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The Retinoblastoma Family Protein p130 as a Negative Regulator of Cell Growth and Tumor Progression

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1. Introduction

In the last years, the large amount of genomic sequences obtained after the decodification of the human genome, has made clearer the differences in the patterns of gene expression among the distinct tumor types and the equivalent normal tissues. The identification of a considerable number of differentially expressed gene products has shortened, in some measure, the bridge between correlative and causative data. Correlative genes are genes simply altered as a result of the process of transformation, and they are not responsible of critical effects upon tumor formation. In contrast, causative genes represent the basis of the malignant transformation. They play a decisive role to origin and maintain the transformed state and could be exploited for therapeutic strategies. Oncogenes and tumor suppressor genes are the most important causative genes and for this reason represent critical targets for new anticancer drug development.

Tumorigenesis proceeds through the accumulation of genetic mutations and epigenetic alterations consenting cells to break free from the tight network of controls set to regulate the homeostatic balance between cell proliferation and cell death (Baylin and Herman, 2000; Hanahan and Weinberg, 2000; Knudson, 2001; Herceg and Hainaut, 2007). The elucidation of the human genome sequence, together with the development of novel experimental techniques, has allowed the identification of genetic alterations in tumors in unprecedented details. The genetic events can be associated with the gain and loss of entire chromosomes, specific chromosomal translocations, gene amplifications, deletions or point mutations (Knudson, 1997). In addition to genetic changes, the important results obtained recently on how chromatin-remodeling enzymes controls gene transcription have underscored the

crucial role of epigenetic mechanisms in the initiation and the development of cancer. Epigenetic events, such as modifications of DNA methylation patterns, and changes of chromatin structure have emerged as key mechanisms in malignant transformation (Fearon, 1997; Jones & Baylin, 2002; Baylin, 2005; Boehm & Hahn, 2011). Genetic and epigenetic events can conduct to the gain of oncogenes functions or to the loss of tumor suppressor genes (TSGs) functions, contributing to the acquired features of transformed phenotype. They represent two complementary mechanisms that are implicated in every step of carcinogenesis, from the responses to carcinogen exposures to the progression into malignancy. Autonomous cellular proliferation, immortalization, deficiencies in differentiation, induction of angiogenesis, propensity for invasion, resistance to apoptosis, induction and increased genomic instability are common characteristics of cancer cells. It has become increasingly evident that cancer is fundamentally a disease of failure of regulation of tissue growth; generally, changes in many genes are required to transform a normal cell into a cancer cell. TSGs are a family of genes that promote negative regulation on cancer cell growth inhibiting cell division and survival. Proto-oncogenes are normal genes that could become oncogenes due to mutations or to increased expression and they are able to stimulate cell proliferation and exert positive regulation of cell growth. Therefore, alterations of tumor suppressors and proto-oncogenes that may occur if the genomic integrity is compromised by intrinsic factors or exogenous agents, represent a crucial step in the transformation of a normal cell into a cancer cell (Knudson, 1985; Levine & Puzio-Kuter, 2010; Croce, 2008; Heeg, et al., 2006).

The RB1 gene represents a typical TSG, first identified in a malignant tumor of the retina known as retinoblastoma. When both the alleles of this gene are mutated, the protein (pRB) is inactivated causing the development of retinoblastoma (Knudson, 1971; Murphree & Benedict, 1984; Friend et al., 1986; Fung, et al., 1987; Lee et al., 1987a, 1987b). Retinoblastoma develops in early childhood, typically before the age of 5, and it has one of the highest cure rates of all childhood cancers, with more than 95% of patients surviving into adulthood. Retinoblastoma is a rare type of eye cancerous tumor that develops in the retina's cells. There are two forms of the disease: a heritable and a non-heritable form. In most children with retinoblastoma, the disease affects only one eye (unilateral retinoblastoma), however, one out of three children with retinoblastoma develops cancer in both eyes (bilateral retinoblastoma). Unilateral retinoblastoma represents a sporadic disease, as there is no family history for this cancer, whereas bilateral retinoblastoma represents the hereditary form and it is an autosomal dominant disease. The most common first symptom of retinoblastoma is an abnormal appearance of the pupil called "leukocoria" or "cat's eye reflex", which is a white reflection in the pupil. Other symptoms of retinoblastoma include red and irritated eyes, crossed eyes or strabismus.

In the early 1970s, Knudson postulated a model, referred to as the 'Two-hit hypothesis', with the main goal of clarifying the distinction between the two forms of retinoblastoma. A patient with inherited retinoblastoma, has a first insult already inherited in his/her own DNA, any second insult would lead to cancer, whereas a patient with non-inherited retinoblastoma, must undergo two "hits" before a tumor could develop. The identification of the retinoblastoma gene occurred in 1987 and fully confirmed Knudson's interpretation (Knudson, 1971; Lee et al., 1987a, 1987b). Indirectly, Knudson's work led to the identification of cancer-related genes and so far represents a milestone in carcinogenesis. As discussed previously, the development of cancer depends on multiple "hits" to the DNA, leading to

both the activation of proto-oncogenes and the deactivation of TSGs. The activation/inactivation mechanisms of TSGs and proto-oncogenes are distinctive. Genetic changes can occur at different levels and by different mechanisms. TSGs are inactivated by "loss of function mutations" on the contrary, proto-oncogenes are activated through "gain of function mutations". In cancer cells, tumor suppressors are not functionally working and they lose the ability to control over cell proliferation. Oncogenes, instead, are constitutively activated, leading to continuous signaling which acts positively on cell growth. Unlike oncogenes, TSGs generally follow the 'two-hit hypothesis', which indicates that, before a particular outcome is manifested, both alleles of a specific gene must be affected because if only one is damaged the second can still produce the correct protein. The characteristic mechanism of this activation/inactivation phenomena means that when the cancer is promoted by the inactivation of a TSG, both the alleles of this TSG are usually inactivated whereas, when the cancer is mediated by oncogenes, the mutation of a single copy of the proto-oncogene is sufficient to activate itself, leading to cell transformation. In other words, mutant tumor suppressor's alleles are usually recessive, whereas mutant oncogene alleles are typically dominant.

pRB and the related proteins, p107 and p130, are TSGs and form the retinoblastoma (Rb) gene family. The three members of the Rb gene family have been the focus of great interest, because of their pivotal role as negative regulators of cell cycle progression. Together these proteins are also known as "pocket proteins". The term pocket protein derives from their highly conserved region, the pocket domain, which mediates interaction with viral oncoproteins as well as cellular proteins to exert the biological functions of these proteins (Graña, 1998; Cobrinik, 2005). Several examples of these interactions involving transcription factors as well as enzymes are listed in Table 1. p107 and p130 share homologies throughout the entire length of the protein, whereas their homology with pRB is limited to the conserved A and B domains. The genes are located on different chromosomes and the expression of the proteins is differently regulated throughout the cell cycle (Lee et al., 1987a; Hong et al., 1989; Yeung et al., 1993; Mayol et al., 1993; Paggi et al., 1996; Ichimura et al., 2000). They interact with different E2F proteins, thereby blocking different subsets of gene promoters, but have in common that this interaction is regulated through phosphorylation by cyclin-dependent kinases (cdks) (Hurford et al., 1997; Classon et al., 2000; Stiegler & Giordano, 2001; Sun, 2007). In fact, all the Rb family members exert their function interfering, between the others, with the coordinated regulation of the enzymatic activity of cdks, which are key regulatory factors of the cell cycle progression (Graña & Reddy, 1995; Morgan, 1995 & 1997). The cdks and their heterodimeric cyclin partners represents prime targets for the development of new inhibitors and anticancer therapeutic strategies. During the last decades, several chemical compounds with remarkable cdk inhibitory activity have been described. These molecules are starting to become a significant therapeutic asset in the treatment of cancer. Among the small molecules, peptides, with a comparable cdk inhibitory activity, are emerging as a novel class of drugs for cancer therapy. Cdk2 is considered the prototypic cell cycle kinase. It represents an excellent runner in the development of anticancer therapeutics not only because of its crucial role to pass through the G1 restriction checkpoint and to drive cells into DNA replication but also because its alteration is a pathogenic hallmark of tumorigenesis (McDonald & El-Deiry, 2000; Fischer, 2004; Whittaker et al., 2004; Dai & Grant, 2004; Shapiro, 2006; de Cárcer et al., 2007; Malumbres & Barbacid, 2009; Cirillo, et al., 2011). p130 together with p107 has the ability to inhibit the kinase activity

Rb family protein	Protein partner	Biological function of the protein partner	Biological role of the Rb family protein
pRB	Cyclin D	CDK subunit	Cell cycle
	E2Fs	Transcription factors	Cell cycle
	c-Jun	Transcription factor	Cell cycle
	c-Myc	Transcription factor	Cell cycle
	Spl	Transcription factor	Cell cycle
	Abl	Nuclear tyrosine kinase	Cell cycle
	Che-1	Transcription factor	Cell cycle
	Id-2	Transcription factor-corepressor	Cell cycle
	MCM7	DNA replication licensing factor	Inhibition of DNA replication
	RBAp48	Histone deacetylase complex factor	Growth inhibition
	TAFII250/TFII D	Transcription factor	Transcription
	HDAC1	Histone deacetylase	Transcription
	BRG1	Transcription factor	Transcription
	MyoD	Transcription factor	Muscle differentiation
	HBP1	Transcription factor	Muscle differentiation
	p202	Transcription factor	Muscle differentiation
	NF-IL6	Transcription factor	Adipocyte differentiation
p130	Cyclins A and E	CDK subunits	Cell cycle
	E2Fs	Transcription factors	Cell cycle
	MCM7	DNA replication licensing factor	Inhibition of DNA replication
	HDAC1	Histone deacetylase	Transcription
	HBP1	Transcription factor	Muscle differentiation
p107	Cyclins A and E	CDK subunits	Cell cycle
	E2Fs	Transcription factors	Cell cycle
	c-Myc	Transcription factor	Cell cycle
	Spl	Transcription factor	Cell cycle
	MCM7	DNA replication licensing factor	Inhibition of DNA replication
	HDAC1	Histone deacetylase	Transcription
	MyoD	Transcription factor	Muscle differentiation

Table 1. The biological roles of the Rb family proteins are mainly dependent on their ability to interact and modulate the activities of cellular proteins

of the cdk2/cyclins A and cdk2/cyclins E complexes (Adams, 1996; Woo, 1997; Lacy, 1997; De Luca, 1997). Specifically, p107 is able to inhibit their kinase activity recruiting or mimicking a cyclin-dependent kinase inhibitor (CKI) p21 (Zhu et al., 1995; Adams, 1996). Whereas, p130 is able to physically bind to the Cdk2/Cyclins A and Cdk2/Cyclin E complexes suggesting that part of its growth suppressor function could be mediated by the inhibition of this essential cell cycle kinase. The inhibitory activity of p130 has been attributed to the spacer region (De Luca, 1997). Recently, a 39 amino acid long p130 spacer-derived peptide termed "Spa310" has been identified as responsible of the cdk2-dependent kinase inhibitory activity proving to be an excellent candidate in a mechanism-based approach in cancer therapy (Bagella, 2007; Giordano, 2007a, 2007b).

2. p130, Rb family proteins and LXCXE-like motif

The p130 protein, together with p105 and p107, is a member of the Rb family of tumor suppressors. The three members of this family share high degree of homology and biological functions (Lee et al., 1987a; Ewen et al., 1991; Mayol et al., 1993; Li et al., 1993; Paggi et al., 1996; Mayol & Graña, 1997; Nevins, 1998). All of them are characterized by two highly conserved functional domains termed A and B, which are separated by a spacer region, which differs between all the three Rb family members. They are also called "pocket proteins" because the two domains, A and B, are assembled into a pocket-like structure for the presence of the spacer region (figure 1) (Graña, 1998; Cobrinik, 2005; Du & Pogoriler, 2006; Macaluso et al., 2006; Sun et al., 2007). The pocket domain sequence of all the three pocket members is well known for its ability to interact with proteins containing LXCXE motifs (Lee et al., 1998; Dahiya et al., 2000). The LXCXE domain is composed by a small block of highly conserved amino-acid residues counting the sequence leucine-X-cysteine-X-glutamate, where the letter 'X' indicates any amino acids. A large selection of proteins containing an LXCXE-like sequence is able to interact with the Rb family proteins.

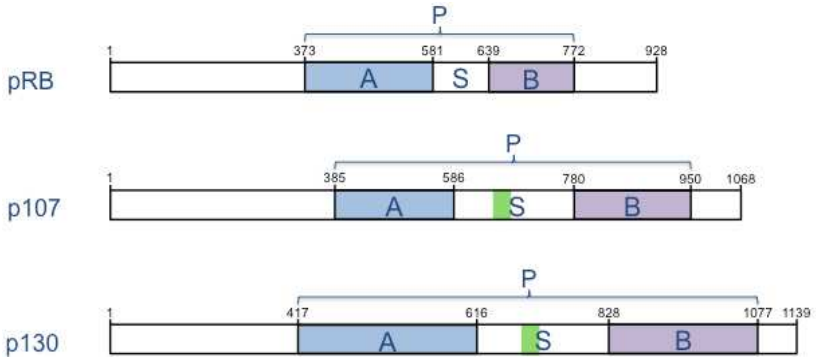

Fig. 1. Schematic diagram of the amino acid sequences of the retinoblastoma family proteins highlighting the relative locations of functional domains within each member (N-terminus to the right, C-terminus to the left). The retinoblastoma family consists of pRb, p107 and p130. P indicates the pocket domain, responsible for most protein–protein interactions, composed by two conserved domains A and B, separated by the spacer region S. The green box specifies the conserved sequence motif, between p107 and p130, responsible for binding the Cdk/Cyclin complexes.

The DNA virus oncoprotein, E1A (the early-region 1A of the human adenovirus type 5), was identified by coimmunoprecipitation with pRB. E1A contains an LXCXE motif that is responsible for this interaction (Whyte, et al. 1989; Nielsch et al., 1991; Rumpf et al., 1999). The pRB pocket domain has been co-crystallized with an LXCXE peptide, allowing localization of the LXCXE binding site on the inside of its B domain sequence (Lee et al., 1998). Also, the other members of the Rb family, p107 and p130, are able to bind E1A through a similar mechanism (Herrmann et al., 1991; Putzer et al., 1997; Lee et al., 2002; Xiao et al., 2003). Together with adenovirus E1A, other DNA virus oncoproteins such as human papillomavirus (HPV) E7 and Simian virus 40 large T antigen, contain LXCXE-like sequences which are used to bind to the Rb family proteins inhibiting their functions and promoting cell transformation and consequently cancer development (Hu et al., 1990; Ciccolini et al., 1994; Jones et al. 1997; Dahiya et al., 2000; Caldeira et al. 2000; Münger et al., 2001; Helt & Galloway, 2003; Caracciolo et al., 2006; Felsani et al., 2006). Moreover, an LXCXE-like motif was also found in several cellular proteins such as, histone deacetylases 1 and 2 (HDAC1 and HDAC2), protein phosphatase 1 (PP1), breast cancer type 1 (BRCA1), and Brahma-Related Gene 1 (BRG1), interacting with Rb family proteins, are involved in their pathways and play important roles for their functions. (Dunaief et al., 1994; Fan et al., 2001; Rayman et al., 2002; Dunaief et al., 2002). The Rb family proteins are essential regulators of the cell cycle. They play a crucial role during the cell cycle, primarily through their ability to bind members of the E2F family and to block the activation of genes involved in cell cycle progression (Moberg et al., 1996; Sidle et al., 1996; Stiegler & Giordano, 1999; Macaluso et al., 2006; Sun et al., 2007). The E2F family members play a major role during the G1/S cell cycle transition. Based on their functions they can be divided in two distinctive groups: transcription activators and repressors (figure 2). The E2F(1-3a) members are activators and promote and help carry out the cell cycle, while the E2F(3b-8) factors are repressors and inhibit the cell cycle. The E2F(1-6) proteins bind to DNA as heterodimers, in association with the dimerization partner DP1 or DP2, increasing the E2F binding stability (Johnson et al., 1993; Zheng et al., 1999; Gaubatz et al., 2000; Cobrinik, 2005; Chen et al., 2009). Although the E2F factors are able to bind the Rb family proteins, they do not possess any LXCXE domains, suggesting that most of them should have a different pocket protein-binding domain. This observation was confirmed in studies focused on mutational analysis. In these studies, the mutation of the LXCXE binding site did not prevent pRB from binding and inactivating the E2F factors, whereas, these mutations inhibited the interactions with HDAC1 and HDAC2. Indeed, as described previously, both HDAC1 and HDAC2 contain an LXCXE-like sequence, and deletions of regions of the proteins containing this sequence preclude their binding to pocket proteins (Dunaief et al., 1994; Magnaghi-Jaulin et al., 1998; Ferreira et al., 1998; Fan et al., 2001). Thus, the LXCXE binding site mutations consent to distinct the ability to bind the E2F factors from the ability to efficiently recruit HDAC1 and HDAC2, suggesting that inhibition of the E2F activity alone is not sufficient to sustain actively repress transcription and consequently cell growth arrest (Dahiya et al., 2000). Therefore, it would seem that effective growth suppression by pocket proteins requires not only the interaction with the E2F factors, but also the recruitment of HDAC1 and HDAC2, providing evidence that the LXCXE binding site is important for their efficient function. Further studies underscored that other chromatin remodeling enzymes such as BRG1 and Brahma (BRM), that are components of the human SWI–SNF nucleosome-remodeling complex, are able to cooperate with the pocket protein-related cell growth suppression (Dunaief et al., 1994; Ferreira et al., 1998; Brehm et al. 1998; Ross et al., 1999; Zhang et al.,

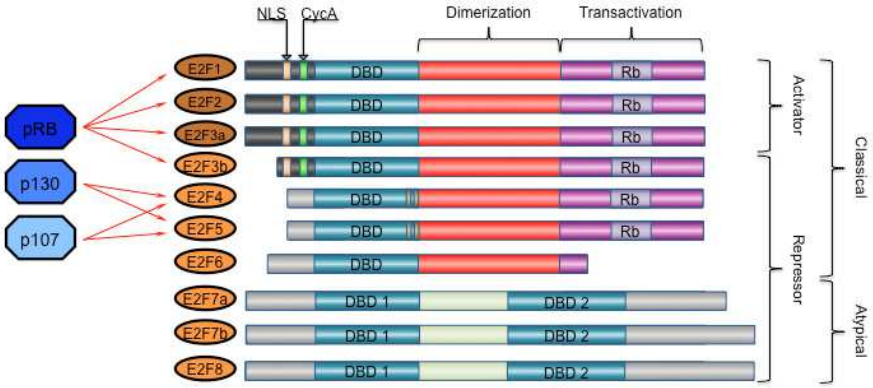

Fig. 2. Structural organization of E2F transcription factors and their interactions with Rb family proteins. E2Fs can be subdivided into activator factors: E2F1, E2F2, E2F3a and repressor factors: E2F3b, E2F4, E2F5, E2F6, E2F7a, E2F7b and E2F8. They can also be divided in Classical and Atypical E2Fs. The most peculiar differences between Classical E2Fs, and Atypical E2Fs are shown: Classical E2Fs (E2F1-6), bind to DNA only after coupling with a second protein, called dimerization partner protein (DP). Through their dimerization domain, they form heterodimers with DP1 and DP2 proteins to allow the binding to DNA. Atypical E2Fs, E2F(7-8), show a duplicated DNA binding domain (DBD) that allow to bind to DNA in a DP-independent manner (as a homodimer). For a review about this class of E2F proteins see: Lammens et al., 2009. The classical E2Fs have also a transactivation domain that contains the Rb family proteins binding motif (Rb). pRB preferentially binds to the activator factors E2F1, E2F2, and E2F3a and the repressor factor E2F3b. p107 and p130 preferentially bind to the repressor factors E2F4 and E2F5. E2F(6-8) do not bind to pocket proteins. NLS and CycA indicate the nuclear localization signal and the Cyclin A binding motif respectively.

2000; Kadam & Emerson, 2003). Several reports showed that active repression mediated by p130 and pRB could involve a molecular mechanism by which condensed chromatin structure is enhanced not only through histone deacetylation but also through methylation. Macaluso and colleagues proposed multimolecular complexes bound to the estrogen receptor-α (ER-α) in breast cancer containing the histone methyl transferase (SUV39H1) and the DNA-(cytosine-5) methyltransferase 1 (*DNMT1)* together with p130-E2F4(5) and HDAC1 suggesting a novel link between p130 and chromatin-modifying enzymes in the transcriptional regulation of the ER-α gene.

In addition, other studies demonstrated that Polycomb group (PcG) proteins, another class of remodel chromatin proteins, interact with Rb family proteins and that these associations are important links between the transcriptional repression activities related to the pocket proteins and polycomb pathways (Dahiya et al. 2001; Bracken et al. 2003; Kotake et al., 2007; Tonini et al., 2004 & 2008). Although the large number of observations underline how the transcriptional repression's mechanisms of the Rb family members have been extensively investigated, so far the contribution of the chromatin remodeling enzymes and pocket proteins to physically repress responsive promoters in G0 and early G1 is still debated, and represents an important unresolved piece of this issue.

3. p130, hypho/hyperphoshorilation and regulation of E2F-responsive genes

One key mechanism controlling the G0/G1 checkpoint is the phosphorylation of the Rb family proteins by the cdk/cyclin complexes. The cdks are serine and threonine kinases and encompass a family divided into two groups based on their roles in cell cycle progression and transcriptional regulation (Lees, 1995; Morgan, 1995 & 1997; Napolitano et al., 2002; Shapiro, 2006). By definition, the cdks are dependent on associations with their activating subunits, termed cyclins for their cyclical expression and degradation. All the Rb family proteins contain several serine or threonine residues that can be recognized and phosphorylated by the cdk/cyclin complexes (Sidle et al., 1996; Mayol & Grana, 1998). In the hypophosphorylated state, the Rb family proteins are active and carry out their role as tumor suppressors binding and inhibiting, as previously described, transcription factors of the E2F family during the G0/G1 phase of the cell cycle. When it is time for a cell to enter the S phase, the cdk/cyclin complexes phosphorylate the pocket proteins, inhibiting their activity. For instance, increased phosphorylation of pRB decreases the affinity to E2F1 that dissociate from the pocket protein and becomes active allowing the progression of the cell cycle. The phosphorylation state of p130 occurs in all the mechanisms of growth regulation associated with this protein, this event is obviously cell cycle regulated as p130 has been shown to be a substrate for the cdk/cyclin complexes (Baldi et al., 1995; Canhoto et al., 2000; Hansen et al., 2001). In comparison to the other Rb family members, the p130 expression levels change during the cell cycle; in fact p130 is the most abundant in the G0 phase (Kiess et al., 1995) and differs from the others also in its phosphorylated status. Indeed, it has been described that p130 undergoes phosphorylation at distinctive sites during the G0 phase in a way that characterizes p130 from the other members of the Rb family proteins (Kiess et al., 1995; Canhoto et al., 2000). p130 is phosphorylated by the Cdk4/Cyclin D or Cdk6/Cyclin D and Cdk2/Cyclin E or Cdk2/Cyclin A complexes and its expression levels fall when the cells enter into the S phase (Baldi et al., 1995; Mayol et al., 1995; Claudio et al., 1996; Dong et al., 1998; Tedesco et al., 2002). *In vivo* phosphorylation mapping of human p130 identified 22 serine and threonine residues, targeted by the kinases Cdk2, Cdk4 and Cdk6 (Hansen et al., 2001). These residues can be divided into four groups. The first group is positioned between the end of the N-terminal region and the beginning of the A domain. It consists of three residues; one is common to all the three Rb family members, one is shared with p107, and the last one is unique to p130. The second group contains six residues that are located in the spacer region; three out of six are unique to p130, the rest are common to p107. The third group is located within the B domain and contains seven residues; six out of seven are unique to p130, one is shared with p107. Finally, the last group is situated in the C-terminal region and contains six residues; two are common to all the three proteins, two are shared with p107 and the remaining two are unique to p130. In total, three out of 22 residues share homology with all the three Rb family members; ten are common to p107, while, twelve are apparently unique to p130 (figure 3). The carboxy-terminal region of p130 is important in coordinating the function of the whole protein. The C-terminus differs in length and similarity to the one of pRB, while it is very comparable to the p107's, considering that, as already extensively mentioned, they are more strictly related to each other. Indeed, the C-terminus of p130 and p107 contains in addition to HADC-1 (Stiegler et al., 1998) and cdk/cyclin complex binding domain (Hansen et al., 2001), independent nuclear localization signals (NLS) that could target reporter proteins to the nucleus (Chestukhin et al., 2002). Hypophosphorylated p130 interacts with the E2F4, and E2F5 transcriptional factors, forming

Fig. 3. Schematic summary of the 22 serine or threonine amino acids, identified by in vivo phosphorylation assays of p130, which are targets for Cdk2/Cyclin A(B) and Cdk4(6)/Cyclin D. A, B and S refer to p130 domains. The red square and the yellow triangle indicate the serine and the threonine residues respectively.

the p130/E2F4(5) repressor complexes. E2F4 and E2F5 are considered poor transcriptional activators due, in part, to their lack of a NLS. The dependence of cellular localization of these E2F transcription factors suggests that p130 may be involved in nucleocytoplasmic trafficking. An accumulation of the p130/E2F4(5) complexes have been shown when cells are quiescent or differentiating, whereas the ability of p130 to bind E2F4(5) is inhibited when cells are entering late G1/S phase of the cell cycle suggesting that the involvement of these complexes is critical during the G0/G1 phase (Dimova and Dyson, 2005). Indeed, E2F4 and E2F5 are expressed throughout the cell cycle, but they are more present in G0/G1 phase, when they can be associated and recruited to the nucleus by p130 in order to form transcriptional repressor complexes (Chestukhin et al., 2002). The p130/E2F4(5) complexes exert their repressive action recruiting to their promoters binding site, the chromatin modulating factors HDAC1, resulting in the removal of acetyl groups from the histones H3 and H4 and generating a compacted chromatin structure that is refractory to the transcription initiation (Smith et al., 1996; Iavarone & Massague, 1999; Takahashi et al., 2000; Ferreira et al., 1998 & 2001; Rayman et al., 2002). A schematic representation of the repressive action of the p130/E2F4(5) complexes is illustrated in figure 4. As showed by several scientific publications, the largest part of the E2F-responsive promoters bound E2F-4 and p130 or in alternative p107, whereas only a limited set of promoters show evidently, an interaction of the pRB/E2F(1–3) complexes (Liu et al., 2005).

In addition, it has been demonstrated that these interactions occur at very low concentration levels (Wells et al., 2000; Takahashi et al., 2000; Morrison et al., 2002; Rayman et al., 2002). Among these, the binding of pRB/E2F(1–3) to the E2F-responsive promoter of Cyclin E represent an important example (Hurford et al., 1997; Le Cam et al., 1999; Polanowska et al., 2001).

As previously indicated, the Cdk4(6)/Cyclin D and Cdk2/cyclin E(A) complexes have been involved in the phosphorylation of all the Rb family proteins (Weinberg, 1995). Although phosphorylation of the Rb family members is very often overturned by dephosphorylation, in particular circumstances, phosphorylation leads to a non-reversible inactivation. Phosphorylation of p130 starts most probably through its C-terminus and leads to the release of HDAC1 binding to the protein (Stiegler et al., 1998; Harbour et al., 1999). The following hyperphosphorylation displaces E2F4(5) from the p130 repressor complexes, leading to the release of the E2F4(5) transcription factors from p130. Unbound E2F4(5) can now migrate to the cytoplasm, while the E2F(1–3) factors are able to bind and activate their responsive promoters. It has been shown that in certain conditions, the E2F(1–3) factors bind to different promoter regions from those made vacant by E2F4(5) (Araki et al., 2003; Zhu et al., 2004). For many promoters, the binding of E2F(1–3) restore histone acetylation by the recruitment of histone acetyltransferases (HATs), which produce a more relaxed chromatin

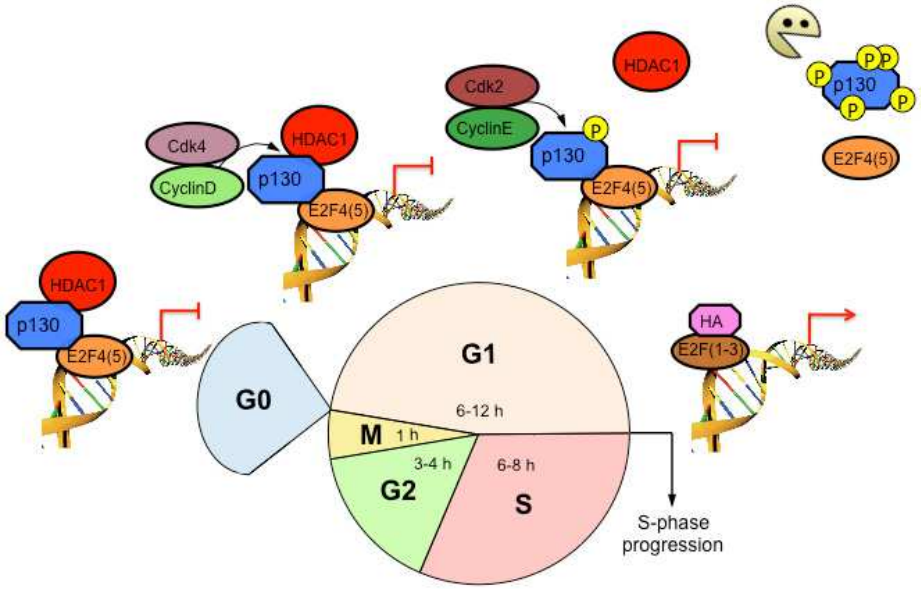

Fig. 4. A simplified view of p130/E2F4(5) complexes at E2F-responsive promoters. For simplicity several cofactors of the complexes (DP proteins or chromatin modifying enzyme) have been ignored. During G0 and early G1, p130 in complex with E2F4(5) is located at E2F-responsive promoters and exert its repressive action recruiting the chromatin modulating factors HDAC1. Phosphorylation of p130 leads to the disruption of the complexes, and E2F-responsive promoters can be activated by E2F(1-3)/HA. Hyperphosphorylated p130 can be degraded through the ubiquitin proteasomal pathway. For more detailed explanations return to text.

state that makes it accessible directly to the transcription factors and allows the cells to proliferate (Ferreira et al., 1998; Takahashi et al., 2000; Taubert et al., 2004). The hyperphosphorylation of p130 leads to its degradation through the ubiquitin proteasomal pathway (Ludlow et al., 1993; Mayol & Grana, 1997 & 1998; Smith et al., 1998; Vuocolo et al., 2003). The ubiquitination of p130 is followed by proteasomal degradation in late G1, which rapidly decreases the expression level of the protein when cells enter in S phase (Tedesco et al., 2002). Thus, p130 is removed when the cells are stimulated to enter a proliferative status confirming that its main relevant function is to arrest cells to G0 phase and to sustain them in this phase when the cells are in a quiescent status or begin to differentiate.

4. p130, cell growth arrest and tumor suppression

One of the most important key factors involved in the origin of a malignant cellular phenotype is the TSGs inactivation. As previously discussed, pRB, p107 and p130, in addition to their similar structural characteristics, share parallel biological functions. The abilities of inhibiting E2F-responsive promoters, recruiting chromatin-remodeling enzymes and actively repressing transcription (Classon & Dyson, 2001; Burkhart & Sage, 2008) confirms that these proteins show extensive overlapping functions and compensatory effects

at cell cycle level. Indeed, fibroblasts lacking one of the three pocket proteins are still able to sustain growth arrest in G0/G1 phase, but, on the other hand, fibroblasts lacking all of the three Rb family proteins lose this biological function (Sage et al., 2000; Dannenberg et al., 2000). Notwithstanding all the Rb family proteins show redundant actions *in vitro*, they clearly have distinct functions in a number of cell types *in vivo*. pRB-deficient mice die during the period of middle gestation showing a large number of anomalies in neural and hematopoietic development, however, $p107^{-/-}$ and $p130^{-/-}$ mice show neonatal lethality with reduced limb and defective chondrocyte growth and endochondral ossification (Clarke et al., 1992; Cobrinik et al., 1996). Given that p107/p130-deficient mice have a normal development during gestation in comparison to $RB^{-/-}$ mice, is reasonable to believe that pocket protein functions cannot be considered completely compensatory (Zhu et al., 1993; Claudio et al., 1994). In addition, other studies highlight elevated proliferation, apoptosis and defective differentiation in liver, brain, muscle, eye, skin, and placenta of pRB-deficient mouse embryos (Liu et al., 2004), whereas p130-/p107-deficient mouse embryos display defects in a different and limited set of tissues (Ruiz et al., 2003; Vanderluit et al., 2004). Although it is possible to speculate with all the statements considered so far, the main reason why the three pocket proteins show redundant effect in some cases whereas, in other cases they lose these compensatory functions is poorly understood. Certainly, given the large spectrum of cells and tissue that have been analyzed, it cannot be excluded that these compensatory effects are more often cell type dependent, but, on the other hand, since p130 owns strict similarity with p107 both in structure and biological functions, it is reasonable to consider that p130 shows a major compensatory effect with p107 in comparison to pRB. For instance, p130-deficient T lymphocytes exhibit normal proliferation *in vitro* and normal cell-mediated immune function *in vivo*, but they show high levels of p107, which is able to replace p130 interacting with E2F4(5) and to form p107/E2F4(5) repressor complexes (Mulligan et al., 1998). Instead, a recent finding demonstrates that p107 and p130 have distinct biological functions to regulate pulmonary epithelial proliferation and survival. In murine models with conditional pocket proteins-deficient lung epithelium, p107 cooperates with pRB to suppress proliferation, however, p130, not being involved in cell growth arrest, exerts a pro-apoptotic function (Simpson et al., 2009). These clinical investigations confirm, as just described above, that although the three proteins share many structural features and are able to work as negative regulators of cell proliferation, they are not temporally and functionally redundant. The inactivation of p130 function can be owed by genetic or epigenetic mechanisms or by the interaction with viral oncoproteins. Numerous melanomas for instance, contain deletion in the chromosomal region (16q12.2) where p130 gene is encoded (Yeung et al., 1993). It has been demonstrated by numerous studies that ectopic expression of human p130 in many human cancer cell lines led to a cell cycle arrest in G1 phase of the cell cycle. For instance, the overexpression of p130 is able to arrest in G1 phase the human T98G glioblastoma cell line, whereas the same cell line does not respond with a G1 arrest after overexpression of the other two members of the Rb family (Claudio et al., 1994). This result is further evidence that the biological functions of the three Rb family proteins are not totally compensatory. The nasopharyngeal HONE-1, cell line displays a strong reduction in the expression level of p130, suggesting a possible involvement of this protein in nasopharyngeal carcinogenesis. Constitutive expression of p130 causes a considerable reduction in HONE-1 cell proliferation and significant changes in cellular morphology (Claudio et al., 1994; Claudio et al., 2000a).

Furthermore, retrovirus-mediated delivery of wild-type p130 shows growth arrest and tumor progression reduction in a lung tumor cell line, H23, and in xeno-transplanted nude mice respectively (Claudio et al., 2000b). The p130 tumor suppressor gene is functionally inactivated in a broad range of cancers. Inactivation of its biological function has been described in different gynecological malignancies. Frequent loss of heterozygosity (LOH) to chromosome 16q12.2, where p130 maps, have been described in ovarian cancer. A large study on ovarian carcinomas displays a drop of the expression level of p130 by 40% and this result correlates inversely with tumor grade (D'Andrilli et al., 2004). In breast cancer, a similar study highlights a reduction of p130 expression level, more recurrent in lobular than in ductal carcinomas, which significantly correlates with estrogen receptor and progesterone receptor-B (Milde-Langosch et al., 2001). Furthermore, p130, in a complex with chromatin-modifying enzymes, takes part in the transcriptional regulation of the ER-α modifying histone acetylation and DNA methylation pattern (Macaluso et al., 2003). A p130 involvement has been also suggested in lung tumor. Low expression level of p130 has been reported in small cell lung cancer (SCLC) and this result inversely correlates with histologic grade, proliferation, and patient survival (Baldi et al., 1996; Helin et al., 1997; Caputi et al., 2002; Cinti et al., 2005). An explanation of p130 deregulation in lung cancer has been recently proposed. CTCF, a chromatin insulator CCCTC-binding factor, is involved in the transcriptional activity of p130 in lung fibroblasts, whereas, in lung cancer cells, a paralog of CTCF, BORIS, impairs the activity of CTCF to control p130 gene transcription (Fiorentino et al., 2011). Furthermore, a conditional triple-knockout murine model able to remove p130, pRB, and p53 in lung epithelial cells, pointed out that loss of p130 leads to a significant increment of cell proliferation and small cell lung cancer (SCLC) development (Schaffer et al., 2010). A deregulation of the p130 biological function has been shown in numerous hematological malignancies. For instance, in AIDS-related non-Hodgkin's lymphomas, an unusual high expression level of p130 has been detected, and, it was found interacting with the HIV-1 Tat protein resulting in deregulation of its tumor suppressor function (Lazzi et al., 2002). Mutations of p130 gene, involving the putative NLS, have been detected in Burkitt's lymphoma cell lines and primary tumors (Cinti et al., 2000). Interestingly, ectopic expression of p130 in the same cell lines recovers growth control (De Falco et al., 2007). Inactivation of the biological function of p130 has also been described in other malignant transformation, such as mesothelioma (Mutti et al., 1998), and nasopharyngeal carcinomas (NPC) (Claudio et al., 1994; Claudio et al., 2000a). Furthermore, an involvement of p130 has been also suggested in retinoblastoma (Bellan et al., 2002).

5. p130, Cdk2 inhibition and Spa310

As extensively previously described the mammalian cell cycle requires the coordinated expression of a family of serine/threonine protein kinases (cdks) that are activated at specific points of the cell cycle by the interaction with their regulatory subunits, cyclins (Graña & Reddy, 1995; Morgan, 1995 & 1997). The active cdk/cyclin complexes phosphorylate target proteins on cdk consensus sites, resulting in changes of their structure that are physiologically crucial for cell cycle progression. Alongside the cdk/cyclin complexes, a family of proteins that exerts cdk inhibitory activity is vital for cell cycle regulation. These proteins called cyclin-dependent kinases inhibitors (CKI) bind to the cdk alone or to the cdk/cyclin complex and regulate the cdk activity. This class of proteins consists of two groups: the INK4 and Cip/Kip proteins. The INK4 members include p15,

p16, p18, p19, which specifically inactivate Cdk4 and Cdk6. They form stable complexes with the two kinases alone before their association with cyclin D (Cánepa, et al., 2007). The second class of inhibitors, the Cip/Kip proteins, includes p21, p27 and p57. Their inhibitory actions occur through the interaction and inactivation of all the G1-cdk/cyclin complexes (Besson et al., 2008). In summary, these CKIs can indirectly inhibit the E2F-mediated transcription through the interaction and inhibition of cdk/cyclin complexes that, maintaining the Rb family proteins in a hypophosphorylated state, allow them to sequester the E2F transcription factors (figure 5). Notwithstanding the wide variety of functions of the pocket proteins is E2F-responsive genes dependent, p130, as well as p107, is able to suppress cell growth through its interaction with two significant cell cycle complexes mentioned

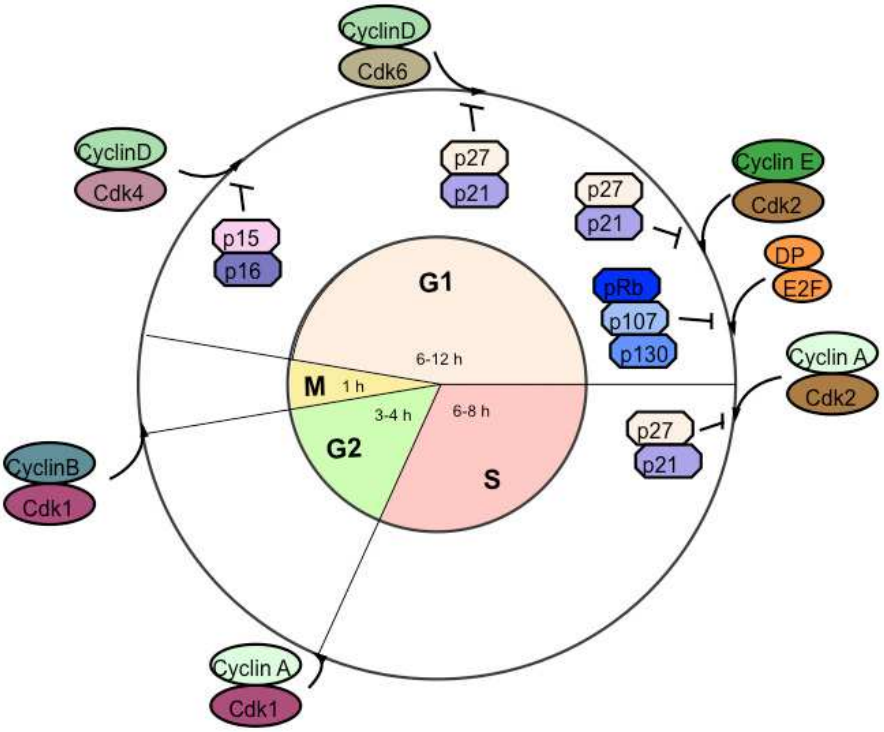

Fig. 5. A schematic representation of the main cdk/cyclin complexes involved in cell cycle control. The passage through the four phases of the cell cycle is regulated by the activities of cdks controlled by the synthesis of the appropriate cyclins during a specific phase of the cell cycle. Cell cycle inhibitory proteins, called cyclin-dependent kinase Inhibitors (CKI), can counteract cdk activity. P15, p16, p21 and p27 represent the main CKIs that specifically prevent accumulated G1-Cdk/Cyclins from acting. The G1-Cdk/Cyclin complexes [(Cdk4(6)/Cyclin D and Cdk2/Cyclin E(A)] control the G1/S checkpoint by the phosphorylation of a variety of proteins. The Rb family proteins represent one key target. Their phosphorylation prevents the binding and inactivation of the E2F transcription factors. The activation of E2Fs allows the transcription of various gene products that are indispensable to trigger S phase.

above, Cdk2/Cyclin A and Cdk2/Cyclin E (Zhu et al., 1995; Lacy & Whyte, 1997). To date, only p130 and p107 are able to bind and inhibit Cdk2/Cyclin A and Cdk2/Cyclin E through independent E2F mechanisms. Certainty, Cyclin E expression is essentially regulated by pRB, as results in pRB deficient cells where Cyclin E levels increase with the parallel disappearance of this protein, but, as mentioned previously, the role of pRB in the regulation of cyclin E occurs through crucial E2F-responsive genes (Le Cam et al., 1999; Polanowska et al., 2001). Inhibition of Cdk2/Cyclin A(E) activity by p130 underlines the fundamental role of p130 during the cell cycle, which is not only the maintenance of a G0 arrest in quiescent or differentiated cells, but also the fact that this protein can exert a control during the transition from G1- to S-phase. This inhibition halts the cells in G1 phase preventing their passing beyond the restriction point G1/S. In order for the cells to progress through G1 phase, p130 as well as all the other related proteins p107 and pRB must be phosphorylated and therefore inactivated by Cdk2/Cyclin A(E) (also by Cdk4(6)/Cyclin D). In this regard, repression of this enzymatic kinase activity by p130 might represent a decisive step to inhibit progression into S-phase. This inhibition can be considered similar to the one performed by the CKI family, and, in certain situations, can work redundantly in support of these proteins. It has been also shown that in p27-/- fibroblasts, an inhibition of Cck2 activity occurs after interaction with p130, which prevents S phase entry. This result confirms that p130, although is not related to the CKI p27, takes its place for the cyclin-dependent kinase inhibition, restoring physiologically cdk regulation (Coats et al., 1999). Moreover, it is hypothesized that the inhibition of the Cdk2 activity by p130 is the result of a direct interaction between specific sequences in the structural domains of this protein and of the kinase. Previously, it has been described that the cdk2-dependent kinase inhibitory activity shown by the pRb2/p130 is specifically confined to the p130 spacer region (De Luca et al., 1997). A recent study identified a polypeptide termed Spa310, which is mainly based on the p130 spacer region. Spa310 consist of 39 amino acids, spanning the p130 spacer region between the 641 and 679 residues (Bagella, 2007; Giordano, 2007a, 2007b). *In vitro* studies confirmed that Spa310 is able to significantly inhibit cdk2-dependent histone phosphorylation. In addition, its ectopic expression in mouse fibroblast shows a significant arrest of proliferation in the G_0/G_1 phase of the cell cycle. Interestingly, the small peptide Spa310 completely maintains the ability, typical of the full-length spacer region of p130, to inhibit cdk2 kinase activity and equally, when introduced into cells, induces growth arrest and inhibits the endogenous cdk2 activity, in an analogous manner to the spacer domain. In addition, Spa310 is also able to reduce human lung tumor growth in xeno-transplanted nude mice suggesting its potential role as a promising new type of mechanism-based drug for the treatment of malignant disorders. This therapeutic approach is focus of great interest in cancer therapy and consequently, pharmacological compounds, like small molecules, or peptides such as Spa310, targeting certain cdks, are potential points of intervention for drug discovery, since they could create rationally designed inhibitors of particular pathways that lead to malignant transformation. Over the last decade, a variety of pharmacological compounds with potent cdk inhibitory and strong anti-tumor activities have been identified and, for some of them, their potential anticancer function has been confirmed in preclinical studies (Dai & Grant, 2004; Shapiro, 2006; de Cárcer et al., 2007; Malumbres & Barbacid, 2009). The development of biological molecules, rather than chemical compounds, represent a larger line of research, since combines the efficacy of arresting cellular proliferation by interacting specifically with peculiar regulators of the cell cycle. The specificity of these compounds, compared to the non-specificity of chemical compounds, would allow the

development of new molecules with better pharmacodynamics, higher patient tolerability and fewer side effects.

6. Conclusions

As broadly discussed in this chapter, a breakdown of the cell cycle caused by an unbalanced perturbation that pushes a cell to stimulate its own growth, resisting to inhibitory signals that might otherwise stop its growth, represents a common hallmark of the malignant transformation and tumor development. The assortment of all the observations here described, taken together with the further evidences available in the large body of literature, supports the scientific relevance of the p130 biological functions in cell cycle control, in cell transformation, and tumor formation.

The development of small molecules with cdk inhibitory activity, as well as the small peptides mimicking, as discussed here, the functional motifs of p130 and the preservation of its cyclin-dependent kinase inhibition, represent key tools to clarify the connections among cdks and TSGs with cell cycle progression and malignant transformation. Meanwhile, additional studies that might elucidate how the loss of function of p130, and the related pocket proteins pRB and p107, merges with the activation or the inactivation of other gene products to develop retinoblastoma or other proliferative disorders, will open up novel horizons for the biology of cancer, which will hopefully lead to the development of innovative pharmacological approaches and efficient therapies.

7. Acknowledgments

This work was supported by grants from "*Fondazione Banco di Sardegna*". I am grateful to Valeria Giola for her editorial assistance.

8. References

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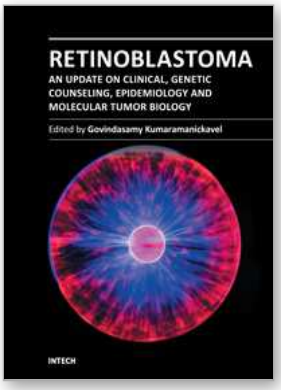

Retinoblastoma: An Update on Clinical, Genetic Counseling, Epidemiology and Molecular Tumor Biology

Edited by Prof. Govindasamy Kumaramanickavel

ISBN 978-953-51-0435-3
Hard cover, 170 pages
Publisher InTech
Published online 28, March, 2012
Published in print edition March, 2012

Retinoblastoma is the first tumor suppressor gene discovered ever. The discovery opened a new avenue in the field of oncology leading to the identification of 35 tumor suppressor genes, till date in our genome. This book is an excellent compilation of both clinical and basic science information that meets the needs of a young clinician and a researcher at the same time. It also has abundant information on recent advances and cutting-edge knowledge in intracellular molecular cross-talking of retinoblastoma protein with various cellular viral-like proteins.

How to reference

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Luigi Bagella (2012). The Retinoblastoma Family Protein p130 as a Negative Regulator of Cell Growth and Tumor Progression, Retinoblastoma: An Update on Clinical, Genetic Counseling, Epidemiology and Molecular Tumor Biology, Prof. Govindasamy Kumaramanickavel (Ed.), ISBN: 978-953-51-0435-3, InTech, Available from: http://www.intechopen.com/books/retinoblastoma-an-update-on-clinical-genetic-counseling-epidemiology-and-molecular-tumor-biology/the-retinoblastoma-family-protein-p130-as-a-negative-regulator-of-cell-growth-and-tumor-progression

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