted the neoplastic phenotype (1, 2).

There is plentiful evidence to support the role of Rbl2/p130 as a tumor suppressor protein. Rbl2/p130 down-regulates the cell cycle and has also been shown to block the cell cycle (30). The expression levels of Rbl2/p130 have been shown to inversely correlate with cancer malignancies by immuno-histochemical analysis in endometrial carcinoma, oral squamous carcinoma and uveal melanoma (5, 30, 34). Rbl2/p130 has been found mutated in several human neoplasias including breast, hepatic, ovarian and prostatic cancer (4, 5, 30). Insertion of wild type Rbl2/p130 in H23 human lung carcinoma via retroviral delivery inhibits tumorigenesis (35).

On the other hand, observations that question the position of Rbl2/p130 as a tumor suppressor include the phenotypically normal development of p130-/- mice (3, 6, 19), whereas Rb knock-out mice in the same genetic background die before day 16 of embryonic development (10, 19). Moreover, p130-/- mice do not show any predisposition to develop cancer (3, 6, 19).

In view of these facts, it is clear that Rbl2/p130 gives a major contribution to tumor progression, whereas its role in tumor initiation remains elusive.

Post-translational modifications of Rbl2/p130

Like other members of the pocket protein family, Rbl2/p130 is phosphorylated in a cell cycle dependent manner (36,37). Rbl2/p130 has three apparent phosphorylation states (38). These phosphorylation states are the result of different CDKs (39). *In vivo* phosphorylation mapping of human Rbl2/p130 has identified 22 serine and threonine residues (Figure 5), which are targets of cyclin A/CDK2, cyclin D/CDK4, cyclin D/CDK6 and non- CDK kinases (40,41). Moreover, it has been observed that Rbl2/p130 is also phosphorylated in the unique loop within the B-domain during G_0 by glycogen synthase kinase 3 (GSK3) (42). Phosphorylation of Rbl2/p130 by GSK3 contributes to the stability of the protein but does not affect the interaction with E2F4 or cyclins (42).

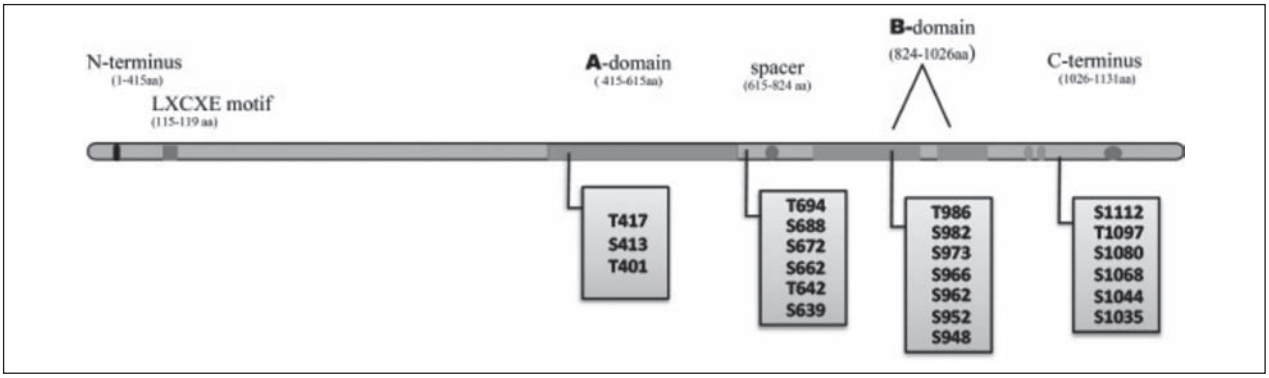

Figure 4. Schematic summary of the location of 22 serine and threonin residues mapped by in vivo phosphorylation assays of Rbl2/p130 (39).

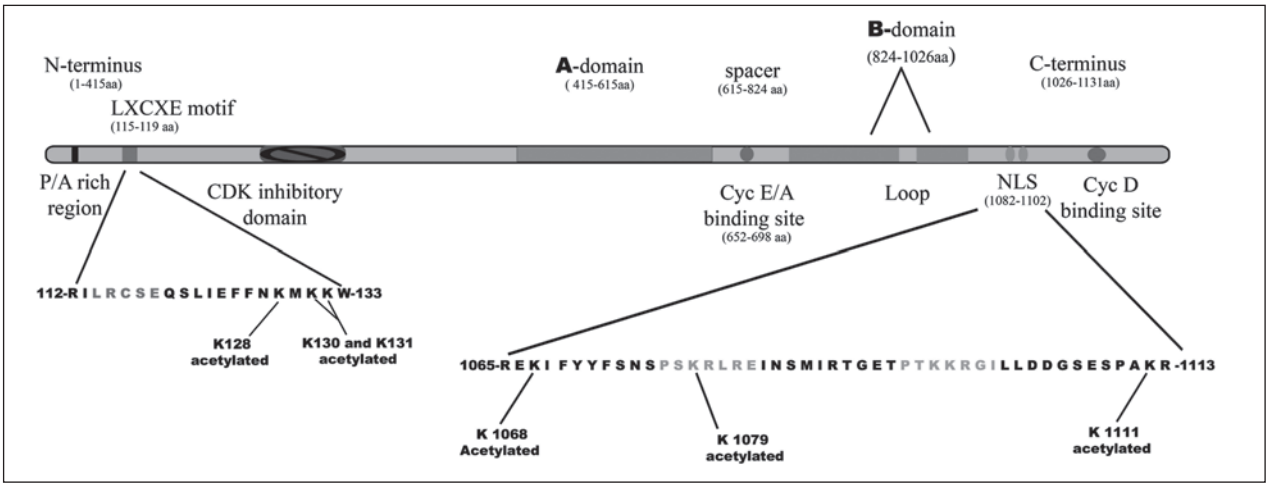

Figure 5. A map of Rbl2/p130 protein showing functionally important domains of the protein, as well as different acetylatable lysine residues.

The hyper-phosphorylated form of Rbl2/p130 is the target of protea- somal degradation through ubiquitination by SCF(Skp2) (43). Degradation of Rbl2/p130 consequently leads to progression through the cell cycle. It has also been observed that protein phosphatase 2A (PP2A) specifically interacts with Rbl2/p130 in the C-terminus of the protein and subsequently dephosphorylates it, whereas inhibition of PP2A by chemical inhibitors resulted in hyper-phosphorylation of Rbl2/p130 (39, 44, 45).

Recently, it was reported with conclusive evidence that Rbl2/p130 is post-translationally acetylated in a cell cycle dependent manner in NIH3T3 cells (46). It was also shown in similar experiments that acetylated and hyperphosphorylated Rbl2/p130 forms were exclusively located in the nucleus from G1 to G2 phase of cell cycle (46). The presence of both modified (acetylated and phosphorylated) forms of Rbl2/p130 during cell cycle emphasizes their mutual interdependence. Moreover, using mass spectrometric analysis of *in vitro* acetylated Rbl2/p130, the same authors identified K1079 in the C-terminus as the major acetylatable lysine residue, whereas K1068 and K1111 were identified as minor acetylation sites. pRb has also been reported to be acetylated mainly in the C-terminal part of the protein (21) . Higher acetylation within the C-terminus of pRb and Rbl2/p130 indicates the functional significance of the domain within the members of retinoblastoma family proteins; likewise the C-terminus and the pocket domain show the greatest homology among members of the pocket proteins (6). Minor acetylation sites were also found in the N-terminal region of Rbl2/p130 at K128, K130 and K131.

It should also be mentioned here that the C-terminus of other important regulatory proteins acting in transcriptional control and cell cycle regulation contains multiple amino acid residues that are subject to posttranslational modifications by otherwise chromatin-modifying enzymes, e. g. p53 (47).

In vitro experimental data presented more recently support the idea that phosphorylation and acetylation of Rbl2/p130 are mutually interdependent (48). It was shown that deacetylated Rb2/p130 is not phosphorylated by CDK4, which raises the possibility that acetylation may be a prerequisite for phosphorylation. The finding that only dephosphorylated Rb2/ p130 is acetylated by p300, while phosphorylation by CDK4 abolishes its acetylation potential, also supported this assumption.

Further evidence to support acetylation as being a prerequisite for phosphorylation includes mutational experiments, where previously identified lysine residues were either changed to arginine (R; which represents the unmodified form of lysine with respect to charge in the lysine side chain) or glutamine (Q;

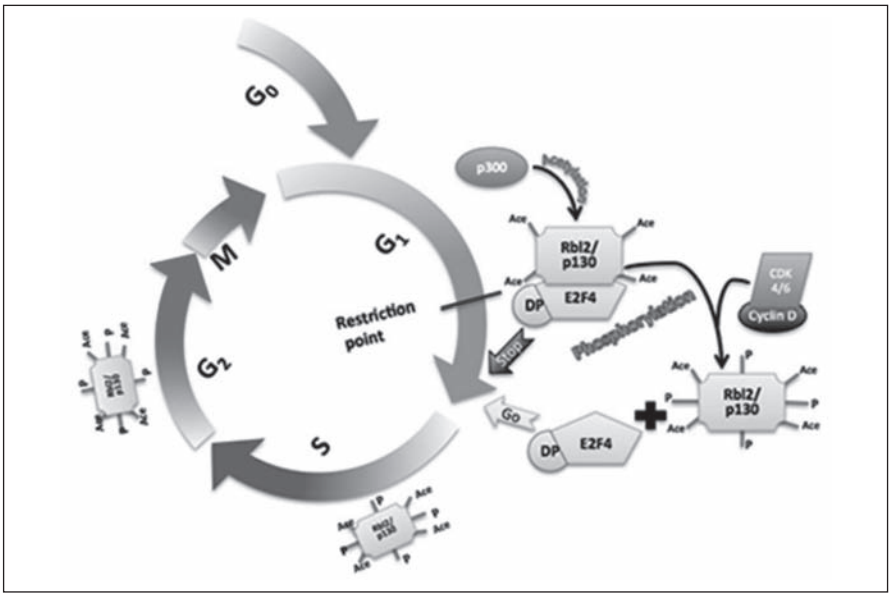

Figure 6. Inter-relation between Rbl2/p130 acetylation and its phosphorylation. Recent evidence indicates that Rbl2/p130 acetylation precedes phosphorylation, and this phenomenon controls an important checkpoint during G1/S transition.

which by contrast mimics the acetylated state of lysine with respect to charge neutralization in the lysine side chain). The 'R' mutants showed strong inhibition of phosphorylation by CDK4; while 'Q' mutants resulted in much less pronounced inhibition of phosphorylation (48).

As discussed earlier, Rbl2/p130 is phosphorylated by CDKs, which predisposes it for ubiquitination-mediated proteosomal degradation (18). It has already been postulated that Rbl2/p130 acetylation might stabilize the protein by protecting it from ubiquitination and subsequent degradation. This also corroborates the finding that hyper-phosphorylated, acetylated forms of Rbl2/p130 persist until into the G2 phase of the cell cycle (46). Despite all this information on Rbl2/p130 acetylation, its role in regulating the cell cycle remained elusive. It is worth mentioning in this context that expression of a mutated form of Rbl2/p130 under a conditional promoter in NIH3T3 cells (lysine1079 mutated to arginine) severely perturbed cell cycle progression, as investigated by FACS analysis; expression of Rb2/p130 mutated at position 1068 (lysine replaced by arginine) did not have any effect on cell cycle progression (Saeed and Loidl, unpublished), supporting a functional role of lysine 1079 acetylation *in vivo*.

Conclusion and future prospects

Despite the fact that Rbl2/p130 is acetylated in a cell cycle dependent manner, the fundamental question how this acetylation affects cell cycle progression remains unanswered. Figure 6 illustrates a possible role for Rb2/p130 acetylation in regulating the cell cycle. As has already been discussed above, losing the ability of Rbl2/p130 to be acetylated also abrogates its phosphorylation potential (48); this suggests an additional regulatory mechanism for controlling the cell cycle. Moreover, this also raises the possibility of a functional role of Rbl2/p130 acetylation in tumor development. *In silico* docking experiments performed on predicted 3-D models of Rbl2/p130 C-terminus with CDK4/cyclin-D complexes has also shown that K1079 is involved in this steric association, and changing this key lysine residue resulted in abrogation of this complex formation (Nadia and Saeed; unpublished data).

Quite recently we showed some preliminary results to the effect that Rbl2/p130 is more highly acetylated in tumor tissues than normal diseased free tissues (49). This finding not only suggests a significant association of Rb2/p130 acetylation with uncontrolled growth (tumor development), but also corroborates our earlier finding that acetylation is a prerequisite for

phosphorylation of Rb2/p130. The discovery of Rbl2/p130 acetylation in tumor tissues has presented a new therapeutic target for disease management and opens a new avenue in cancer research. Thorough studies are required to further elucidate the role of Rbl2/p130 acetylation in tumor development and cancer progression.

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Received: 12.2.2015
Accepted: 19.5.2015
Address: Muhammad Saeed, PhD
Department of Biosciences,
COMSATS Institute of Information Technology (CIIT),
Park Road Chak Shahzad, 44000 Islamabad, Pakistan
E-mail: muhammad.saeed@comsats.edu.pk