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Cell death, cell growth and cell cycle regulation by the Retinoblastoma family

TESIS DOCTORAL

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CERTIFICAN

Que D. Yuri Chiodo, Licenciado en Biotecnología por la Università degli Studi di Palermo y Máster en Biotecnología Medica y Farmacéutica por la Università degli Studi di Modena e Reggio Emilia, ha realizado bajo nuestra dirección el trabajo de Tesis Doctoral que lleva por título: Cell death, cell growth and cell cycle regulation by the Retinoblastoma family.

Revisado el presente trabajo, expresamos nuestra conformidad para la presentación del mismo en el Departamento de Bioquímica de la Universidad Autónoma de Madrid, por considerar que reúne los requisitos necesarios para ser sometido a su evaluación ante el tribunal correspondiente, para optar al grado de Doctor en Biociencias Moleculares.

En Madrid, a 29 de Marzo de 2017

Dr. Miguel Campanero García
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Dr. Juan Miguel Redondo Moya
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A chi c’è, a chi ci sarà e a chi non c’è più…

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Abstract

The Retinoblastoma gene (*RB1*) was the first tumor suppressor gene to be cloned. Its encoded protein, the Retinoblastoma protein (pRB) is a member of the Retinoblastoma family, which is constituted also by p107 and p130. This family plays a critical role in the control of the progression through the different phases of the cell cycle. pRB is particularly involved in the G1-to-S transition and, although its role in cell cycle regulation is well defined, its role in the regulation of cell death remains controversial. In this work, we have performed a systematic and global study of the Retinoblastoma family members and of the various functional domains of pRB in the regulation of E2F activity, cell cycle progression, DNA replication, and in the regulation of cell death triggered by ionizing radiation (IR). While all family members inhibited E2F activity and DNA replication, and accumulated cells in G0/G1 in the short-term, pRB was the only member of the family that inhibited IR-induced cell death and arrested cells in the G0/G1 cell cycle phase in the long-term. We have found a strong correlation between the capacity of pRB to induce a long-term arrest of the cell cycle and its capacity to inhibit cell death, suggesting that a sustained arrest of the cell cycle in G0/G1 is necessary and sufficient to inhibit cell death caused by DNA damage. These data suggest that pRB may not play a direct role in the regulation of cell death induced by DNA damage and that cell survival might be the consequence of its main function, the arrest of the cell cycle until DNA damage has been eliminated. We have forced the expression of various domains of pRB and the results obtained strongly suggest that a correct folding of pRB, integrating its small pocket and carboxyl terminus into the large pocket, is critically required to efficiently inhibit IR-induced cell death and to block cell cycle progression in the long-term. Finally, we have created *in-silico* a 3-dimensional model of full-length pRB that shows the presence of an amino acid from the carboxyl terminus (R798) in the soil of the pocket, next to an amino acid from the A-domain (K530). The analysis of the conformational changes induced by inactivating mutations in the A- or in the B-domain showed that the carboxyl terminus was more severely affected than the small pocket. The *in-silico* analysis therefore supports the notion that the functional pRB large pocket is the result of the structural integration of the small pocket and the carboxyl terminus.

Resumen

El gen Retinoblastoma (*RB1*) fue el primer gen supresor de tumores clonado y codifica la proteína Retinoblastoma (pRB). Esta proteína es el miembro fundador de la familia Retinoblastoma, que también está constituida por p107 y p130. Esta familia juega un papel crítico en el control de la progresión a través de las diferentes fases del ciclo celular. pRB está particularmente implicado en la transición de G1 a S y, aunque su papel en la regulación del ciclo celular está bien definido, su papel en la regulación de la muerte celular sigue siendo controvertida. En este trabajo hemos realizado un estudio sistemático y global de los miembros de la familia de Retinoblastoma y de los diferentes dominios funcionales de pRB en la regulación de la actividad de E2F, la progresión del ciclo celular, la replicación del ADN y en la regulación de la muerte celular provocada por la radiación ionizante (IR). Mientras que todos los miembros de la familia Retinoblastoma inhibieron la actividad de E2F y la replicación del ADN, y favorecieron la acumulación de las células en G0/G1 a corto plazo, pRB fue el único miembro de la familia que inhibió la muerte celular inducida por IR y detuvo las células en la fase G0/G1 a largo plazo. Hemos encontrado una fuerte correlación entre la capacidad de pRB para inducir una detención a largo plazo del ciclo celular y su capacidad para inhibir la muerte celular, lo que sugiere que una detención sostenida del ciclo celular en G0/G1 es necesaria y suficiente para inhibir la muerte celular causada por daño del ADN. Estos datos sugieren que pRB no desempeña un papel directo en la regulación de la muerte celular inducida por daño del ADN y que la supervivencia celular sería la consecuencia de su función principal, la detención del ciclo celular hasta que el daño del ADN ha sido eliminado. Hemos forzado la expresión de diversos dominios de pRB y los resultados obtenidos sugieren fuertemente que un plegamiento de pRB que permita la integración del dominio llamado *bolsillo pequeño* con la región carboxilo terminal es esencial para inhibir eficientemente la muerte celular inducida por IR y para bloquear la progresión del ciclo celular a largo plazo. Por último, hemos creado *in silico* un modelo tridimensional de pRB completa que muestra la presencia de un aminoácido del extremo carboxilo (R798) en el suelo del bolsillo, junto a un aminoácido del dominio A (K530). El análisis de los cambios conformacionales inducidos por mutaciones inactivadoras en el dominio A o en el dominio B mostró que el extremo carboxilo estaba más gravemente afectado que el bolsillo pequeño. El análisis *in silico* por lo tanto apoya la noción de que el denominado *bolsillo grande* de pRB es el resultado de la integración estructural del *bolsillo pequeño* con el dominio carboxilo terminal. En conjunto, nuestros datos sugieren que esta integración estructural podría dar lugar a la formación de un dominio funcional en pRB necesario para la parada sostenida del ciclo celular y la inhibición de muerte que no estaría presente en p107 y p130.

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Abbreviations

ATM	Ataxia telangiectasia-mutated protein kinase
ATR	ATM and Rad3-related protein kinase
Bak	Bcl-2 homologous antagonist/killer protein
Bax	Bcl-2-associated X protein
BRCA1	Breast cancer growth suppressor protein 1
BRG1	Brahma-related gene-1
c-Abl	Abelson leukemia oncogene cellular homolog
Caspase	Cysteine proteases with aspartate specificity
CD95	Cluster of differentiation 95
CD95L	Cluster of differentiation 95 ligand
CDKs	Cyclin dependent kinases
Chk	Checkpoint kinase
CHX	Cycloheximide
CIP/KIP family	CDK-interacting protein/Kinase-inhibitory protein family
CKIs	Cyclin-dependent kinase inhibitors
C-ter	Carboxyl terminus
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DP	DRTF1-polypeptide
DRTF1	Database of Rice Transcription Factor-1
E1A	Adenovirus early region 1A
E2F	E2 transcription factor
E7	Human papilloma virus-16 early region 7
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2´-deoxyuridine
Fas	First Apoptotic Signal
FasL	First Apoptotic Signal Ligand
G1 phase	Gap phase 1
G2 phase	Gap phase 2
GFP	Green fluorescent protein
HA	Hemagglutinin
HDAC	Histone deacetylases
HDM2	Human double minute 2
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HU	Hydroxyurea
INK4 family	Inhibitor of CDK4 family
IR	Ionizing radiation
M phase	Mitosis phase
Mad2	Mitotic arrest deficient 2
Mdm2	Murine double minute 2
MEFs	Mouse embryonic fibroblasts
Mim	Mimosine
Noc	Nocodazole
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulphonyl fluoride
PNUTS	Phosphatase Nuclear Targeting Subunit
pp32	Phosphoprotein 32
pRB	Retinoblastoma protein
RB NPC	pRB mutated in N-terminus, pocket and C-terminus
RB C-ter	pRB carboxyl terminus
RB SE	pRB carboxyl terminus from the SspI site to the end
RB ΔI	Retinoblastoma protein truncated in ICE-site
RB-MI	Retinoblastoma protein mutated in ICE-site
RB-PMS	Phosphorylation site mutated Retinoblastoma protein
S phase	Synthesis phase
SDS	Sodium dodecyl sulphate
SV40	Simian Virus 40
TFIIH	Transcription factor II Human
TNF family	Tumor necrosis factor family
Tub	Tubulin
WCE	Whole cell extract buffer

Introduction

1. Retinoblastoma family and cell cycle control

1.1. Cell cycle overview

The cell cycle is a highly-controlled series of events that ensures correct replication of the DNA during S phase and guarantees equal distribution of identical chromosome copies into two daughter cells during M phase (Heichman and Roberts 1994; Wuarin and Nurse 1996). It is possible to identify separated phases in the cell cycle: 1) The Gap phase 1 (G1 phase) in which cells spend most of the time and prepare themselves for DNA replication; 2) the Synthesis phase (or S phase) in which cells replicate their DNA; the Gap phase 2 (G2 phase) in which cells duplicate all the other cellular components and resumes its growth in preparation for division; and 4) the mitosis phase (or M phase) which consists in the physical separation of the genetic material and the cellular components (the cytokinesis).

The unidirectional progression along the different phases of the cell cycle is strictly regulated by the "cell cycle control system" or "checkpoint control". Each checkpoint serves as a potential point in the cell cycle that ensures the progression to the next phase only if favorable conditions are met.

The G1 checkpoint (also known as Restriction checkpoint) regulates the entry through the cell cycle. Depending internal and/or external condition, the Restriction point can delay G1 phase, lead cells in a quiescent state (G0 phase) or allow cell cycle progression, promoting the transition from G1 to S. The G2/M checkpoint, also known as the DNA damage checkpoint, ensures that the cell has correctly completed the replication of the DNA during the S phase and properly achieves all the necessary changes in G2 phases. Accordingly, the G2/M checkpoint monitors if the cell is ready to divide. Finally, the Metaphase checkpoint controls the metaphase-to-anaphase transition in Mitosis, ensuring sister chromatid separation and driving for completion of Mitosis and Cytokinesis. The existence of an intra-S phase checkpoint, involved mostly in DNA damage response, has been also hypothesized (Nyberg et al. 2002; Bartek et al. 2004; Zhu et al. 2004). Once the cell splits into its two daughter cells, they enter again in G1 and the cycle continues.

The importance of a correct control of cell cycle progression is underlined by the observation that tumor cells typically acquire alterations to genes whose products are involved in cell cycle regulation. Not by chance, uncontrolled cell proliferation is considered a hallmark of cancer (Sherr 1996; Hanahan and Weinberg 2000). A great number of proteins are involved in the molecular mechanisms that control cell cycle progression and the Retinoblastoma family of proteins plays a key role in regulating the transition through the different phases of the cell cycle.

1.2. The Retinoblastoma family of proteins: pRB, p107 and p130

The Retinoblastoma family of proteins, or RB family, has a collective role in cell cycle control and tumor suppression. This family of proteins, known also as the pocket proteins, includes three members: Retinoblastoma protein (pRB), p107 and p130 (Cobrinik 2005; Henley and Dick 2012). Although the three proteins show high homology, p107 and p130 share more structural and functional similarity than pRB.

The most highly conserved region, the so-called “small pocket”, is characterized by two distinct functional domains (A and B domain), separated by a flexible spacer (Classon and Harlow 2002). p107 and p130 contain a longer spacer region than pRB, that allows them to stably interact with proteins involved in cell cycle regulation, such as the Cyclin-dependent kinase complexes (Zhu et al. 1995; Lacy and Whyte 1997; Woo et al. 1997). Furthermore, they present an insertion in the B domain of the small pocket sensitive to phosphorylation and that, at least for p130, could be useful to maintain its stability (Litovchick et al. 2004).

The small pocket is the minimal region capable of interacting with proteins that possess the LXCXE peptide motif such as viral oncoproteins (including adenovirus E1A, simian virus 40 tumor antigen, and Human Papillomavirus E7 protein), D-type cyclins, and proteins with chromatin-modifying activity, like histone deacetylase, HDACs (Ludlow and Skuse 1995) (Dowdy et al. 1993; Ewen et al. 1993) (Brehm et al. 1998).

The combination of the small pocket and the carboxyl terminal domain is called the “large pocket”, that is the minimal growth suppression domain found in RB family proteins (Qin et al. 1992; Sellers et al. 1995). An intact large pocket is necessary and sufficient for the interaction and inhibition of E2F family of transcription factors (Hiebert et al. 1992; Qin et al. 1992; Flemington et al. 1993; Helin et al. 1993).

E2F family members regulate the expression of genes involved in cell cycle progression. The inhibition of their transcriptional transactivation activity through their binding to the pocket proteins, emphasizes the role of pRB, p107 and p130 in cell cycle progression’s control (Dyson 1998; Dimova and Dyson 2005). Furthermore, the disruption of this interaction by viral oncoproteins, reinforces the relevance of the pocket proteins in avoiding cellular transformation (Whyte et al. 1988).

The carboxyl terminal region of pRB harbors two domains that are absent from p107 and p130: an E2F1-specific binding site, involved in regulation of apoptosis (Dick and Dyson 2003), and a short peptide region able to interact with Cyclin/CDKs complexes or Protein Phosphatase 1 (PP1) (Dunaief et al. 2002). In contrast, the amino terminal region of p107 and p130 contain a kinase inhibitor domain that is absent from pRB (Woo et al. 1997).

The ability of pRB, p107 and p130 to employ their different functional domains, allow these proteins to interact and regulate a great number of binding partners and to properly perform their cellular functions.

1.3. Retinoblastoma family in cell cycle control

The importance of the role of Retinoblastoma family proteins in cell cycle control was perceived since Chellappan and colleges' description of E2F transcription factors as a cellular target of Retinoblastoma protein (Chellappan et al. 1991), and previous finding of the interaction between virus oncoproteins and pocket proteins (Ludlow and Skuse 1995).

Pocket proteins regulate the E2F family of transcription factors.

Although the levels of pRB, p107 and p130 changed along the cell cycle, all the three pocket proteins have a direct role in cell cycle regulation (Henley and Dick 2012). They have in common the ability to bind, and regulate, E2F transcription factors (Chellappan et al. 1991; Hiebert et al. 1992; Flemington et al. 1993; Helin et al. 1993; Dyson 1998).

To date, eight different members of the E2F family, and two DP binding partners (DP1 and DP2), have been described. E2F1, E2F2 and E2F3a have a more potent transcriptional activity than the other members of the family; for this reason, they are classified as the activator E2Fs. In contrast, E2F3b, E2F4 and E2F5 are the repressor E2Fs because of their limited activation potential. Furthermore, E2F4 and E2F5 lack a functional nuclear localization signal and they rely on pocket proteins for their translocation to the nucleus. E2F6 does not have a transactivation domain or a pocket proteins binding domain. It regulates cell cycle in a pocket proteins' independent manner, possibly through its association to proteins of the Polycomb group. Less is known about the mechanism of action of E2F7 and E2F8 (Dimova and Dyson 2005).

The high expression of p130 in resting cells (G0 phase of the cell cycle) is considered a reliable marker of quiescence, in which p130 complexes mainly with E2F4. Although p107 is almost undetectable, pRB expression is present at lower level than p130 and preferentially binds to repressor E2F3b (Moberg et al. 1996; Hurford et al. 1997). To ensure the maintenance of the quiescent state, pRB also represses t-RNA and r-RNA expression in G0, while p130 represses r-RNA transcription (Hannan et al. 2000).

Following appropriate stimuli, and according with optimal external condition detected by the G1 checkpoint, cells can progress to the next phase of the cell cycle (G1 phase), in which the three pocket proteins are detectable and interact with E2F transcription factors.

In early G1, the most abundant pocket protein/E2F complex present is composed by p130 and E2F4. The inhibition of E2F responsive genes mediated by p130 is maintained until mid-G1 phase, when it is replaced by p107 at E2F responsive promoters, until late G1 (Moberg et al. 1996; Takahashi et al. 2000; Wells et al. 2000). At this point of the cell cycle, pRB increases its levels and associates with activator E2Fs, inhibiting their transactivation capacity (Hiebert et al. 1992; Flemington et al. 1993; Helin et al. 1993).

E2F responsive genes include mostly genes whose expression is required for cell cycle progression from G1-to-S phase, including cell cycle regulators, components of the replication machinery and enzymes involved in nucleotide biosynthesis. Although it is not known how pocket protein/E2F complexes selectively interact with specific promoters, it is widely accepted that pRB, p107 and p130 negatively regulate the cell cycle mainly through the inhibition of E2F factors (Dyson 1998; Henley and Dick 2012). This notion is further supported by the necessity of some viral oncoproteins to disrupt pocket protein/E2F complexes to induce expression of E2F responsive genes and cellular transformation (Whyte et al. 1988).

The ability of the pocket proteins to bind E2F transcription factors depends on their phosphorylation state.

The main kinases involved in pocket proteins phosphorylation are the cyclin-dependent Kinases, or CDKs. Cyclins are regulatory subunits required for CDKs activation, but the additional interaction with the CDK-activating kinase (CAT) is needed also for the full activation of the complex. Although the levels of CDKs are normally constant throughout the cell cycle, the expression of the different cyclins changes along the cell cycle: D-type cyclins are expressed in late G1 and interact with CDK4 and 6; Cyclin E is present during G1-to-S transition and complexes with CDK2; CDK2 and CDK1 can also form complexes with Cyclin A early in S phase to promote DNA replication; and Cyclin B is expressed in Mitosis and binds and actives CDK1(Malumbres and Barbacid 2005).

Not surprisingly, cells present different levels of control of Cyclin-dependent kinase activity to ensure correct progression through the different phases of the cell cycle. One of the best characterized mechanisms involves their association with cyclin-dependent kinase inhibitors (CKIs). There are two families of CKIs: the CIP/KIP family, which consists of p21, p27 and p57, and the INK4 family, which consists of p16, p15, p18 and p19. The main difference between these two families of inhibitors is that CIP/KIP family members can inhibit any Cyclin/CDK complex that is found in G1, whereas INK4 proteins only block the kinase activity of Cyclin D-bound CDK4 and CDK6 (Sherr and Roberts 1999; Vidal and Koff 2000; Besson et al. 2008).

In addition, p107 and p130 contain a dedicated domain for CDK inhibition that is absent from pRB (De Luca et al. 1997; Woo et al. 1997). However, pRB can also inhibit CDK activity through its capacity to interfere with p27 proteolysis in G1 (Ji et al. 2004; Binne et al. 2007).

The G1-to-S transition requires the correct activation of specific Cyclin/CDKs complexes and the sequential phosphorylation of the pocket proteins by them to release E2F factors and activate the expression of E2F responsive genes. Pocket proteins' phosphorylation starts at late G1 by active Cyclin D/CDK4 or 6 kinase complex (Dowdy et al. 1993). The release of activator E2Fs allows the expression of Cyclin E and the following formation of active complex Cyclin E/CDK2. At this point, the most import mechanism to avoid uncontrolled entry in S phase is mediated by p27. Once DNA replication has been started, the activation of Cyclin E/CDK2 induces phosphorylation and subsequent degradation of p27 (Ji et al. 2004; Binne et al. 2007). This event allows full activation of Cyclin E/CDK2, which completes pocket proteins' phosphorylation and totally disrupts their interaction with E2F family members (Sherr and Roberts 1999; Malumbres and Barbacid 2005). Sequential phosphorylation of the pocket proteins is necessary because phosphorylation mediated by Cyclin D/CDK4 or 6 is not enough to complete their inactivation and because CDK2 cannot bind unphosphorylated proteins as substrate (Lundberg and Weinberg 1998).

To avoid genome instability that may result in cell transformation, once cells start the replication of the DNA, the cell cycle progresses until cell division is completed. Release of E2F transcription factors from pocket proteins coincides with the "point of no return" in which cells are obligated to complete the rest of the cell cycle (Yao et al. 2008). Again, pocket proteins play an important role in G1-to-S progression's control, avoiding reverse direction of it.

Recently Cappell et al. propose the idea of a "window of reversibility" in which cells can return to a quiescent state and that the inactivation of the anaphase-promoting complex/cyclosome (APC^{Cdh1}) has the necessary characteristics to be the point of no return for cell-cycle entry, in a pocket proteins independent way (Cappell et al. 2016).

Pocket proteins have also a role in Mitosis.

Overexpression of some mitotic checkpoint genes (Emi1 and Mad2) has been observed in pRB deficient cells. The finding that these genes are target of E2F transcription factors, suggests a possible role of pocket proteins in Mitosis (Sotillo et al. 2007; Weaver et al. 2007). Besides these E2F-dependent effects, deficiency of all the three pocket proteins results in mitotic errors, due to a defective chromosome structure in pericentromeric region (Gonzalo et al. 2005; Manning et al. 2010).

At the end of Mitosis, specific phosphatases induce pocket proteins' dephosphorylation and consequently activation of their growth suppression functions, thus leading cells to correctly complete cell division into two daughter cells (Ludlow et al. 1993). Finally, cells enter again in G1 phase and the cycle can proceed.

1.4. Retinoblastoma family in DNA damage response.

Alterations in DNA damage response (DDR) pathways are often associated with tumor transformation (Sancar et al. 2004). DNA damage response is a highly-controlled process that arrests cell cycle progression when an injury could affect DNA integrity. The activation of cell cycle checkpoints avoids progression to the next phase of the cell cycle and allows the activation of the DNA repair system: if the damage is repaired, cell cycle can be resumed. Otherwise, cells undergo programmed cell death, or apoptosis (Weinert 1998a; Weinert 1998b; Nyberg et al. 2002).

DNA double-strand brakes recruit and activate ATM and ATR kinases, which, in turn, activate cell cycle checkpoint kinase 1 and 2 (Chk1 and Chk2). Phosphorylation of p53 by Chk1 and 2 decreases its binding to the E3-Ubiquitin ligase HDM2 (Mdm2 in mice) and therefore reduce p53 degradation. The increase of p53 levels promotes its tetramerization and subsequent translocation to the nucleus, where it induces expression of the CIP/KIP family member p21. Consequently, p21 inhibits kinase activity of the specific Cyclin/CDKs complexes present at the time of the injury, and prevents pocket proteins' phosphorylation. Pocket proteins acquire again cell growth suppression's function, preventing progression of the cell cycle (Brugarolas et al. 1999).

In addition, p21 can be induced also through p53-independent mechanisms (Macleod et al. 1995; Galanos et al. 2016). Furthermore, Chk1 can also inhibits activity of Cdc25 family members (phosphatases that activate Cyclin-dependent kinases of M and S phase) in response to DNA damaging agents (Xiao et al. 2003).

In summary, DDR actives a controlled sequence of molecular events that inhibit the activity of Cyclin/CDKs complexes, restore the cell growth suppression function of pocket proteins and promote a pause in the cell cycle. If the DNA repair system fixes the injury, cells restart their progression through the cell cycle. Conversely, if the damage cannot be repaired, cells will activate their suicide (Cerqueira et al. 2009; Yata and Esashi 2009).

There is also evidence of a regulatory function of p107 and p130 in DDR, but their contribution has been studied in much less detail (Jackson et al. 2005). Nevertheless, it seems plausible that the contribution of pocket proteins to DDR could mediate, at least in part, their capacity to suppress cellular transformation.

2. Focusing in pRB: pRB as a multifunctional protein

2.1. From a target of viral oncoproteins to a cell cycle regulator

In human, the tumor suppressor Retinoblastoma protein (pRB) is the product of the *RB1* gene, located in chromosome 13q14. It was identified as a gene whose loss is responsible for the predisposition to retinoblastoma and osteosarcoma (Friend et al. 1986) and it was the first tumor suppressor gene to be cloned (Fung et al. 1987; Lee et al. 1987).

Retinoblastoma protein was identified as an important target of viral oncoproteins. In 1988, for the first time, it was described that simian virus 40 (SV40) large tumor antigen formed a specific complex with the product of the retinoblastoma susceptibility gene (DeCaprio et al. 1988). In the same year, the association with another oncoprotein, the adenovirus E1A protein, was characterized (Whyte et al. 1988) and the first evidence of pRB tumor suppression's capacity was obtained, analyzing the suppression of the neoplastic phenotype mediated by pRB in human cancer cells (Huang et al. 1988). The Human papilloma virus-16 E7 oncoprotein was also found to bind to Retinoblastoma protein (Dyson et al. 1989; Munger et al. 1989; Lee et al. 1998). Of note, the region of interaction of these viral oncoproteins in pRB sequence is a common site of mutations in human tumor cells (Hu et al. 1990; Huang et al. 1990). Further studies identified amino acids 379-792 as the minimal binding domain for these viral oncoproteins in pRB (Kaelin et al. 1990). These studies showed for the first time a physical link between an oncoprotein and a tumor suppressor protein.

To explain the growth suppression properties of pRB, a model suggesting a role for pRB in cell cycle regulation was proposed (DeCaprio et al. 1989). The new concept of pRB as a cell cycle regulator protein, encouraged additional research along this line, and, in fact, Chellappan and coworkers described in 1991 that the E2F transcription factor was a cellular target for pRB (Chellappan et al. 1991). This work also confirmed the cell cycle-dependent phosphorylation of pRB. The role of pRB was further clarified that year by showing that RB acted in a specific point of the G1 phase of the cell cycle; exogenous pRB failed to inhibit DNA replication in cells synchronized in late G1 or in G1/S, suggesting that pRB does not inhibit DNA synthesis once DNA replication is initiated (Goodrich et al. 1991).

The evidence that viral oncoproteins shared the ability to disrupt the interaction between pRB and E2F proteins (Chellappan et al. 1992), reinforced the importance of pRB in cell cycle regulation. E1A, the SV40 large tumor antigen and E7 disrupted pRB/E2F interaction and stimulated a proliferative state. The consequent loss of cell cycle control may be the initiation of the oncogenic process in a manner analogous to

the inactivating mutations founded in the *RB1* gene. Not by chance, analysis of human cervical carcinoma cell lines expressing E7 protein or carrying *RB1* mutations revealed no formation of pRB/E2F complexes (Chellappan et al. 1992).

The demonstration that pRB was a negative regulator of E2F transcription activity (Hiebert et al. 1992; Flemington et al. 1993; Helin et al. 1993) and a target of phosphorylation mediated by Cyclin/CDK complexes (Hinds et al. 1992; Dowdy et al. 1993; Ewen et al. 1993) further supported the notion that pRB was involved in cell cycle control.

The study of non-functional and deletion mutants of *RB1* opened a new understanding of the protein's functions, focusing on the relevance of a proper structure to allow correct performance of the biological properties pRB (Templeton et al. 1991; Qian et al. 1992). The minimal growth suppression domain of the protein (Qian et al. 1992) and the region necessary for its interaction with E2F proteins (Hiebert 1993) were identified soon. Furthermore, an amino-terminal deletion of pRB (RB 379-928) showed enhanced growth suppression function (Xu et al. 1994). A single-point mutation in *E2F1* sequence was found that destroyed its interaction with pRB and that increased cells replication (Shan et al. 1996).

In contrast with the idea that a stable pRB/E2F interaction was necessary and sufficient for cell growth's inhibition, Sellers and colleagues suggested that this interaction was not required for pRB to activate transcription, promote differentiation and suppress tumor cells growth (Sellers et al. 1998). Moreover, although a low-penetrance pRB mutant, RB 661W (Onadim et al. 1992; Lohmann et al. 1994), was defective in E2F binding (or at least less efficient than the wild-type protein), it suppressed cell growth (Whitaker et al. 1998). In this regard, pRB can also inhibit G1-to-S progression in an E2F-independent way, by increasing p27 expression (Alexander and Hinds 2001; Ji et al. 2004); inducing PML nuclear bodies formation (and inhibiting their effect on cell proliferation, differentiation and survival) (Fang et al. 2002); suppressing pro-proliferative Ras signaling (Lee et al. 1999); or augmenting expression of differentiation-associated genes (Sellers et al. 1998; Thomas et al. 2001).

The knowledge of the role of pRB in cell cycle control has become more detailed and complex since its discovery. The characterization of the other two members of the RB family, p107 (Ewen et al. 1991) and p130 (Baldi et al. 1996), and the overlapping functions of these proteins in important biological processes (Cobrinik et al. 1996; Lee et al. 1996; Hurford et al. 1997; Classon et al. 2000; Sage et al. 2000), are just some of the features that make the study of the Retinoblastoma protein fascinating.

2.2. pRB as a scaffold: the matchmaker hypothesis

The three-dimensional structure of the small pocket (Lee et al. 1998) suggests that pRB can simultaneously bind to more than one target, and that only a correct conformation of the protein can facilitate optimal interactions with its different partners and proper fulfillment of the different biological functions. To date, more than 150 different partners of pRB have been identified and this number could increase year after year (Morris and Dyson 2001; Goodrich 2006; Dyson 2016). The ability of pRB to interact and regulate such a great number of proteins raises the question of how many different functions might be able to perform this protein.

It was described that the carboxyl-terminal end of pRB regulates nuclear activity of the c-Abl tyrosine kinase, suggesting a “matchmaker function” of pRB (Welch and Wang 1993; Welch and Wang 1995a). pRB was detected in a trimeric complex with E2F and c-Abl and, supporting the matchmaker hypothesis, it was proposed that the biological activity of pRB not only depends on the inhibition of its targets, but also on its ability to properly assemble specific protein complexes (Welch and Wang 1995b). In fact, simultaneous overexpression in a pRB deficient cell line of full length wild-type pRB and a mutated carboxyl-terminal fragment of pRB (RB SEΔ), that loss the amino acids involved in E2F binding but not the ability to inhibit the kinase activity of c-Abl, showed a dominant-negative function of RB SEΔ over pRB function in cell growth inhibition (Welch and Wang 1995b).

Retinoblastoma protein also forms a complex with the ATP-dependent helicase BRG1 (Khavari et al. 1993; Dunaief et al. 1994). This interaction cooperates in the induction of cell cycle arrest (Dunaief et al. 1994; Strober et al. 1996; Strobeck et al. 2000; Zhang and Dean 2001) and in the repression of E2F1 (Trouche et al. 1997). The relevance of this interaction was confirmed by the discovery in human cancer cell lines of BRG1 mutations that destroy pRB capacity to arrest the cell cycle (Bartlett et al. 2011). In the context of chromatin remodeling, pRB also recruits histone deacetylase proteins (Brehm et al. 1998). The interaction between the small pocket of pRB and the LXCXE motif of HDACs provided a plausible mechanism for the active repression of E2F responsive genes by pRB (Brehm et al. 1998; Magnaghi-Jaulin et al. 1998; Dahiya et al. 2000).

2.3. pRB in embryo development

Before starting the description of pRB as a tumor suppressor protein and its functions in cell death, it is important to mention its functions during embryonic development. In fact, although *pRb* is ubiquitously expressed in adult mouse tissues,

its expression is temporally and spatially controlled during embryogenesis (Classon and Harlow 2002).

In 1992, three independent groups reported a mouse strain in which one allele of *Rb1* was disrupted. Contrary to human patients, *Rb1*$^{+/-}$ mice showed no predisposition to develop retinoblastoma tumors, but some mice displayed pituitary tumor. In contrast, *Rb1*$^{-/-}$ embryos died between days 14 and 15 of gestation and showed defects in hematopoiesis and neurogenesis associated to strong E2F-dependent cell death, both in central and in peripheral nervous system (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). These observations strongly suggested that pRB played a critical anti-apoptotic role during embryonic development and prompted the generation of *Rb1* and *E2fs* double knock-out mice in which loss of *Rb1* with *E2f1* decreased cell death only in the central nervous system, whereas loss of *Rb1* and *E2f3*$^{-/-}$ diminished cell death only in the peripheral nervous system. The defects in DNA replication and apoptosis found in *Rb1*$^{-/-}$ embryos were inhibited only partially in *Rb1*$^{-/-}$*;E2f2*$^{-/-}$ embryos (Saavedra et al. 2002).

Regarding the major repressive E2F family member, *E2f4*, its loss suppressed the development of both pituitary and thyroid tumors in *pRb* heterozygous mice, and reduced alteration in both gene expression and proliferation observed in *pRb* deficient cells. In *Rb1*$^{-/-}$*;E2f4*$^{-/-}$ mice, p107 and p130 inhibit the activator members of E2F, suggesting that these pocket proteins compensate the absence of pRB to suppress tumor formation (Lee et al. 2002). To support this hypothesis, Dannenberg and co-authors generated chimeric mice with loss of the different pocket proteins genes, and they confirmed that *pRb/p107* or *pRb/p130* deficient mice were highly prone to develop tumors in different tissues (Dannenberg et al. 2004).

Remarkably, the expression of an *E2f* binding-deficient *Rb* protein, analogous to low-penetrance human pRB mutant RB 661W, partially rescued the developmental defects in *Rb* nullizygous mice (Sun et al. 2006).

All these data underline the complexity of pRB function in development. The engineering of conditional *pRb*-KO mice allowed a better understanding of the role of pRb in tissue differentiation. The affected tissues seemed to try a differentiation program in the absence of pRb, as determined by the induction of early lineage-specific markers, but failed to reach correct terminal differentiation (Goodrich 2006). These observations were also confirmed in mouse embryonic fibroblasts (MEFs) *in vitro*: cells established from *pRb*-deficient mice embryos showed defect in myogenesis and adipogenesis as well as in limb development (Cobrinik et al. 1996), confirming the importance of pRB in proliferation, apoptosis and differentiation during development.

3. pRB is a tumor suppressor protein

3.1. pRB pathway in cancer

Uncontrolled cell proliferation is a hallmark of cancer (Sherr 1996; Kaelin 1997; Hanahan and Weinberg 2000) and cell cycle regulators are frequently mutated in human cancers (Malumbres and Barbacid 2001). Supporting Knudson's "Two-hit hypothesis", (Knudson 1971; Knudson et al. 1975), germline mutations of the *RB1* gene causes the highly penetrant hereditary retinoblastoma, as the result of bi-allelic loss of this gene in embryonic retinoblasts (Cavenee et al. 1983; Dunn et al. 1988). *RB1* was found mutated later in various cancer types. In addition, although many human tumors retain wild-type *RB1*, they present mutations that indirectly affect pRB activity as a "master regulator" of cell cycle progression (Kaelin 1997; Malumbres and Barbacid 2001; Classon and Harlow 2002; Zhu et al. 2015). Thus, in the multistep nature of cancer (Vogelstein and Kinzler 1993), inactivation of cell cycle control systems is necessary for tumor transformation and underscores the importance of the maintenance of these systems for cancer prevention.

Amplification of *CCND1* or *CDK4*, translocation of *CDK6* and deletion of INK4 family proteins-encoding genes (*CDKN2*) or *RB1* deletion are the most common chromosomal alterations observed in human cancers. Furthermore, methylation of *CDKN2A* or *RB1* promoters, and their consequent inactivation, are additional alterations identified in cell cycle regulators in human cancers. (Sherr 1996; Kaelin 1997; Malumbres and Barbacid 2001; Dyson 2016). Together, these findings strongly support the notion that loss of cell cycle control, in which pRB plays a key role, is a crucial step in cell transformation.

More evidences of pRB role as a tumor suppressor protein are described with respect to p107 and p130. However, it was suggested tissue-specific tumor suppression activity of both p107 and p130, by the observation that *pRb/p107*- or *pRb/p130*-deficient mice are more susceptible to tumor development than *pRb* null mice (Dannenberg et al. 2004). Moreover, p107 presents tumor suppression ability in *pRb*-deficient epidermis (Lara et al. 2008), while p130 could be involved in retinoblastoma tumor development (Tosi et al. 2005).

Thus, all these observations strongly suggest tumor suppressor activity of pocket proteins.

3.2. pRB in DNA damage response

As mentioned above, the DNA damage response induces a pause on cell cycle progression if any of the cell cycle checkpoints detects an injury on DNA. pRB plays a fundamental role in cell cycle arrest induced by DNA damage, revealing the tumor

suppressor role of this protein. Defects in G1 phase arrest in response to gamma-irradiation observed in *Rb1*$^{-/-}$ MEFs underline the importance of pRb in DNA damage-induced cell cycle arrest (Harrington et al. 1998). Moreover, pRb is also dephosphorylated and therefore activated during S-phase to inhibit cell cycle progression in MEFs treated with various genotoxic stimuli to inhibit cell cycle progression (Knudsen et al. 2000). Furthermore, human tumor cell lines with defective cell cycle-dependent activation of pRB (like the U2OS osteosarcoma cell line) also appear to be defective for pRB activation in response to DNA damage, displaying a phenotypic behavior similar to pRB-defective cells (Broceno et al. 2002). In this regard, pRB controls both replication and re-replication (Barbie et al. 2004). In fact, pRB is recruited to focal replication foci after DNA damage to block early firing of replication origins in S phase (Blow and Hodgson 2002; Avni et al. 2003).

Defects in pRB pathway regulation also increase the levels of the E2F's target Mad2, a protein whose overexpression is associated with mitotic defects that lead to aneuploidy and tumorigenesis (Hernando et al. 2004; Sotillo et al. 2007). These findings reinforce the idea that the pRB pathway plays a critical role in G2 checkpoint control after DNA damage.

Finally, pRB is a required mediator of the interaction between Topoisomerase II-β and BRCA1 for the correct activation of their DNA repair function in cells treated with the cytotoxic anticancer drug Etoposide (Xiao and Goodrich 2005). These results confirm the role of pRB in DNA damage response and tumor suppression.

3.3. pRB in senescence

Cellular senescence (or simply senescence) was described for the first time as a mechanism to explain the limited proliferation of normal cells in culture (Hayflick 1965). Further studies revealed the linking of telomere shortening to replicative senescence (Harley et al. 1990), a process that drives cells in an irreversible no proliferative state. In fact, senescent cells, although metabolically active, stop proliferation in an irreversible way. p27 and p16 are often overexpressed and cells usually become resistant to cell death stimuli. Nowadays, the molecular mechanisms involved in this biological process have not been totally characterized; however, the p53-p21 and p16-pRB pathways seem to play a fundamental role in senescence induction and maintenance (Campisi and d'Adda di Fagagna 2007; Fiorentino et al. 2009).

Different causes of senescence have been described, including telomere-dependent senescence, DNA-damage-initiated senescence, senescence caused by chromatin perturbation, and oncogene-induced senescence (Campisi and d'Adda di Fagagna 2007). In addition, several findings reveal an important role of senescence as

a mechanism to limit the risk of neoplastic transformation, strongly suggesting a tumor suppressor property of cellular senescence (Campisi 2001).

Focusing on the Retinoblastoma protein, pRB re-expression in some tumor cells was found to induce senescence and inhibit telomerase activity (Xu et al. 1997). Thus, pRB may play a causal role in senescence induction by eliciting p27 accumulation (Alexander and Hinds 2001). Moreover, the recruitment of heterochromatin proteins and pRB to E2F-responsive promoters causes their stable repression, coinciding with senescence-associated heterochromatic foci (SAHF) formation, underlying the importance of pRB in senescence induction and revealing new mechanisms of tumor suppression by pRB (Narita et al. 2003).

To note, p107 and p130 were found to play distinct roles in the regulation of senescence: while p107 was required for initiation of accelerated senescence in the pRB-null DU45 human prostate cancer cell line, the knock down of p130 promoted premature senescence in these cells even in the absence of genotoxic stimuli (Lehmann et al. 2008). In contrast, other reports propose that p130 could be a master regulator for the sustained arrest of the cell cycle (Helmbold et al. 2006; Fiorentino et al. 2009).

3.4. How cancer cells escape from pRB regulation of the cell cycle?

The Retinoblastoma protein regulates G1-to-S progression of the cell cycle by inhibiting E2F activity and by recruiting chromatin-modifying enzymes that further repress transcription. As a scaffold, pRB promotes correct interactions between different proteins to best perform important biological functions: it is involved in embryo development and tissue differentiation, in DNA replication and it concurs in DNA damage response and regulation of apoptosis.

Not surprisingly, cancer cells exploit pRB loss-of-function for oncogenic transformation. *RB1* gene mutations are found in both hereditary and sporadic cancer types. In addition, oncoviral proteins block pRB interaction with E2F factors to promote uncontrolled cell proliferation. Alterations in proteins involved in pRB phosphoregulation promote defects in cell cycle progression's control and, finally, interference with Retinoblastoma pathway could also support cancer cells' evasion from apoptosis (Chau and Wang 2003).

4. pRB in programmed cell death regulation

4.1. Cell death: overview

Cell death is the biological process in which a cell, or a group of cells, quit all their biological functions. It is possible to distinguish a programmed cell death, which includes apoptosis and autophagy, and a non-physiological cell death or necrosis (Fuchs and Steller 2015).

The word “autophagy” was coined by the discoverer of lysosomes, Christian de Duve (De Duve 1963) and refers to a cellular process that guarantee correct degradation and recycling of unnecessary cellular components. The formation of a double-membrane vesicle, the autophagosome, and its fusion with a lysosome, allows the correct execution of autophagy. In the context of programmed cell death, it is a current matter of debate if autophagy acts directly in dying cells or as a survival mechanism (Tsujimoto and Shimizu 2005).

The first description of apoptosis dates to 1842, thanks to the work of Carl Vogt and later description of Walther Flemming in 1885. It was in 1972, with the works of Kerr, Wyllie and Currie (Kerr et al. 1972) and Brenner, Horvitz and Sulston (awarded in 2002 with the Nobel prize in medicine), when the term apoptosis acquired new interest. Apoptosis is a highly controlled cellular process, with different morphologically identifiable stages, that allows the “suicide” of a single cell. During the firsts steps, cell loses contact with its tissue of origin. Irreversible condensation (pyknosis) and fragmentation of the chromatin (karyorrhexis) precede cell contraction, ruffling and blebbing of the plasmatic membrane and subsequent formation of apoptotic bodies. These are recognized by macrophages, which complete their degradation.

From a molecular point of view, p53 is a major regulator of the “intrinsic pathway” of apoptosis. Once the apoptotic program is active, p53 induces the expression of pro-apoptotic BH1-2-3 proteins (Bax, Bak) and BH3 only proteins (Bim, Bid, Puma, Noxa), and inhibits expression of the anti-apoptotic proteins Bcl2 and BclxL. The previously mentioned pro-apoptotic proteins displace Bcl2 and BclxL from mitochondrial membrane causing the Mitochondrial Membrane Permeabilization (MMP). The drop of mitochondria’s membrane potential and the following stretch of the mitochondria’s crests and disruption of its membrane, allow the release to the cytoplasm of apoptosis-inducing factor (AIF), cytochrome c and apoptotic protease activating factor-1 (Apaf-1). Apoptosome, the result of the interaction between cytochrome C and Apaf-1, triggers caspase 9 activation, which in turn, active the executioner caspase 3.

Caspase 3 is active also in the “extrinsic pathway” or “death ligand pathway”. One of these pathways is mediated by Fas/FasL. Fas ligand (FasL or CD95L) is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family.

The binding to its receptor, FAS receptor (FasR)- also known as apoptosis antigen 1 (APO-1 or APT)- cluster of differentiation 95 (CD95) or tumor necrosis factor receptor superfamily member 6 (TNFRSF6), promotes receptor's tetramerization and subsequent recruitment of Fas-associated-death-domain (FADD). FADD, in turn, recruits and activate caspase 8 in the so-called DISC-complex (Death-inducing signaling complex), which activates caspase 3.

The main differences between apoptosis and necrosis is that necrosis is caused by external factors of the cell or tissue, that characterize it as a non-physiological-passive process. While apoptosis is a genetically regulated mechanism, in which chromatin is condensed and specifically cleaved in internucleosomal sites, necrosis is characterized by chromatin flocculation. Furthermore, in necrosis there is no formation of cellular bodies, and the complete lysis of groups of cells evoke an inflammatory state.

The theory most widely accepted today is that the death program would be running all the time, if it was not because it is continually thwarted by the survival-promoting or anti-death stimuli.

4.2. pRB and apoptosis regulation: anti-apoptotic or pro-apoptotic functions?

While the role of pRB in cell cycle regulation and cell growth inhibition has been extensively investigated and characterized, its role in apoptosis regulation has not been characterized in much detail. Indeed, its role in apoptosis regulation is still controversial (Indovina et al. 2015).

Massive apoptosis detected in *Rb1*$^{-/-}$ mice during embryogenesis suggested, at first, an anti-apoptotic function of pRb (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Chau and Wang 2003). Furthermore, *pRb1*$^{-/-}$ MEFs not only showed inappropriate S phase entry, but also predisposition to apoptosis (Almasan et al. 1995). According to an anti-apoptotic role, forced expression of pRB in pRB deficient cells inhibited ionizing radiation-induced apoptosis (Haas-Kogan et al. 1995).

In contrast, a pro-apoptotic role of pRB has been found also. Indeed, forced expression of a phospho-resistant mutant version of pRB (RB-PSM) in C33A cells (a pRB deficient tumor cell line) increased cisplatin-induced cell death (Knudsen et al. 1999). However, wt pRB and RB-PSM failed to inhibit C33A proliferation (Knudsen et al. 1999), therefore suggesting that cell cycle inhibition and apoptosis induction by hypo-phosphorylated pRB are mechanistically unrelated. Furthermore, under DNA damage condition, pRB was found associated with E2F1 in transcriptionally active pro-apoptotic promoters (Ianari et al. 2009), suggesting again a pro-apoptotic role of pRB under genotoxic stress. Consistent also with a pro-apoptotic role of pRB, the formation

of a trimeric complex pRB-HDM2-p53 was reported in which pRB binding to HDM2 inhibits its ubiquitinase activity and promotes p53 stabilization (Hsieh et al. 1999).

The interaction between pRB and HDM2 was also described to support an anti-apoptotic function of pRB. Janicke and colleagues identified a specific caspase-dependent cleavage at the carboxyl terminus of pRB and suggested that the loss of the last 42 amino acids could be one of the first steps of the apoptosis program (Janicke et al. 1996). Since HDM2 interacts with the carboxyl terminus of pRB (Xiao et al. 1995), Janicke and colleagues proposed that the lack of HDM2 interaction with cleaved pRB might promote HDM2 degradation and therefore activate p53-dependent apoptosis (Janicke et al. 1996). Supporting this hypothesis, the use of a cleavage-resistant pRB mutant (RB MI) in *Rb1*$^{-/-}$ MEFs, attenuated death induced by TNF. However, no effects were observed in a leukemia cell line, Jurkat, treated with anti-CD95 (Tan et al. 1997). The description of a pro-apoptotic function of the c-Abl tyrosine kinase (Theis and Roemer 1998; Wang 2000) and pRB inhibitory effect on this kinase (Welch and Wang 1993) are also consistent with an anti-apoptotic function of pRB.

A second E2F-binding site was found at the carboxyl terminus of pRB that was specific for E2F1 (Dick and Dyson 2003), the only known member of the E2F family with pro-apoptotic capacity (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994; DeGregori et al. 1997; Lazzerini Denchi and Helin 2005). While mutation of several residues in the small pocket of pRB (RB-ΔE2F-G) did not affect its capacity to repress E2F1 transactivation of pro-apoptotic gene promoters following DNA damage, it impaired its transcriptional repressive capacity on other E2F family members (Dick and Dyson 2003). It seems that acetylation of Lysine 873/874 of pRB, that occurs in response to DNA damage, regulates this specific interaction (Markham et al. 2006). Further characterization of this interaction identified pRB residues Methionine 851 and Valine 852 as necessary for the interaction: a pRB mutant in which both amino acids were substituted with an Alanine (RB ΔS) loss the capacity to inhibit the apoptosis induced by E2F1 overexpression, suggesting again an anti-apoptotic role of pRB (Julian et al. 2008). E2F1 binding at pRB carboxyl terminal specific site is maintained even in the hyper-phosphorylated (and presumably inactive) pRB form (Cecchini and Dick 2011).

Thus, apoptotic regulation mediated by Retinoblastoma protein could be mediated by specific phosphorylation events. However contradictory information increases the complexity of this theme.

In fact, there are additional conflicting data on the effect of pRB phosphorylation over its capacity to regulate apoptosis.

The capacity of pRB to regulate apoptosis might be controlled by specific phosphorylation of Serine 567. This residue is phosphorylated only under hyper-proliferative conditions and it is a signal for pRB degradation and cell death, supporting the notion that pRB expression is required to inhibit both cell proliferation and death (Ma et al. 2003). Furthermore, hyper-phosphorylated pRB inhibits the pro-apoptotic function of nuclear phosphoprotein pp32 (ANP32A), suggesting the possibility that cancer cells could gain both proliferative and survival advantages in the presence of inactive, hyper-phosphorylated pRB (Adegbola and Pasternack 2005).

In contrast, a pro-apoptotic function was proposed again upon the finding that pRB interacted with the pro-apoptotic protein Bax in the mitochondria. Induction of pRb expression in MEF increased apoptosis in cells treated with TNFα plus cycloheximide and this apoptosis was reduced in $Bax^{-/-}$ MEFs, suggesting that pRb induces apoptosis directly at the mitochondria though Bax interaction (Hilgendorf et al. 2013). This interaction appeared to be promoted by phosphorylation of pRB at Serine 807 (Antonucci et al. 2014). Supporting also a pro-apoptotic role for pRB, the knock-down of Phosphatase Nuclear Targeting Subunit (PNUTS) increased apoptosis only in pRB-expressing tumor cell lines, coinciding with E2F1 dissociation from pRB and the induction of caspase 8 activity (De Leon et al. 2008).

Finally, although some experiments suggest that pRB silencing decreases apoptosis induced by genotoxic agents in T98G human glioblastoma cells and in U2OS human osteosarcoma cells (Ianari et al. 2009), the loss of Retinoblastoma in breast cancer patients increases sensitivity to antimetabolite treatment and it is the only predictive factor of good clinical outcome (Derenzini et al. 2008).

It seems therefore that pRB can either promote apoptosis or inhibit apoptosis depending on cellular settings and environmental conditions (Bosco and Knudsen 2005; Masselli and Wang 2006; Indovina et al. 2015). Very little is known however about the role of p107 or p130 in apoptosis. Indeed, to the best of our knowledge, there is no published information about the role of p107 in apoptosis and there is very little information about the role of p130 in this process. The presence of p130 in human retinoblastoma samples is associated with increase of apoptotic index (Bellan et al. 2002) and p130 promotes radiation-induced cell death in the glioblastoma hamster cell line HJC12 (Pucci et al. 2002). However, the overexpression of p130 in CAOV-3 ovarian cell line can inhibit apoptosis triggered by camptothecin and doxorubicin (Tonini et al. 2004), suggesting, as for pRB, a dual role of p130 in cell death regulation regarding the cellular context and the environmental condition.

In conclusion, great efforts have been made to reveal Retinoblastoma functions in cell biology, but the molecular mechanisms involved in these functions are not

completely understood yet (Dyson 2016). A better knowledge of pRB role in apoptosis regulation and the use of new technologies to characterize better the heterogeneity of cancer biology could promote new therapeutic strategies aimed to the Retinoblastoma pathway (Knudsen and Wang 2010).

In this study, we wanted to perform a comprehensive structure-function analysis to define the major domains of pRB involved in the regulation of E2F activity, cell cycle progression, proliferation and cell death. Our findings strongly suggest that cell death inhibition by pRB requires a prolonged cell cycle arrest that cannot be provided by p107 or p130.

Objectives

The main objectives of this thesis are:

1- To determine the role of the Retinoblastoma family members in cell death regulation.

2- To assess the contribution of the different functional domains of pRB to cell death regulation.

3- To investigate whether cell death regulation by pRB is related to its capacity to inhibit E2F activity, cell cycle progression, or DNA replication.

4- To generate a 3-dimensional model of full-length pRB for structural analysis.

Materials and Methods

1. Plasmids

pcDNA3-HA-RB wt, pcDNA3-HA-RB 1-792, and pcDNA3-RB 379-928 were created by subcloning HA-RB wt, HA-RB 1-792, and RB 379-928 cDNA fragments from pSG5L-HA-RB, pSG5L-HA-RB 1-792, and pSG5L-RB 379-928, respectively into pcDNA3. pSG5L-RB 379-928, pSG5L-empty, pSG5L-HA-RB wt, pSG5L-HA-RB Δ503, pSG5L-HA-RB Δ529, pSG5L-HA-RB Δ535, pSG5L-HA-RB Δ542, pSG5L-HA-RB Δ651, pSG5L-HA-RB Δ657, pSG5L-HA-RB Δ678, pSG5L-HA-RB Δ685, pSG5L-HA-RB 661W, and pSG5L-HA-RB 567L were previously described (Sellers et al. 1998) and generously provided by W. Sellers (Dana-Farber Cancer Institute, Boston MA). To generate pcDNA3-p107-HA and pcDNA3-HA-p130, p107-HA and HA-130 were excised from pCMVp107-HA (Zhu et al. 1993) and pcDNA1-HA-p130 (Vairo et al. 1995) and subcloned into pcDNA3. pCMVp107-HA and pcDNA1-HA-p130 were a gift from M. Ewen; Dana-Farber Cancer Institute.

pcDNA3-HA-RB/p130 C-ter, pcDNA3-HA-p130/RB C-ter and pcDNA3-HA-RB C-ter were generated in the laboratory. SacII restriction site in nucleotide position 50-55 of RB wt (ID: 5925) was eliminated in pcDNA3-HA-RB wt by site-directed mutagenesis, introducing a silent mutation (primer sense 5' – CCGCCGCCGCTGCCGCAGCGGAACCCCCGGCAC – 3'; primer antisense 5' - GTGCCGGGGGTTCCGCTGCGGCAGCGGCGGCGG – 3'). Subsequently, a SacII restriction site was introduced in nucleotide position 2356-2361 of RB wt by site-directed mutagenesis, introducing two silent mutations (primer sense 5' - CACCAATACCTCACATTCCGCGGAGCCCTTACAAGTTTCC – 3'; primer antisense 5' – GGAAACTTGTAAGGGCTCCGCGGAATGTGAGGTATTGGTG – 3'). Digestion of pcDNA3-HA-RB wt with BamHI and SacII excised a cDNA fragment of RB from nucleotide 1 to nucleotide 2359 and maintained in the plasmid the carboxyl terminal nucleotide sequence (nucleotides 2360-2787). Site-directed mutagenesis was used also to introduce a SacII restriction site in nucleotide position 3049-3054 of p130 (ID: 5934) in pcDNA3-HA-p130 (primer sense 5' – CATCTACATCAAACAGCCGCGGACATTTGCCATGAAG – 3'; primer antisense 5' – CTTCATGGCAAATGTCCGCGGCTGTTTGATGTAGATG – 3'). This mutagenesis introduced I1017P and K1018R mutations (P and R are the same amino acids present in wt pRB). This plasmid was digested with BamHI and SacII to excise a p130 cDNA fragment from nucleotide 1 to nucleotide 3052 and to maintain the carboxyl terminal sequence of p130 in the plasmid (nucleotides 3053-3420). To generate pcDNA3-HA-RB/p130, nucleotides 1-2359 from RB were ligated to plasmid pcDNA3-HA-p130 previously mutated and digested with BamHI and SacII. pcDNA3-HA-p130/RB was generated by ligation of p130 nucleotides 1-3052 to plasmid pcDNA3-HA-RB

previously mutated and digested with BamHI and SacII. pcDNA3-HA-RB C-ter was generated by ligating a BamHI-AgeI-SacII adapter to pcDNA3-HA-RB previously mutated and digested with BamHI and SacII. pcDNA3-HA-RB ΔS was generated by site-directed mutagenesis of plasmid pcDNA3-HA-RB wt using oligonucleotides 5' - GAGAAGTTCCAGAAAATAAATCAGGCGGCA TGTAACAGCGACCGTGTG – 3' and 5' – CACACGGTCGCTGTTACATGCCGCCTGATTTATTTTCTGGAACTTCTC – 3'. The mutation consists in two amino acids substitution: M851A and V852A. pcDNA3-HA-RB ΔI (harboring a Stop-codon in nucleotide 2659 of RB) was generated by PCR using pcDNA3-HA-RB wt as template plasmid and the following oligonucleotides 5' – ACTGTTGGATCCATGCCGCCCAAAACCCCCCGAAAAACGG – 3' and 5' – TGACAACTCGAG TCA ATCTGCTTCATCTGATCCTTC – 3'. pcDNA3-HA-RB 13S was generated by site-directed mutagenesis of plasmid pcDNA3-HA-RB wt using oligonucleotides 5'-GCAGTATGCTTCCACCAATATGGATGCACCACCATTGTCACCAATACCTC – 3', 5'-GAGGTATTGGTGACAATGGTGGTGCATCCATATTGGTGGAAGCATACTGC – 3', 5'-AAAAATGACTCCAAGCTCAACAATCTTAGTATCAGAGCCATGGGAAATTGGTGAAT CATTC – 3', and 5' – GAATGATTCACCAATTTCCCATGGCTCTGATACTAAGATTGTTGAGCTTGGAGTCA TTTTT – 3'.

pcDNA3-HA-p27 was generated by subcloning p27 cDNA from pcDNA3-p27 (kindly provided by C. Calés; Instituto de Investigaciones Biomédicas Alberto Sols, CSIC-UAM, Madrid, Spain) into pcDNA3-HA. pcDNA3-HA-p21 was generated by subcloning from pRcCMVp21 (Adams et al. 1996), a generous gift from W.G. Kaelin (Dana-Farber Cancer Institute). pcDNA3-HA-BRG1 was generated from pSV-BRG1, (kindly provided from B. Belandia; Instituto de Investigaciones Biomédicas Alberto Sols), and pcDNA3-HA-BRG1 DN (K785R; Khavari et al. 1993) was generated by site-directed mutagenesis of plasmid pcDNA3-HA-BRG1, using the following oligonucleotides: 5' – GAGATGGGCCTGGGGCGAACCATCCAGACCATCG – 3' and 5' – CGATGGTCTGGATGGTTCGCCCCAGGCCCATCTC – 3'.

pcDNA3-9E10, pcDNA3-9E10-RB wt and pcDNA3-9E10-RB NPC were generously provided by S. Mittnacht (Cancer Institute, University College London, United Kingdom).

pcDNA3-HA-NLS-Stop was generated introducing the SV40 Large T antigen nuclear localization signal as previously described (Kalderon et al. 1984). The RB 1-792 cDNA fragment from excised from pcDNA3-HA-RB 1-792 and subcloned into this plasmid to obtain pcDNA3-HA-NLS-RB 1-792.

pCMV-BamNeo-flag-RB wt and pCMV-BamNeo-flag RB C-ter were generated in the laboratory from plasmid pcDNA3-flag-RB wt and pcDNA3-flag-RB C-ter, respectively (in turn generated in the laboratory). pCMV-BamNeo-flag-RB SE was kindly provided by J. Wang (Center for Molecular Genetics, and Cancer Center, University of California, San Diego, California, USA).

pBB14 plasmid was provided from L. W. Enquist (Department of Molecular Biology, Princeton University, NJ). This plasmid expresses a variant of the Green Fluorescent Protein (US9-GFP) able to anchorage to cellular membranes.

BG-Luc 3xE2F-DHFR wt, BG-Luc 3xE2F-DHFR mut, pGL2- E2F1 promoter wt, pGL2-prom E2F promoter mutated, pGL2-CDC6 promoter used in luciferase assays were previously described (Campanero et al. 2000). pGL2-p73 promoter that was generated by subcloning from pGL3-p73 promoter (a generous gift from M.C. Marín Vieira; Universidad de León, Leon, Spain). pRL-null (Promega; E-2271) was used for transfection normalization.

2. Cell culture and transfection

Saos2 cells were obtained from ATCC and grown at 37°C in a 5% CO_2 humidified incubator, in Dulbecco's modified Eagle media (DMEM; Gibco, Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS; Gibco- Thermo Fisher Scientific), 100 mM Glutamine (Gibco- Thermo Fisher Scientific), 100 U/ml Penicillin (Penilevel; Laboratorios ERN, S.A.) and 100 µg/ml Streptomycin sulfate (Laboratorio Reig Jofré, S.A.).

Transfection was performed with the Calcium Phosphate method, as previously described (Campanero et al. 2000). Co-transfection with a GFP-encoding plasmid (pBB14) was performed to select transfected cells by flow cytometry.

For experiments of cell cycle synchronization, cells were treated with 0.4 mM L-Mimosine (M-0253; Sigma-Aldrich), 1mM Hydroxyurea (H-8627; Sigma-Aldrich), 25 ng/ml Nocodazole (M-1404; Sigma-Aldrich) or 10 µg/ml Cycloheximide (C-1988; Sigma-Aldrich).

3. Immunoblot analysis and antibodies

To prepare protein extracts, cells were centrifuged 5 minutes at 500xg; cell pellets were suspended in 4 pellet volumes of WCE buffer (20 mM Hepes pH 7.9, 1 mM EDTA, 400 mM NaCl, 25% Glycerol; supplemented with 0.5% NP40, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM NaF, 1 µg/ml Pepstatin A, 1 µg/ml Leupeptin, 1 µg/ml Bestatin, and 2 µg/ml Aprotinin) and left 30 minutes on ice (vortexing occasionally). After 15 minutes of centrifugation at maximum speed at 4 °C, supernatants were collected and stored at -80°C.

Cytoplasmic/nuclear fractionation was performed using the NP-40 method. Cells were centrifuged 5 minutes at 500xg; cell pellets were suspended in 4 pellet volumes of Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0,1 mM EDTA, 0,1 mM EGTA; 1 mM Na_3VO_4, 1 mM PMSF, 1 mM NaF, 1 µg/ml Pepstatin A, 1 µg/ml Leupeptin, 1 µg/ml Bestatin and 2 µg/ml Aprotinin) and left 15 minutes on ice. NP-40 was added (0.6%) and the samples were vortexed and centrifuged 20 seconds at 14,000xg. The supernatant was transferred into a new tube and centrifuged at maximum speed for 15 minutes at 4°C. The new supernatant was transferred into a new tube and used as cytosolic fraction after adding Glycerol to reach a final concentration of 20%. The pellet obtained after the brief centrifugation was washed twice in Buffer A and suspended in 2.7 pellet volumes of Buffer B (20 mM Hepes pH 7.9, 20 % Glycerol, 1,5 mM $MgCl_2$ 0.2 mM EDTA; 1 mM Na_3VO_4, 1 mM PMSF, 1 mM NaF, 1 µg/ml Pepstatin A, 1 µg/ml Leupeptin, 1 µg/ml Bestatin and 2 µg/ml Aprotinin) before adding 0.3 pellet volumes of 4.2 M NaCl. Samples were maintained 20 minutes on ice, vortexing occasionally. The supernatant obtained from a final centrifugation at 14,000xg for 10 minutes at 4°C was used as the nuclear extract.

Protein concentration was measured using the Lowry method (DC Protein Assay Reagent kit, Bio-Rad 500-0116). Proteins (50 µg) were fractionated by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membranes (Amersham™ Protran™ 0,45 µm NC; GE Health Care Life Science). The following primary antibodies were used: anti-HA.11 (Covance); anti-RB (c-ter) (sc50X; Santa Cruz Biotechnology); anti-RB (pocket) (554136; BD Pharmigen); anti-αTubulin (T-9026, Sigma-Aldrich); anti-TFIIH (sc293X; Santa Cruz Biotechnology). Primary antibodies were detected with goat anti-mouse IgG antibody (IRDye 800CW_926-32210; LI-COR), donkey anti-rabbit IgG antibody (IRDye800CW_926-32213; LI-COR), or goat anti-mouse IgG antibody (IRDye 680_926-32220), employing LI-COR Odyssey technology. Immunoblot bands quantification was performed with Image Studio Lite Ver 5.2 (LI-COR).

4. Immunofluorescence

Cells were seeded in 10-mm-diameter cover glasses; fixed with 4% Formaldehyde (Riedel-de Haën, 15512), 4% Saccharose (Riedel-de Haën, 16104) in 1xPBS for 15 minutes at RT; and permeabilized using 0.1% Triton®X-100 (T-8787; Sigma-Aldrich) in 1xPBS for 5 minutes at RT. Blocking was performed for 30 minutes at RT in blocking solution (5% BSA and 5% goat serum in 1xPBS). Anti HA-11 (Covance) or anti-RB (c-ter) (sc50X; Santa Cruz Biotechnology) were used as primary antibodies and detected, respectively, with goat anti-mouse Alexa Fluor® 546 conjugate (A-11030; Molecular probers- Thermo Fischer Scientific) or goat anti-rabbit Alexa Fluor® 546 conjugate (A-11035; Molecular probers- Thermo Fischer Scientific). Nuclei were stained with DAPI (D-1306; Molecular probes- Thermo Fischer Scientific).

Images were obtained with a SP5 Leica confocal microscope equipped with a 63x magnification lens.

5. Cell cycle analysis, cell proliferation assay and cell growth assay

For cell cycle analysis, cells were fixed in ice cold 70% ethanol and stained with a solution of 69 mM Propidium Iodide (S-4170; Sigma-Aldrich) in 38 mM sodium citrate. 10 µg/ml RNase (R-6513; Sigma-Aldrich) was added and samples were incubated 30 minutes at 37°C. DNA profiles were analyzed by flow-cytometry using FACS-Can or FACS-Canto II (Becton Dickinson) and Cell Quest or FACS-Diva software (Becton Dickinson), respectively. Flowing Software 2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland) was employed for cell cycle analysis and representation of cell cycle histograms, while ModFit LT software (Verity software house) was employed for cell cycle's phases quantification. At least 10.000 events (or 10.000 GFP-positive events in the case of transfected cells) were evaluated.

Proliferation was determined by flow cytometry analysis of EdU incorporation. Cells were incubated with 10 µM EdU in complete DMEM medium for 2 hours at 37°C in a 5% CO_2 humidified incubator. Cells were stained following manufacturer's instructions (Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit; Molecular Probes- Thermo Fischer Scientific). Due to incompatibility of Click-iT kit with GFP fluorescence, a GFP-Tag Polyclonal Antibody was used (A-6455; Molecular Probes- Thermo Fisher Scientific) and detected with Alexa Fluor® 488-conjugated goat anti-mouse IgG secondary antibody (A-11029; Molecular Probes- Thermo Fischer Scientific). Samples acquisition was performed with FACS-Canto II (Becton Dickinson) and FACS-Diva software (Becton Dickinson) was used for the analysis. At least 10.000 GFP-positive events were evaluated.

Cell growth was assessed by counting viable cells (unstained by trypan blue) using a hemocytometer. Cells (100.000) were seeded in six-well plates treated 24 hours later with various drugs and subjected to ionizing radiation (IR;15 Gy) 24h later. The effects of ionizing radiation on cell growth were determined after 72 hours.

6. Luciferase assay

Luciferase assays were performed using Dual-Luciferase® Reporter Assay System (E-1910; Promega) following manufacturer's instructions. Cells were collected and lysed with 100 µl of Passive Lysis Buffer 1X (PLB 1X) and 50 µl were employed for the analysis. Analysis was performed with GloMax® 96 Microplate Luminometer (Promega), using 25 µl of LARII buffer, to stimulate Luciferase emission, and 25 µl of Stop and Glo buffer, to inactivate Luciferase and allow Renilla emission.

7. Nuclei fragmentation assay

Cells were plated on 22-mm^2 cover glasses and subjected 24h later to IR (15 Gy) using the biological irradiator SHEPHERD MARK I. Cells were fixed in ice cold 70% ethanol 72 later, washed with 1xPBS, and stained with a 69 mM Propidium Iodide (S-4170; Sigma-Aldrich) solution in 38 mM sodium citrate, diluted four times in 1xPBS. Transfected cells were exposed to IR (15 Gy) 48 hours after transfection. Images were acquired with an Axiophot Zeiss microscope and a 20X magnification lens, using DP-manager and DP-controller software. For each experiment, 200 cells or 200 GFP+ cells (transfected cells) were counted.

8. Bioinformatic study and analysis

Bioinformatic studies and analysis were performed at the Bioinformatic unit of the Spanish National Centre for Cardiovascular Research (CNIC). I-Tasser software suite v5.0 (Yang et al. 2015) was employed for modeling with homology, whereas Disopred v3.16 program (Jones and Cozzetto 2015) was used to predict secondary structure, disordered regions and protein binding profiles.

The loopmodel tool (Mandell et al. 2009; Stein and Kortemme 2013) of Rosetta suite v3.5 release 2015.38.58158 (www.rosettacommons.org) was used to close the gap between the N and A domains of pRB-prerefine and B and C domains of pRB_B-Ctail in the final model of the protein. For refinement, the relax tool (Nivon et al. 2013; Conway et al. 2014) of Rosetta suite v3.5 release 2015.38.58158 (www.rosettacommons.org) was used.

The representation of the pRB model was created with PyMOL 1.8.x.

9. Figures' elaboration and statistical analysis

Figures were elaborated with Adobe Photoshop CS6 and Adobe Illustrator CS6. Graphs and ANOVA test were generated with GraphPad Prism 6 (GraphPad Software, Inc.) considering significant results with a p-value <0.05.

Results

1. Effects of RB family members in cell cycle arrest and cell death inhibition

To compare the activities of the pocket proteins, pRB, p107 and p130, we forced their expression in the osteosarcoma cell line Saos2 because this cell line is considered as a good model for the study of pRB function in the context of cell cycle and apoptosis regulation. The Saos2 cell line was derived from a primary osteosarcoma of an 11 years-old Caucasian girl in 1973 (Fogh et al. 1977). This cell line is p53 deficient and expresses a truncated, non-functional form of pRB, but, responds to forced expression of wild-type (Thomas et al. 2004) pRB, as observed by changes in morphology and inhibition of growth rate, colony formation in soft agar, and tumorigenicity in nude mice (Huang et al. 1988; Shew et al. 1990). In 1995 Haas-Kogan et al. showed that ionizing radiation (IR) induced apoptosis in a time- and dose-dependent manner in Saos2 cells and that forced expression of wt pRB decreased IR-induced apoptosis in these cells (Haas-Kogan et al. 1995).

To confirm the sensitivity of Saos2 cells to IR, we exposed them to a 15Gy dose and analyzed their cell cycle distribution after several periods of time. We found an accumulation of cells in G2/M 16-24h post-IR (probably because these cells are also p53-negative), followed by an increase of a sub-G0 population, indicative of cell death, particularly 72h after IR (Fig. 1A).

To determine the role of pRB in cell death regulation, we transfected a pRB-encoding pcDNA3 vector or the empty vector together with a green fluorescent protein (GFP)-encoding plasmid in Saos2 cells and exposed them to 15 Gy of IR. Cell cycle analysis 72h after the treatment showed that RB-expressing cells (GFP^+) accumulated in G0/G1 and showed an almost negligible sub-G0 population (Fig. 1B, bottom-right panel), whereas the non-transfected cells (GFP^-) showed an almost negligible G0/G1 population and substantial G2/M and sub-G0 populations (Fig. 1B, bottom-left panel) that were comparable to those observed in cells transfected with an empty pcDNA3 plasmid (Fig. 1B, top panels). These results suggested that, as previously described (Haas-Kogan et al. 1995), pRB inhibits IR-induced cell death. We confirmed these results analyzing nuclei fragmentation in irradiated cells. Indeed, the overexpression of wt pRB in IR-treated cells sharply decreased the presence of fragmented nuclei in GFP^+ cells relative to control transfected GFP^+ cells (Fig. 1C, 1D).

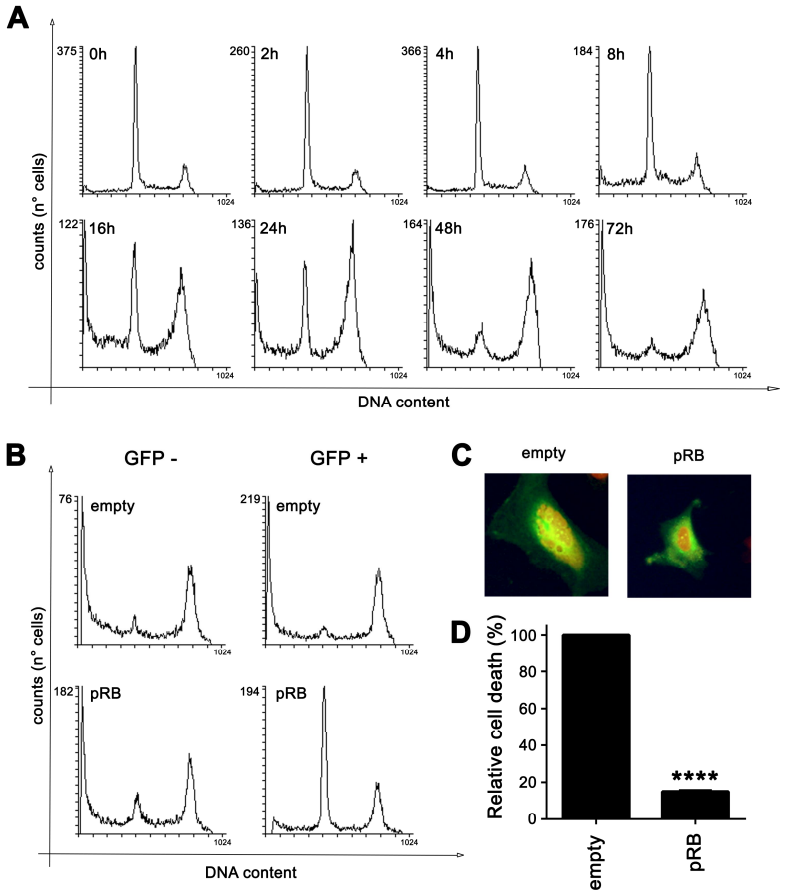

Figure 1. The Retinoblastoma protein inhibits IR-induced apoptosis in Saos2 cells. (A) Saos2 cells were co-transfected with pCDNA3 and a GFP-encoding plasmid (pBB14) to analyze the cell cycle in transfected cells. Cells were irradiated (15 Gy) 48 hours after transfection and their cell cycle distribution was analyzed by flow cytometry 2 hours, 4 hours, 8 hours, 16 hours, 24 hours, 48 hours and 72 hours later. **(B)** Saos2 cells were co-transfected with pBB14 plus either pRB-encoding or empty pcDNA3 to analyze the cell cycle in transfected (GFP+) cells and in non-transfected cells (GFP-). Cells were irradiated (15 Gy) 48 hours after transfection and their cell cycle distribution was analyzed by flow cytometry 72 hours later. **(C)** Representative images of nuclei fragmentation and (D) percentage of GFP+ cells with fragmented nuclei in cells treated as in (B) (n=3; media ± SEM). ****p<0.0001 vs empty; by one-way ANOVA with Tukey's multiple comparisons test.

Next step was to compare the activity of the pocket proteins (Fig. 2A) in cell proliferation, cell cycle control, E2F activity regulation, and cell death. The expression of HA-tagged pRB, p107, and p130 in transiently transfected Saos2 cells was confirmed by immunoblotting (Fig. 2B). As expected, forced expression of pRB inhibited cell proliferation (Fig. 2C) and induced accumulation of cells in the G0/G1 phase of the cell cycle relative to control cells (Fig. 2D). The overexpression of either p107 or p130 strongly inhibited cell proliferation and accumulated cells in G0/G1, but less markedly than pRB expression (Fig. 2C, 2D). Using a reporter plasmid with three copies of the wt E2F-binding site in the dihydrofolate reductase (*DHFR*) promoter, we found that the three pocket proteins

inhibited E2F activity similarly (Fig. 2E, filled bars). As expected, none of these proteins affected significantly the activity of a reporter driven by a mutant version of the E2F sites unable to bind E2F (Fig. 2E, empty bars).

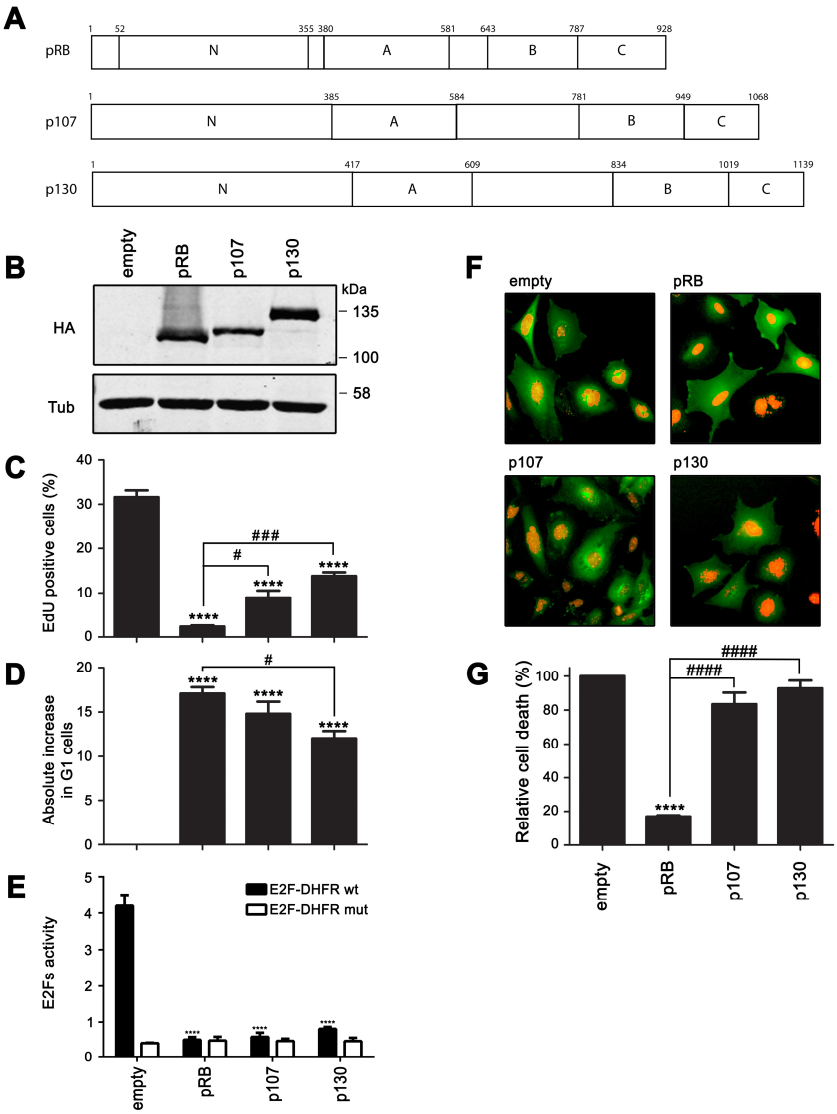

Figure 2. pRB is the only pocket family member that inhibits IR-induced cell death. **(A)** Schematic representation of pRB, p107 and p130. **(B-D)** Saos2 cells were co-transfected with pBB14 plus vectors encoding the indicated HA-tagged pocket proteins. **(B)** Representative anti-HA and Tubulin (Tub) immunoblots (n=3). **(C)** Quantification of cell proliferation as assessed by flow cytometry evaluation of EdU incorporation, and **(D)** cell cycle analysis, represented as absolute increase in G1 cells relative to control-transfected cells, 48 hours after transfection (n=3; mean + SEM). **(E)** The indicated E2F-DHFR luciferase reporter plasmids were co-transfected with pRL-null in asynchronously growing Saos2 cells in the presence of plasmids encoding the indicated pocket proteins or empty vector. 48 hours later, cell extracts were prepared and firefly and renilla luciferase assays were performed. Firefly luciferase values were normalized for renilla activity. Luciferase activity is shown relative to that in the presence of the empty vector (mean ± SEM; n=3). **(F)** Representative images of nuclear fragmentation and **(G)** percentage of GFP+ cells with fragmented nuclei 72h after IR exposure (15 Gy). Data are shown relative to empty vector as mean ± SEM (n=3). ****p<0.0001 vs empty; #p<0.05, ###p<0.001, ####p<0.0001 vs pRB; by one-way ANOVA with Bonferroni's multiple comparisons test (C, E) or with Tukey's multiple comparisons test (D, H).

We also assessed the capacity of the three pocket proteins to regulate cell death. While pRB forced expression sharply decreased IR-induced apoptosis in Saos2 cells, the overexpression of either p107 or p130 did not significantly inhibit or induce cell death (Fig. 2F, 2G). Together, these data indicate that while the pocket proteins share the capacity to inhibit E2F activity, cell cycle progression, and cell proliferation, pRB is the only pocket family member that possesses the capacity to inhibit cell death in a situation of DNA damage.

2. The ability of pRB in cell death inhibition is not regulated by phosphorylation

The control of cell cycle progression by pRB is strictly regulated by its phosphorylation state (Macdonald and Dick 2012; Rubin 2013). However, the phospho-regulation of pRB in cell death control is highly controversial (Dou et al. 1995; Adegbola and Pasternack 2005; De Leon et al. 2008; Lentine et al. 2012; Antonucci et al. 2014). For this reason, we investigated the effect of the overexpression of a phospho-defective mutant of pRB (RB-NPC) in which all the putative CDK phosphorylation sites (Fig. 3A) were substituted by Alanine (Chew et al. 1998). Immunoblot analysis showed that pRB-wt and pRB-NPC levels were similar in transiently transfected Saos2 (Fig. 3B).

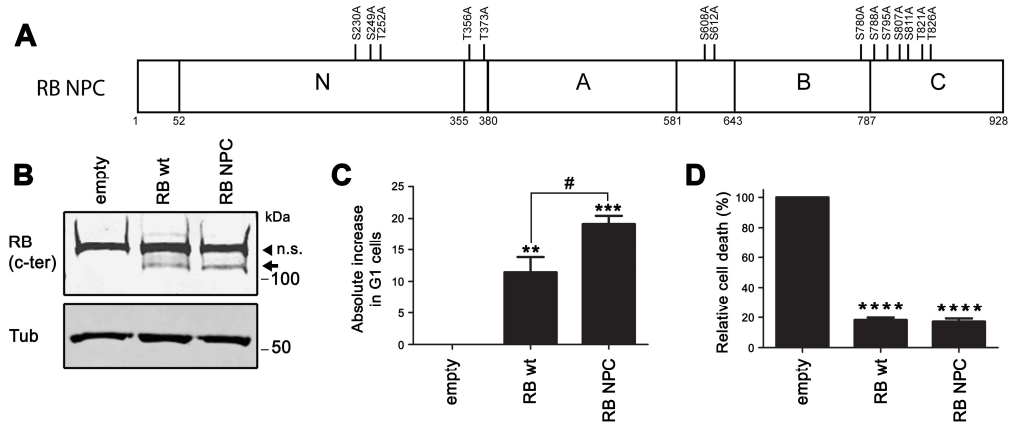

Figure 3. Phospho-resistant pRB inhibits IR-induced cell death. (A) Schematic representation of RB NPC showing positions mutated to Ala. Saos2 cells were co-transfected with pBB14 plus vectors encoding the indicated proteins or empty vector. **(B)** Representative anti-pRB (c-ter) immunoblot of these cells; the arrow and the arrowhead indicate the overexpressed proteins and a non-specific (ns) protein, respectively. Tubulin (Tub) was used as loading control. **(C)** Cell cycle analysis, represented as absolute increase in G1 cells relative to control-transfected cells, 48 hours after transfection (n=3; mean ± SEM). **(D)** Cells were exposed to IR (15 Gy) 48h after transfection and the percentage of GFP+ cells with fragmented nuclei 72h after IR exposure was determined; data are shown relative to empty vector as mean ± SEM (n=3). (C, D) **p<0.01, ***p<0.001, ****p<0.0001 vs empty; #p<0.05 vs pRB; by one-way ANOVA with Tukey's multiple comparisons test.

Forced expression of either pRB-wt or pRB-NPC accumulated cells in G0/G1 relative to control cells, but the phospho-defective mutant showed a significantly stronger effect ($p<0.05$) in G1 phase arrest than pRB-wt (Fig. 3C). However, both proteins inhibited IR-induced cell death with similar efficiency (Fig. 3D). Our data therefore indicate that phospho-deficient pRB, not only fails to promote apoptosis, but also inhibits cell death as efficiently as pRB-wt.

3. The inhibition of cell cycle progression and cell death by pRB is not mediated by BRG1

Brahma-related gene 1 or BRG1, is a member of the SWI/SNF family of proteins. Members of this family have helicase and ATPase activities that allow alterations in chromatin structure around genes that are directly regulated by these proteins. Following its isolation in 1993 (Khavari et al. 1993), BRG1 was found in complex with pRB (Dunaief et al. 1994; Strober et al. 1996) and it was described that BRG1 is required for pRB-mediated cell cycle arrest (Strobeck et al. 2000). We therefore hypothesized that BRG1 may be required for cell cycle arrest and inhibition of IR-induced cell death by pRB. If our hypothesis was correct, a dominant-negative version of BRG1 should inhibit the capacity of pRB to arrest cells in G1 and to inhibit IR-induced cell death.

To challenge this hypothesis, we transiently transfected Saos2 cells with a plasmid encoding a previously described (Khavari et al. 1993) dominant-negative version of BRG1 (BRG1 DN) or wt BRG1 together with pBB14 and a plasmid encoding wt pRB. Expression of these proteins was confirmed by immunoblot analysis (Fig. 4A). The expression of wt BRG1 or that of BRG1 DN did not substantially modify the DNA profile of the cells or the percentage of cell survival following IR exposure in the absence of ectopic pRB, and also failed to suppress the inhibition of cell cycle progression or IR-induced cell death by pRB (Fig. 4B, 4C). These data therefore strongly suggest that BRG1 does not mediate these inhibitory activities of pRB.

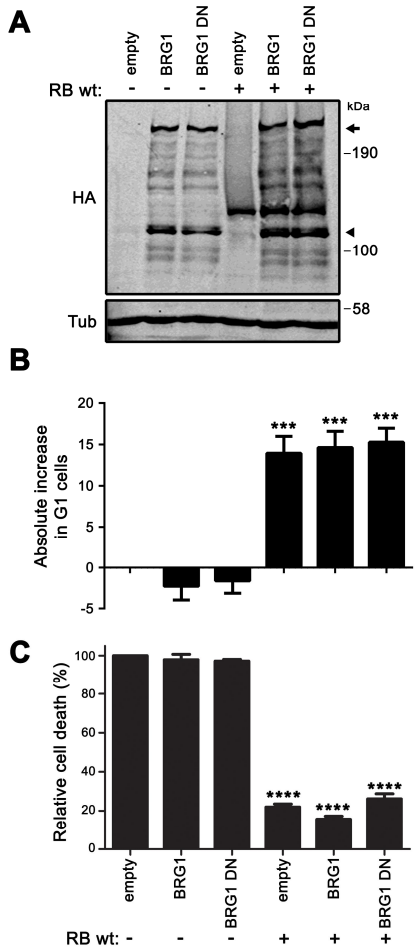

Figure 4 Effects of BRG1 and BRG1 DN over expression in Saos2 cell line. Saos2 cells were transfected with pBB14 and plasmids encoding the indicated proteins. **(A)** Representative anti-HA immunoblot showing the expression of pRB (arrowhead) and that of either wt BRG1 or BRG1 DN (arrow) 48 hours after transfection (n=3). Tubulin (Tub) was used as loading control. **(B)** Cell cycle analysis, represented as absolute increase in G1 cells relative to empty vector, evaluated 48 hours after transfection (mean ± SEM; n=3). **(C)** Percentage of GFP+ cells with fragmented nuclei 72h after exposure to IR (15 Gy). Data are shown relative to empty vector (media ± SEM; n=3). ***p<0.001, ****p<0.0001 vs empty; by one-way ANOVA with Tukey's multiple comparisons test.

4. Study of the effects of the different pRB functional domains in cell cycle arrest and cell death inhibition

Although the three pocket proteins share structural properties, the specific capacity of pRB to inhibit cell death could be potentially attributed to structural features unique to pRB. Indeed, p107 and p130 are more closely related to each other by sequence similarity than to pRB (Classon and Dyson 2001). The small pocket is the region with the highest homology between the three proteins and alterations of its sequence affect its activity in transcriptional regulation, differentiation induction and tumor growth suppression (Sellers et al. 1998). In contrast, the carboxyl terminus of pRB is the least homologous domain to p107 and p130 (Hasan et al. 2013).

4.1. The carboxyl terminal domain of pRB is necessary but not sufficient to inhibit cell death

To understand the role of the various domains of pRB in cell cycle arrest and cell death inhibition, we used pRB mutant derivatives lacking one or more functional domains. The effect of the wild-type protein (RB wt) was compared with that of mutants lacking either the carboxyl terminal (RB 1-792) or the amino terminal domain (RB 379-928) or with that of two fragments of the carboxyl terminus of the protein, RB SE (768-928) (Welch and Wang 1995b) and RB C-ter (786-928) (Fig 5A).

After transient transfection of Saos2 cells with plasmids encoding these proteins, we used western blotting to confirm their expression (Fig. 5B, 5C). We observed that RB 1-792 loss the capacity to inhibit cell proliferation (Fig. 5D), to arrest the cell cycle in G1 (Fig. 5E), to inhibit E2F activity (Fig. 5F) and to substantially protect cells from IR-induced death (Fig. 5G). Conversely, pRB large pocket (RB 379-928) inhibited all these processes as efficiently as the wild-type protein (RB wt) (Fig. 5D, 5G). Of note, the carboxyl terminal fragments of pRB (RB SE or RB C-ter) inhibited E2F activity as sharply as wt pRB (Fig. 5J) and substantially inhibited cell proliferation and cell cycle progression, but not as markedly as RB wt (Fig. 5H, 5I). However, RB SE and RB C-ter failed to inhibit IR-induced cell death (Fig. 5K).

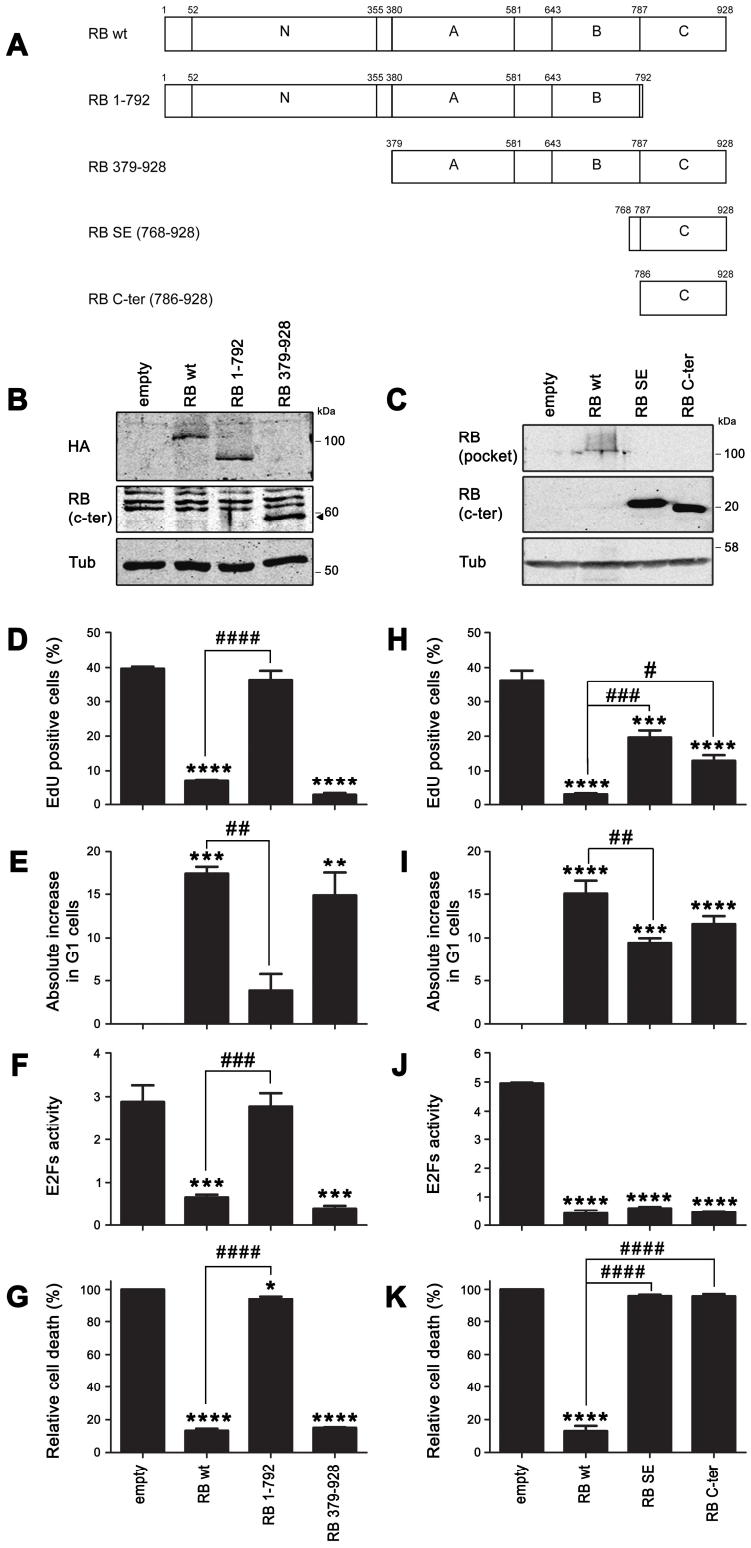

Figure 5. Functional analysis of pRB domains. (A) Schematic representation of pRB and indicated mutant derivatives RB 1-792, RB 379-928, RB SE and RB C-ter. Saos2 cells were co-transfected with pBB14 plus empty vector or **(D-G)** pcDNA3 encoding HA-tagged versions of the indicated proteins or **(H-K)** pCMV encoding the indicated proteins. **(B, C)** Representative anti-HA, anti-pRB (c-ter) or anti-pRB (pocket) immunoblots of these cells (n=3); the arrowhead indicates the position of RB 379-928. Tubulin (Tub) was used as loading control. **(D, H)** Quantification of cell proliferation as assessed by flow cytometry evaluation of EdU incorporation, and **(E, I)** cell cycle analysis, represented as absolute increase in G1 cells relative to control-transfected cells, 48 hours after transfection (n=3; mean ± SEM). **(F, J)** The wt E2F-DHFR luciferase reporter plasmid was co-transfected with pRL-null in asynchronously growing Saos-2 cells in the presence of plasmids encoding the indicated proteins or empty vector. 48 hours later, cell extracts were prepared and firefly and renilla luciferase assays were performed. Firefly luciferase values were normalized for renilla activity. Luciferase activity is shown relative to that in the presence of the empty vector (mean ± SEM; n=3). **(G, K)** Cells were exposed to IR (15 Gy) 48h after transfection and the percentage of GFP+ cells with fragmented nuclei 72h after IR exposure was determined. Data are shown relative to empty vector as mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs empty; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs pRB; by one-way ANOVA with Bonferroni's multiple comparisons test (D, F, H, J) or with Tukey's multiple comparisons test (E, G, I, K).

To complete our analysis of the pRB carboxyl terminal fragments RB SE and RB C-ter, we studied their subcellular localization. Cytoplasmic/nuclear fractionation showed a preferentially nuclear localization of the wild-type protein (RB wt), whereas similar levels of both RB SE and RB C-ter were detected in cytosolic and nuclear fractions (Fig. 6A, 6B). Confocal microscopy analysis of RB staining provided similar results (Fig. 6C).

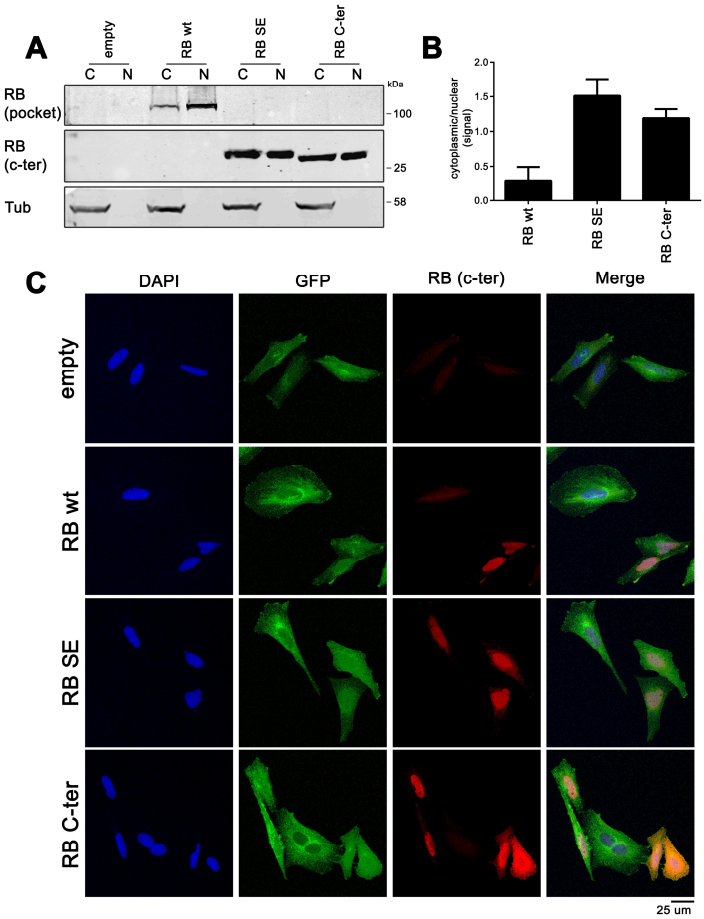

Figure 6. Sub-cellular localization of carboxyl terminal fragments of pRB. **(A)** Representative anti-pRB (pocket) or anti-pRB (c-ter) immunoblots of cytoplasmic and nuclear fractions of Saos2 cells transfected with pBB14 plus empty vector or pCMV encoding the indicated proteins. Tubulin (Tub) was used as loading control of cytoplasmic extracts. **(B)** Quantification of the cytoplasmic/nuclear ratio of densitometric values from these immunoblots shown as mean ± SEM (n=3). **(C)** Representative confocal microscopy images (n=3) of Saos2 cells transfected as in (A) and stained with anti-RB (c-ter) and DAPI. GFP auto-fluorescence is also shown. Scale bar, 25µm.

All these data allowed us to conclude that the amino terminus of pRB is not required for the regulation of cell cycle progression and cell death inhibition and that the carboxyl terminus is sufficient to block E2F activity and to inhibit cell cycle progression. Finally, the carboxyl terminus of pRB is necessary, but not sufficient, to inhibit IR-induced cell death.

4.2. Forced RB 1-792 expression in the nucleus does not recover its capacity to inhibit cell cycle progression or cell death

A bipartite nuclear localization signal (NLS), important for its biological activities, has been found in the carboxyl terminus of Retinoblastoma protein between amino acids 860-876 (Zacksenhaus et al. 1993). The pRB mutant derivative RB 1-792, which loss the capacity to arrest the cell cycle and to inhibit cell death, consequently lacks this NLS. We therefore investigated whether the lack of activity of this mutant could be attributed to its sequestration in the cytosol. Once confirmed the expression of RB wt and RB 1-792 in transiently transfected Saos2 cells (Fig. 7A), we determined their levels in cytosolic and nuclear fractions of these cells. While RB wt was detected preferentially in the nuclear fraction, RB 1-792 was preferentially detected in the cytosolic fraction (Fig. 7B, 7C). To force a nuclear localization of RB 1-792 we cloned the NLS of SV40 large tumor antigen (Kalderon et al. 1984) in the amino terminus of this mutant. The expression levels of this novel mutant, NLS-RB 1-792, were similar to those of RB 1-792 (Fig. 7A) but it was preferentially located in the nuclear fraction (Fig. 7B, 7C). Similar results were obtained by confocal microscopy analysis of RB staining in the same cells; RB wt and NLS-RB 1-792 were located mostly in the nucleus, whereas RB 1-792 was found both in the cytosol and in the nucleus (Fig. 7D). Although NLS-RB 1-792 showed a subcellular distribution similar to that of RB wt, it was as inactive as RB 1-792 arresting the cell cycle in G1 (Fig. 7E) or inhibiting IR-induced cell death (Fig. 7F).

These data therefore indicate that the pRB carboxyl terminus plays a critical role in cell growth and cell death inhibition regardless its contribution to the preferential location of pRB in the nucleus.

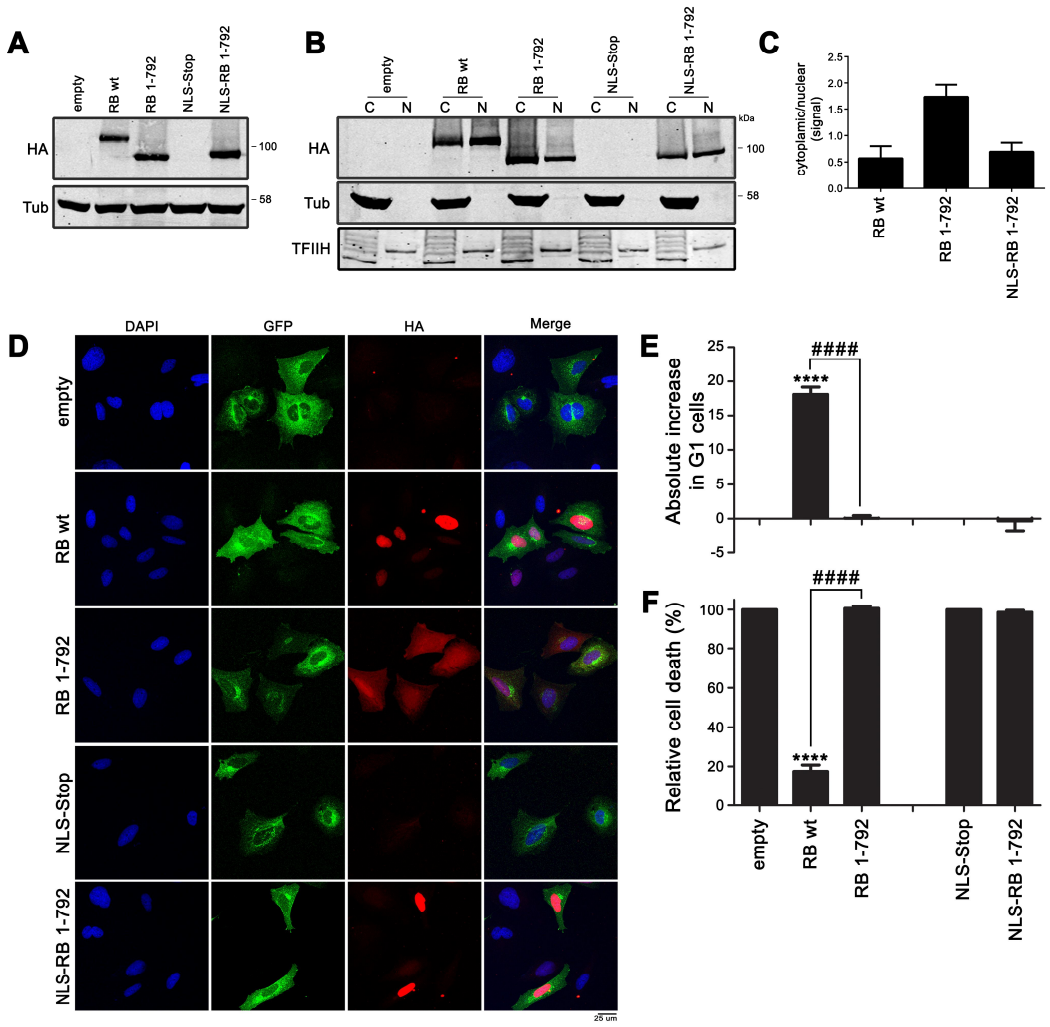

Figure 7. The restoration of RB 1-792 nuclear location does not recover its capacity to inhibit cell cycle progression or cell death. Representative anti-HA immunoblots of Saos2 cells transfected with plasmids encoding the indicated proteins employing **(A)** whole-cell extracts or **(B)** cytosolic and nuclear fractions of the same cells (n=3). Tubulin (Tub) was used as loading control of whole-cell extracts and cytosolic fractions, whereas TFIIH was used as loading control of nuclear extracts. **(C)** Quantification of the cytoplasmic/nuclear ratio of densitometric values from these immunoblots after normalization with the corresponding loading control (n=3; mean ± SEM). **(D)** Saos2 cells were co-transfected with pBB14 and vectors encoding the indicated proteins. Representative confocal microscopy images (n=3) of anti-HA immunofluorescence, GFP autofluorescence and DAPI staining are shown. Scale bar, 25µm. **(E)** Quantification of cell cycle analysis in the same cells, represented as absolute increase in G1 cells relative to control-transfected cells (empty), 48 hours after transfection (n=3; mean ± SEM). **(F)** Cells transfected as in (E) were exposed to IR (15 Gy) 48h later and the percentage of GFP+ cells with fragmented nuclei was determined after 72h. Data are shown relative to empty vector as mean ± SEM (n=3). ****p<0.0001 vs empty; ####p<0.0001 vs pRB; by one-way ANOVA with Tukey's multiple comparisons test.

4.3. The carboxyl terminus of pRB does not confer cell death inhibition activity to p130

We have shown that pRB is the only pocket family member with capacity to inhibit cell death and that its carboxyl terminus is required for this activity. Since the carboxyl terminus of pRB is the least homologous domain to p107 and p130, we constructed chimeric pRB/p130 and p130/pRB proteins exchanging only their carboxyl terminal fragments (Fig. 8A), and compared their functional activity with the non-chimeric wt pRB and p130 and with RB 1-792.

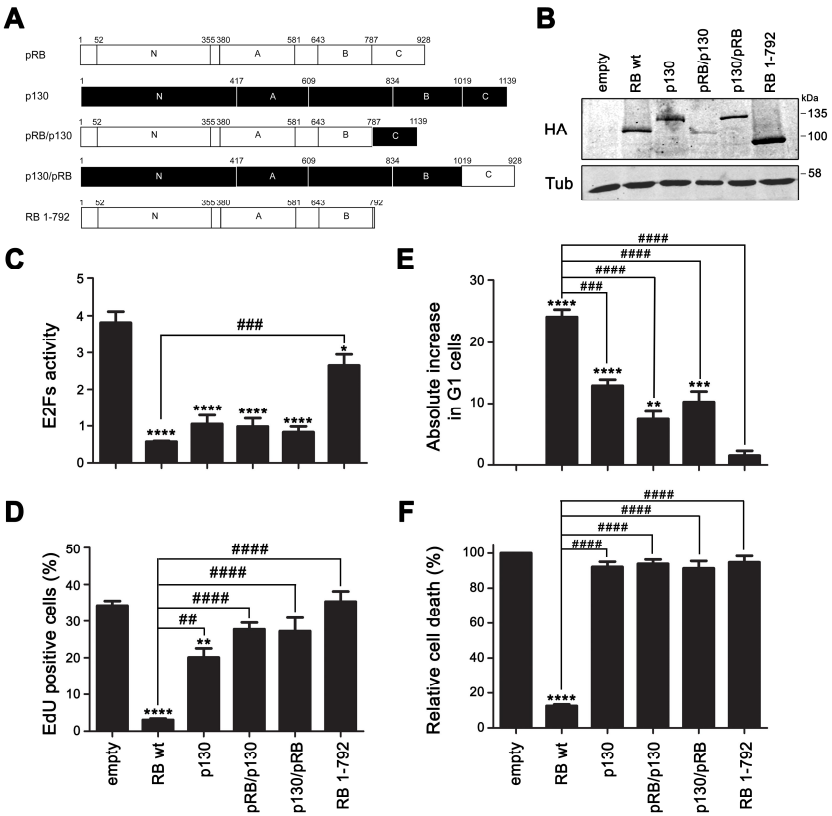

Figure 8. Characterization of chimeric pRB/p130 proteins. **(A)** Schematic representation of pRB, p130, RB 1-792 and chimeric pRB/p130 and p130/pRB proteins. **(B)** Representative anti-HA immunoblot of Saos2 cells transfected with pBB14 plus pcDNA3 encoding the indicated HA-tagged proteins or empty vector (n=3). Tubulin (Tub) was used as loading control. **(C)** The wt E2F-DHFR luciferase reporter plasmid was co-transfected with pRL-null in Saos-2 cells in the presence of plasmids encoding the indicated proteins or empty vector. 48 hours later, cell extracts were prepared and firefly and renilla luciferase assays were performed. Firefly luciferase values were normalized for renilla activity. Luciferase activity is shown relative to that in the presence of the empty vector (mean ± SEM; n=3). **(D-F)** Saos2 cells were transfected as in (B) and cell proliferation **(D)** and cell cycle distribution **(E)** were determined by flow cytometry evaluation of percentage of EdU incorporation (mean ± SEM; n=3) and propidium iodide staining, respectively, in GFP+ cells 48 hours after transfection. Data in **(D)** represent the absolute increase in G1 cells relative to empty vector (mean ± SEM; n=3). **(F)** Cells were exposed to IR (15 Gy) 48h after transfection and the percentage of GFP+ cells with fragmented nuclei was determined 72h after IR exposure. Data are shown relative to empty vector as mean ± SEM (n=3). **p<0.01, ***p<0.001, ****p<0.0001 vs empty; ##p<0.01, ###p<0.001, ####p<0.0001 vs pRB; by one-way ANOVA with Bonferroni's multiple comparisons test (C, D) or with Tukey's multiple comparisons test (E, F).

Western blotting of Saos2 cells transiently transfected with the different HA-tagged pocket proteins expression plasmids showed that all proteins were expressed at detectable levels (Fig. 8B). Although pRB/p130 levels were lower than those of wt proteins, this chimera inhibited E2F transactivation activity as efficiently as the wt proteins or as the p130/pRB chimera (Fig. 8C) and arrested cells in G1 as modestly as wt p130 (Fig. 8D), suggesting that the chimeric proteins were not completely misfolded. However, both chimeras failed to significantly inhibit cell proliferation and IR-induced cell death (Fig. 8E, 8F). Together, these results suggest that pRB cell growth and cell death inhibitory activities are mediated by a very precise configuration of the small pocket and the carboxyl terminus.

4.4. The association of the carboxyl terminus of pRB with E2F1, c-Abl or HDM2 is not required for inhibition of IR-induced cell death

In addition to a general binding site for E2F factors in pRB, involving the large pocket, an E2F1-specific binding site was found in the carboxyl terminus of pRB (Dick and Dyson 2003). While wt pRB blocked the proapoptotic activity of E2F1, RB ΔS (M851A; V852A) a mutant derivative of this binding site, failed to inhibit E2F1-induced apoptosis (Julian et al. 2008). c-Abl, a proapoptotic protein tyrosine kinase positively regulated by ATM in response to DNA damage, is negatively regulated by pRB (Wang 2000). A mutant pRB protein unable to bind c-Abl, RB 13S, was constructed (Whitaker et al. 1998). Caspases cleave the c-terminal 42 amino acid peptide of pRB during apoptosis induction and RB ΔI, a mutant RB protein that mimics the caspase-cleaved product, bound E2F1 but not MDM2 (Janicke et al. 1996).

To investigate whether the association of pRB with E2F1, c-Abl or HDM2 (the human ortholog of MDM2) mediates its capacity to inhibit cell death, we compared the functional activity of wt pRB with that of RB ΔS, RB 13S, and RB ΔI. We used site-directed mutagenesis to create HA-tagged versions of the three mutant derivatives (Fig. 9A) and forced their expression in Saos2 cells. Their expression levels were similar to those of wt pRB and they arrested cells in G1 and inhibited IR-induced cell death as efficiently as wt pRB (Fig 9B-9J).

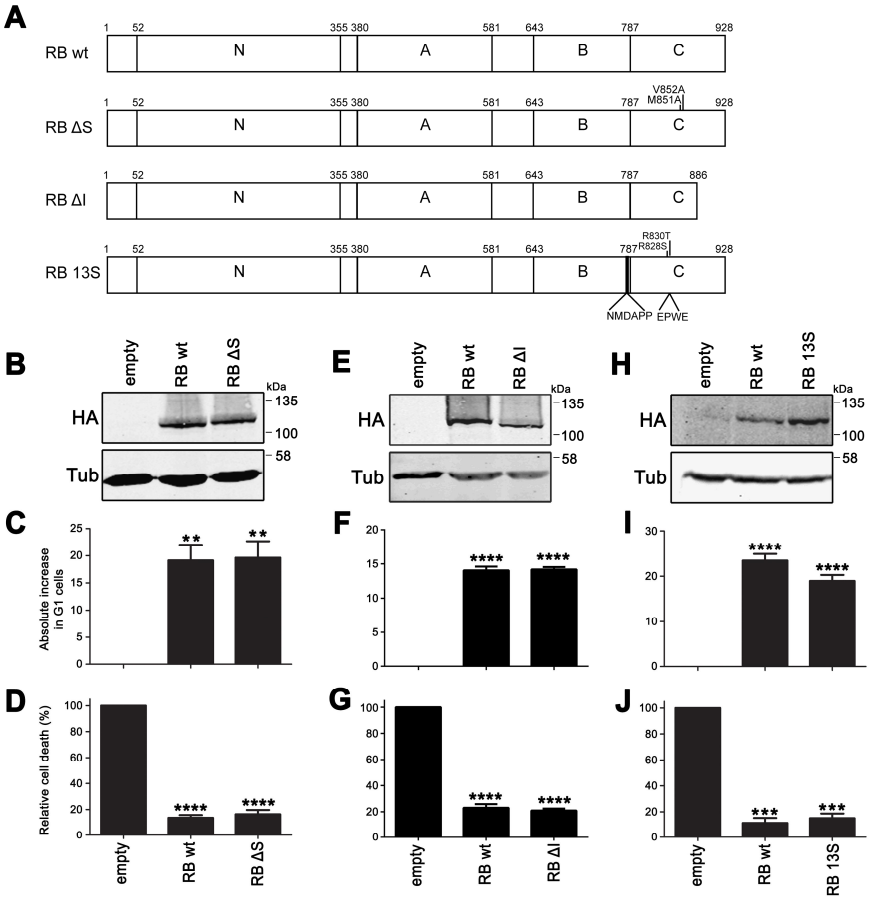

Figure 9. Cell cycle and cell death regulation by RB ΔS, RB ΔI and RB 13S. (A) Schematic representation of RB wt, RB ΔS, RB ΔI and RB 13S, indicating the mutations introduced in these proteins. **(B-J)** Saos2 cells were transfected with pBB14 plus vectors encoding the indicated HA-tagged proteins. **(B, E, H)** Representative anti-HA immunoblots of these cells (n=3). Tubulin (Tub) was used as loading control. **(C, F, I)** Cell cycle analysis, represented as absolute increase in G1 cells relative to empty vector, evaluated 48 hours after transfection in GFP+ cells (n=3; mean ± SEM). **(D, G and J)** Cells were exposed to IR (15 Gy) 48h after transfection and the percentage of GFP+ cells with fragmented nuclei was determined 72h after IR exposure. Data are shown relative to empty vector as mean + SEM (n=3 for RB ΔS and RB ΔI; n=2 for RB 13S). **p<0.01, ***p<0.001, ****p<0.0001 vs empty; by one-way ANOVA with Tukey's multiple comparisons test.

In addition, RB ΔS and RB 13S inhibited DNA replication and E2F transactivation capacity as efficiently as wt pRB (Fig. 10). Together, these data strongly suggest that the association of pRB c-terminal with E2F1, c-Abl, or HDM2 does not mediate cell death inhibition and cell growth inhibition capacities of pRB.

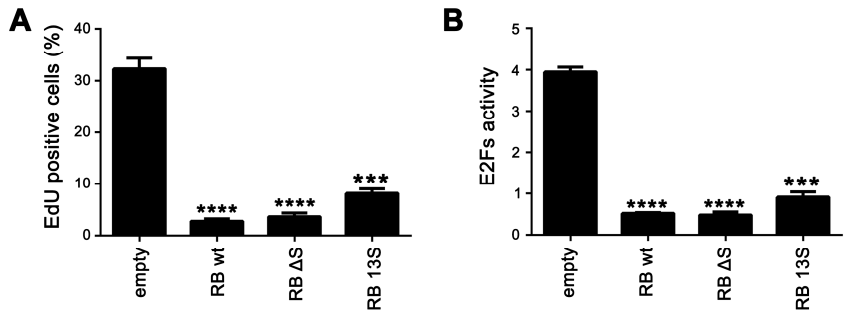

Figure 10. Regulation of DNA replication and E2F transactivation activity by RB ΔS and RB 13S. (A) Saos2 cells were transfected with pBB14 plus vectors encoding the indicated HA-tagged proteins and cell proliferation was assessed by flow cytometry evaluation of the percentage of EdU incorporation in GFP+ cells 48 hours after transfection (mean ± SEM; n=3). **(B)** The wt E2F-DHFR luciferase reporter plasmid was co-transfected with pRL-null in Saos2 cells in the presence of plasmids encoding the indicated proteins or empty vector. 48 hours later, cell extracts were prepared and firefly and renilla luciferase assays were performed. Firefly luciferase values were normalized for renilla activity. Luciferase activity is shown relative to that in the presence of the empty vector (mean ± SEM; n=3). ***p<0.001, ****p<0.0001 vs empty; by one-way ANOVA, Bonferroni's multiple comparisons test.

4.5. The small pocket is critical for pRB inhibition of cell death

The small pocket does not inhibit cell growth (Qin et al. 1992) but is required for cell growth suppression (Sellers et al. 1998). Accordingly, we hypothesized that although RB 1-792 failed to inhibit IR-induced cell death, it could be critical for cell death inhibition. To study the contribution of the small pocket to cell death inhibition, we used a set of mutants in which residues in the pRB sequence that are highly conserved in p107 and p130 were replaced with the flexible linker's sequence NAAIRS (Wilson et al. 1985; Sellers et al. 1998) (Fig.11A). Mutants were named according to the first substituted amino acid; for example, residues 503-508 were replaced with NAAIRS in RB Δ503.

We transiently transfected Saos2 cells with plasmids encoding HA-tagged versions of pRB mutants and analyzed their expression by Western blotting of whole cell extracts. All mutants were produced at comparable levels (Fig.11B). Analysis of the capacity of these mutants to inhibit cell proliferation (Fig.11C), arrest cells in G1 (Fig.11D) and inhibit E2F transactivation activity (Fig.11E) identified three groups of mutants according to their efficiency relative to wt pRB; RB Δ503, RB Δ529 and RB Δ685 acted like the wt pRB; RB Δ657 and RB Δ678 loss these activities; and RB Δ535, RB Δ542 and RB Δ651 showed an intermediate effect (Fig. 11C, 11E). We then analyzed the capacity of selected mutants representative of each group (RB Δ685, RB Δ657 and RB Δ651) to inhibit IR-induced cell death and found that RB Δ685 inhibited cell death as efficiently as

wt pRB, whereas RB Δ657 failed to prevent nuclei fragmentation and RB Δ651 showed an intermediate activity (Fig.11F, 11G).

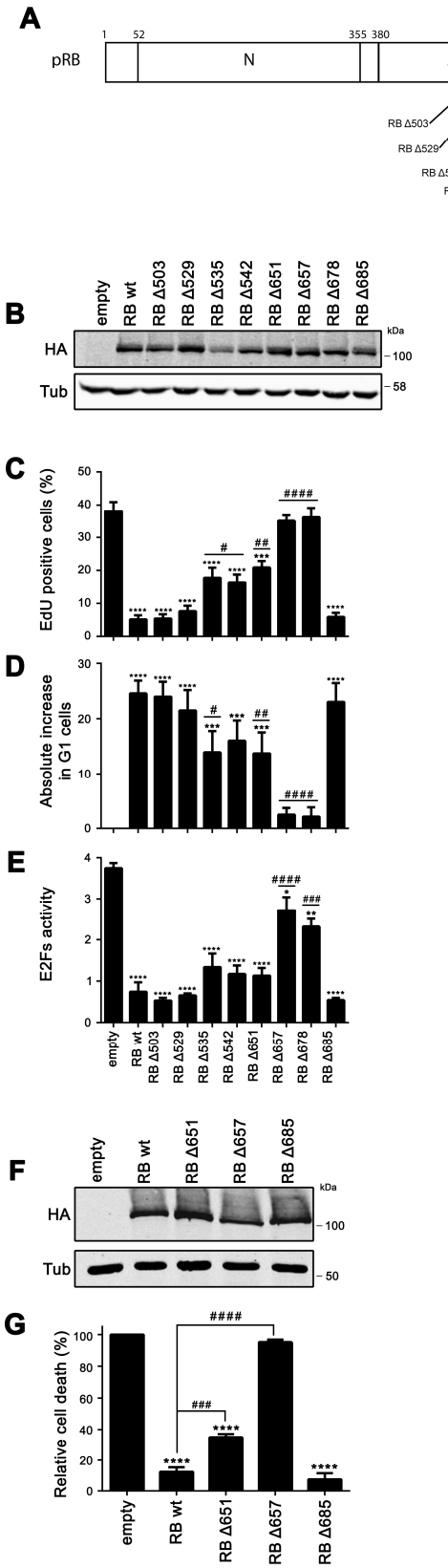

Figure 11. Functional characterization of pRB small pocket mutants. **(A)** Schematic representation of the localization of the different mutants in pRB. (B-D) Saos2 cells were co-transfected with pBB14 and plasmids encoding the indicated HA-tagged proteins. (B) Representative anti-HA and Tubulin (Tub) immunoblots of these cells (n=3). (C) Quantification of cell proliferation by flow cytometry evaluation of the percentage of GFP+ cells incorporating EdU 48h after transfection (mean ± SEM; n=3), and (D) cell cycle analysis of GFP+ cells, represented as absolute increase in G1 cells relative to empty vector, evaluated 48 hours after transfection (mean ± SEM; n=3). (E) The wt E2F-DHFR luciferase reporter plasmid was co-transfected with pRL-null in Saos2 cells in the presence of plasmids encoding the indicated proteins or empty vector and firefly and renilla luciferase assays were performed 48h later. Firefly luciferase values were normalized for renilla activity. Luciferase activity is shown relative to that in the presence of the empty vector (mean ± SEM; n=3). (F-G) Saos2 cells were co-transfected with pBB14 and plasmids encoding the indicated HA-tagged proteins. (F) Representative anti-HA and Tubulin (Tub) immunoblots of these cells (n=3). (G) Cells were exposed to IR (15 Gy) 48h after transfection and the percentage of GFP+ cells with fragmented nuclei was determined 72h after IR exposure. Data are shown relative to empty vector as mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs empty; #<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 vs pRB; by one-way ANOVA with Bonferroni's multiple comparisons test (C and E) or with Tukey's multiple comparisons test (D and G).

These data therefore indicate that not only the c-terminal of pRB is required for cell death inhibition but also its small pocket. To further confirm the relevance of the small pocket in cell death inhibition, we assessed the capacity of pRB mutants in the A and B domain identified in human tumors: the low-penetrance mutant RB R661W (Onadim et al. 1992; Lohmann et al. 1994) and the non-functional RB S567L (Yandell et al. 1989) (Fig. 12A). We transiently transfected plasmids encoding these mutants in Saos2 cells and observed that both mutants were expressed (Fig. 12B). Like RB Δ651, the low-penetrance mutant RB R661W inhibited cell cycle progression and IR-induced cell death, but less efficiently than RB wt (Fig. 12C, 12D). In contrast, RB S567L failed to inhibit cell cycle progression and cell death (Fig. 12C, 12D).

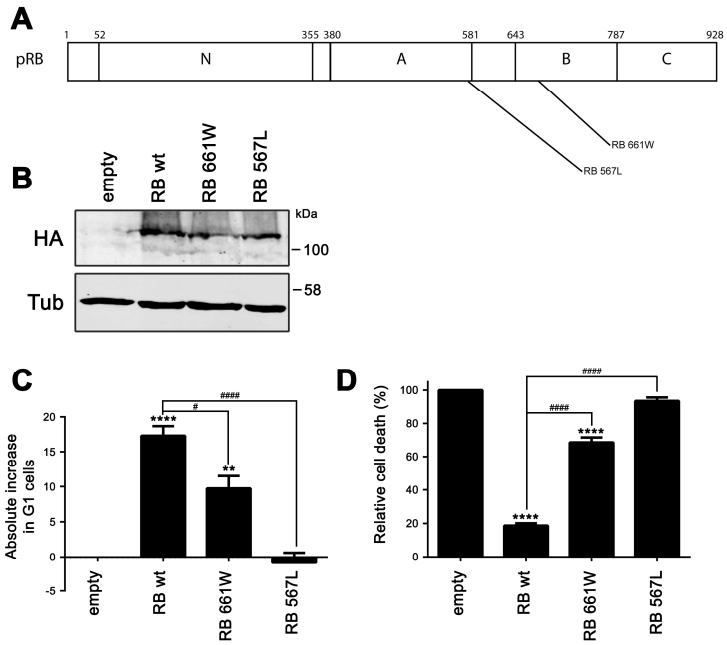

Figure 12- Functional characterization of RB 661W and RB 567L mutants. **(A)** Schematic representation of the localization of RB 661W and RB 567L mutants in pRB. **(B-D)** Saos2 cells were co-transfected with pBB14 and plasmids encoding the indicated HA-tagged proteins. **(B)** Representative anti-HA and Tubulin (Tub) immunoblots of these cells (n=3). **(C)** Cell cycle analysis of GFP+ cells, represented as absolute increase in G1 cells relative to empty vector, evaluated 48 hours after transfection (mean ± SEM; n=3). **(D)** Cells were exposed to IR (15 Gy) 48h after transfection and the percentage of GFP+ cells with fragmented nuclei was determined 72h after IR exposure. Data are shown relative to empty vector as mean ± SEM (n=3). **p<0.01, ***p<0.001, ****p<0.0001 vs empty; #p<0.05 vs pRB; by one-way ANOVA with Tukey's multiple comparisons test.

5. Correlation between long-term cell cycle arrest in G0/G1 and cell death inhibition

How pRB inhibits cell death? Why the over expression of p107, p130 or the Carboxyl terminus of pRB induces an increase in G1, but fail to inhibit IR-induced cell death? Are cell growth and cell death regulation independently regulated or is there a correlation between cell cycle arrest and cell death inhibition?

5.1. Permanent G0/G1 arrest induced by drugs avoid nuclei fragmentation

Our data show a tight correlation between the inhibitory activities of pRB in cell proliferation and cell death. We therefore hypothesized that pRB-independent means of cell cycle inhibition might also inhibit IR-induced cell death. To challenge this hypothesis, we used a pharmacological approach to either arrest or synchronize pRB-deficient Saos2 cells in different phases of the cell cycle and assess their sensitivity to IR.

Low concentration of the protein synthesis inhibitor cycloheximide (CHX) arrests cells in G1 (Hung et al. 1996; Liu et al. 2010). We therefore assess its capacity to stably arrest cells in G1 and to inhibit IR-induced cell death. Although the DNA profile of cells treated 24h with CHX did not show a marked G1 arrest, its capacity to block cell cycle progression in G1 was evident when cells previously synchronized with Nocodazole were released for 24h in the presence of CHX (Fig. 13A). Contrary to previous observations showing that CHX did not induce cell death in Saos2 cells (Morimoto et al. 1999), we found that CHX modestly induced death of these cells, as evidenced by the presence of a sub-G1 population in CHX-treated cells (Fig. 13A) and by the decrease in the number of cells after 3 days of CHX treatment (Fig. 13B). Despite its modest toxicity, cells arrested by CHX treatment were resistant to IR-induced cell death (Fig. 13C).

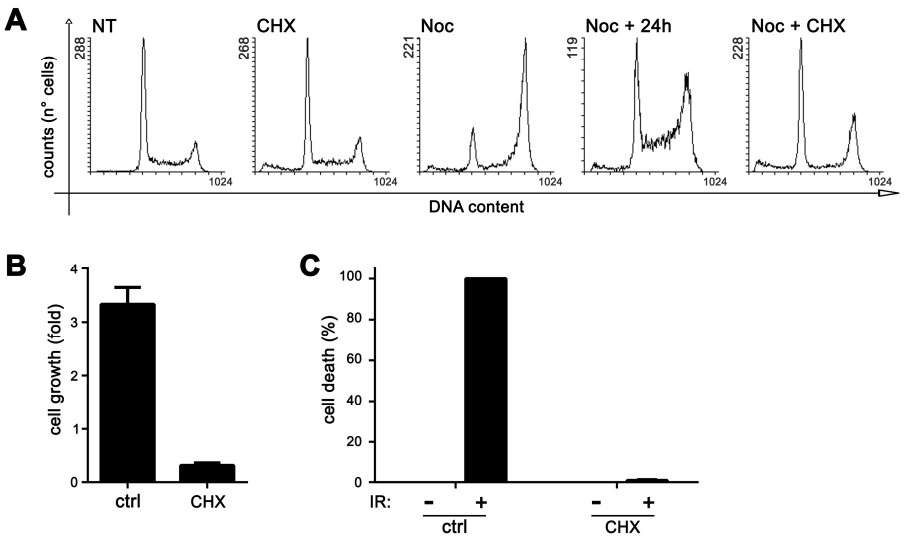

Figure 13. Cell cycle arrest by cycloheximide blocks IR-induced cell death. Saos2 cells were treated with 10 µg/ml cycloheximide (CHX) or in the absence of stimuli (NT) and subjected to ionizing radiation (15 Gy). Alternatively, Saos2 cells were treated 24h with 25 ng/ml nocodazole (Noc); treated with nocodazole, washed and cultured without additional stimuli for 6h (Noc+6h) or 24h (Noc+24h); or washed and treated for additional 24h with 10 µg/ml CHX (Noc+CHX). **(A)** Representative DNA profiles of these cells. **(B)** Cells were irradiated 24h after CHX addition and the effect on cell growth of indicated treatments was evaluated 3 days later by counting viable cells in the presence of trypan blue. Data show cell numbers at the end of the experiment relative to numbers immediately before exposure to IR (media ± SEM; n=3). **(C)** Percentage of cells with fragmented nuclei 72 hours after irradiation (IR) or in non-irradiated cells (NO IR) (media ± SEM; n=3).

Our data show a correlation of stable cell cycle arrest and resistance to IR-induced cell death, but we cannot rule out that CHX inhibits apoptosis by blocking the expression of pro-apoptotic proteins. We therefore assessed the capacity of additional drugs that block cell cycle progression without inhibiting protein synthesis. In particular, we used mimosine (Mim), a tyrosine-related alkaloid that arrests cells in the late G1 phase of the cell cycle by inhibiting DNA replication initiation; hydroxyurea (HU), an antineoplastic drug that inhibits DNA synthesis and induces cell death in S phase and arrests surviving cells in this phase; and nocodazole (Noc), an antineoplastic agent that inhibits microtubules polymerization and arrests cells in G2/M before killing them.

As expected, cells treated for 24h with Mim, HU, or Noc were arrested in late G1, S, or G2/M respectively, whereas cells treated sequentially with Noc and Mim or with Mim and HU were arrested late in G1 or at the G1/S transition, respectively (Fig. 14A). Similarly, cells cultured 6h in the absence of drugs after 24h of treatment with Mim, HU, or Noc were synchronized early in S, late in S, or early in G1, respectively (Fig. 14A).

Long-term treatment (4 days) of cells with Mim or HU not only blocked cell proliferation but also induced cell death in a major fraction of the cells and therefore decreased the total number of cells in culture (Fig. 14B, empty bars). Of note, irradiation of surviving cells, stably arrested in G1 (Mim) or early in S (HU), did not decrease the number of cells any further (Fig. 14B, filled bars), suggesting that stably arrested cells may be resistant to IR-induced cell death.

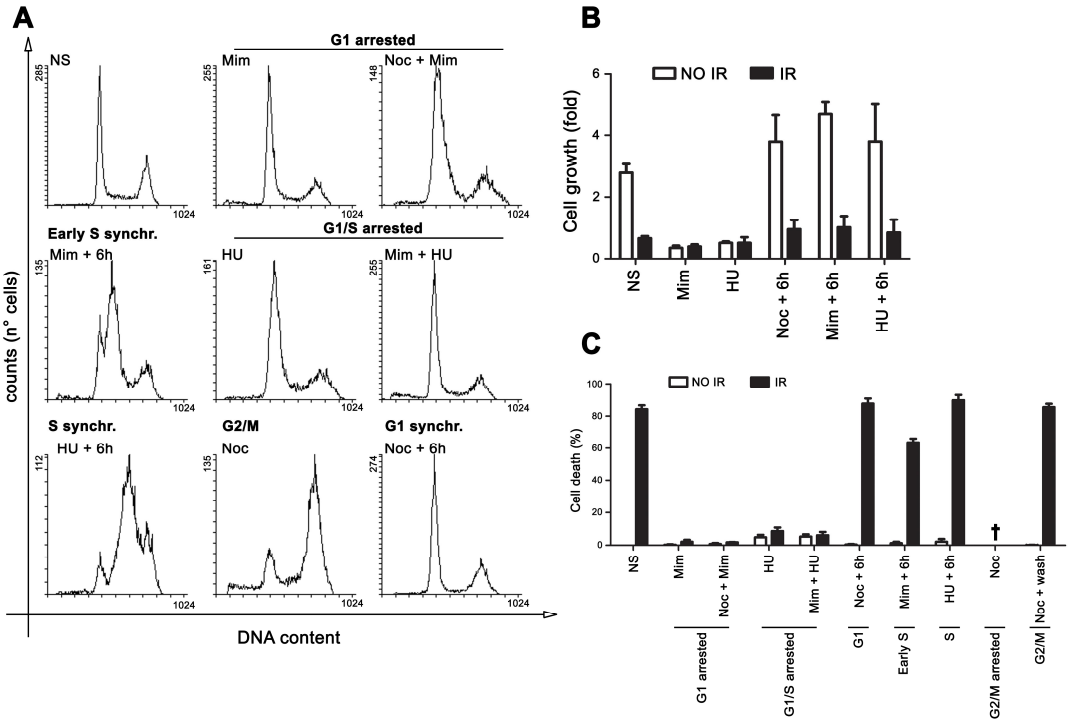

Figure 14. Saos2 cells stably arrested with mimosine or hydroxyurea are resistant to IR-induced cell death. Saos2 cells were either arrested or synchronized in different cell cycle phases using various drugs combinations and exposed to IR (15 Gy). Cells treated with 0.4 mM L-mimosine (Mim), 1mM hydroxyurea (HU), or 25 ng/ml nocodazole (Noc) for 4 days to stably arrest them late in G1, in S, or in G2/M, respectively, were irradiated 30h after the beginning of the treatment. Alternatively, cells were treated with 0.4 mM L-mimosine, 1mM hydroxyurea, or 25 ng/ml nocodazole for 24h, washed to eliminate the stimuli, and exposed to IR 6h later to irradiate cells synchronized early in S (Mim+6h), late in S (HU+6h) or early in G1 (Noc+6h), respectively. Finally, cells were treated with 0.4 mM L-mimosine or 25 ng/ml nocodazole for 24h, washed to eliminate these stimuli, and exposed to IR after an additional treatment with 1mM hydroxyurea (Mim+HU) or 0.4 mM L-mimosine (Noc+Mim) for another 24h to irradiate cells stably arrested late in G1 or at the G1-S transition, respectively. **(A)** Representative DNA profiles of these cells immediately before IR treatment (n=3). **(B)** The effect on cell growth of the indicated treatments was evaluated 3 days after irradiation by counting viable cells in the presence of trypan blue. Data show cell numbers at the end of the experiment relative to numbers immediately before exposure to IR (media ± SEM; n=3). **(C)** Percentage of cells with fragmented nuclei 3 days after irradiation (media ± SEM; n=3). NS, non-synchronized cells; NO IR, non-irradiated cells; IR, irradiated cells; †, no cells survived.

Accordingly, cells stably arrested late in G1 (Mim and Noc+Mim), early in S (HU), or at the G1/S transition (Mim+HU) were extremely resistant to IR-induced cell death, as determined by the near absence of fragmented nuclei in these cells (Fig. 14C). In contrast, irradiation of cells synchronized, but not stably arrested, early in G1 (Noc+6h), early in S (Mim+6h), or late in S (HU+6h) sharply decreased the number of cells in culture relative to non-irradiated cells (Fig. 14B) and markedly induced nuclei fragmentation (Fig. 14C), indicating that non-stably arrested cells were sensitive to IR.

We also determined the sensitivity of cells treated for 24h with Mim and then released for different periods to time and found that regardless the cell cycle phase in the G1-S transition, most non-stably arrested cells showed fragmented nuclei upon irradiation (Fig. 15). Finally, cells synchronized in G2/M (treated 24h with Noc and then washed) were also sensitive to IR-induced cell death (Fig. 14C). We could not assess the sensitivity of cells stably arrested in G2/M because no cells survived a 4-day treatment with nocodazole (Fig. 14C).

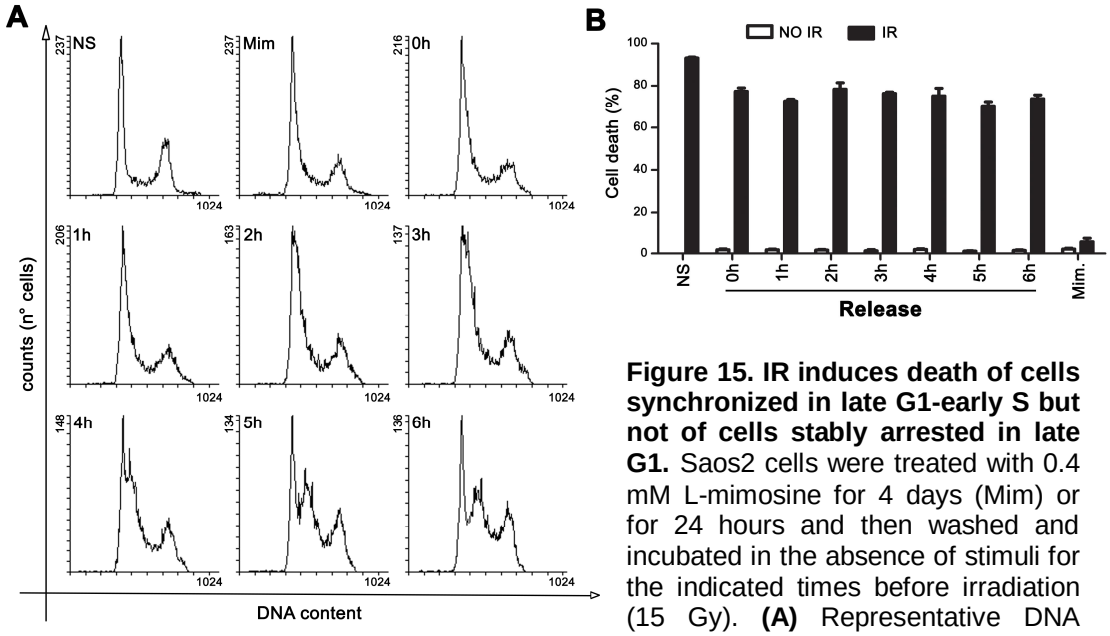

Figure 15. IR induces death of cells synchronized in late G1-early S but not of cells stably arrested in late G1. Saos2 cells were treated with 0.4 mM L-mimosine for 4 days (Mim) or for 24 hours and then washed and incubated in the absence of stimuli for the indicated times before irradiation (15 Gy). **(A)** Representative DNA profiles of these cells immediately before irradiation. **(B)** Percentage of cells with fragmented nuclei 72 hours after irradiation exposure (IR) and in the non-irradiated population (NO IR) (media ± SEM; n=3). NS, non-synchronized cells.

Together, our data strongly support the idea that a permanent arrest in the G1 phase of the cell cycle is necessary and sufficient to inhibit IR-induced cell death.

5.2. pRB is the only pocket protein that arrest cells in G0/G1 in the long-term

Since a pharmacological long-term arrest in the G1 phase of the cell cycle inhibits IR-induced cell death, we investigated whether pRB family members and pRB carboxyl terminus have the capacity to induce a long-term cell cycle arrest. We therefore analyzed the short-term and long-term (48h and 5 days after transfection, respectively) functional consequences of forcing the expression of pRB family members and mutant derivatives of pRB. We included in this analysis the three members of the Retinoblastoma family and the two pRB carboxyl terminal domain proteins, (RB SE and RB C-ter). We selected 5 days after transfection as a long-term time point because we have assessed cell death in IR-treated cells 5 days after transfection.

Plasmids encoding HA-tagged Retinoblastoma family members were transiently transfected in Saos2 cells and the expression of these proteins was detected both 48h and 5 days (Fig.16A). Although all the pocket proteins induced increase in G1 phase of the cell cycle at short time, only pRB retained the capacity to arrest cells after 5 days (Fig.16B). Forced expression of RB SE or RB C-ter was also detected in the short- and in the long-term (Fig. 16C) and, like p107 and p130, both proteins inhibited the cell cycle 48h after transfection, but failed to arrest cells in G1 in the long-term (Fig. 16D).

To confirm that pRB is the only pocket protein capable of stably arrest the cell cycle in G1, we used an alternative experimental approach taking advantage of the capacity of nocodazole to block the cell cycle in G2/M unless cells are previously arrested in G1 (Whitaker et al. 1998). We transiently transfected Saos2 cells with plasmids encoding each pocket protein and treated them two days later with Nocodazole for another 24h. The expression of these proteins was determined at the end of the experiment and we found that it was not affected by the treatment (Fig. 16E). As expected, nocodazole induced accumulation of control-transfected cells in G2/M (Fig. 16F). While forced expression of pRB blocked nocodazole-induced accumulation of cells in G2/M, the overexpression of p107 or p130 poorly inhibited the arrest in G2/M induced by nocodazole (Fig. 16F). Similarly, the ectopic expression of RB SE or RB C-ter was not affected by nocodazole (Fig. 16G) and failed to block nocodazole-induced arrest in G2/M (Fig. 16H).

Together, these data indicate that the accumulation of cells in G0/G1 induced by p107, p130, RB SE, or RB C-ter in the short-term is not the

consequence of a permanent arrest of the cell cycle and further support the notion that long-term cell cycle arrest in G0/G1 inhibits IR-induced cell death.

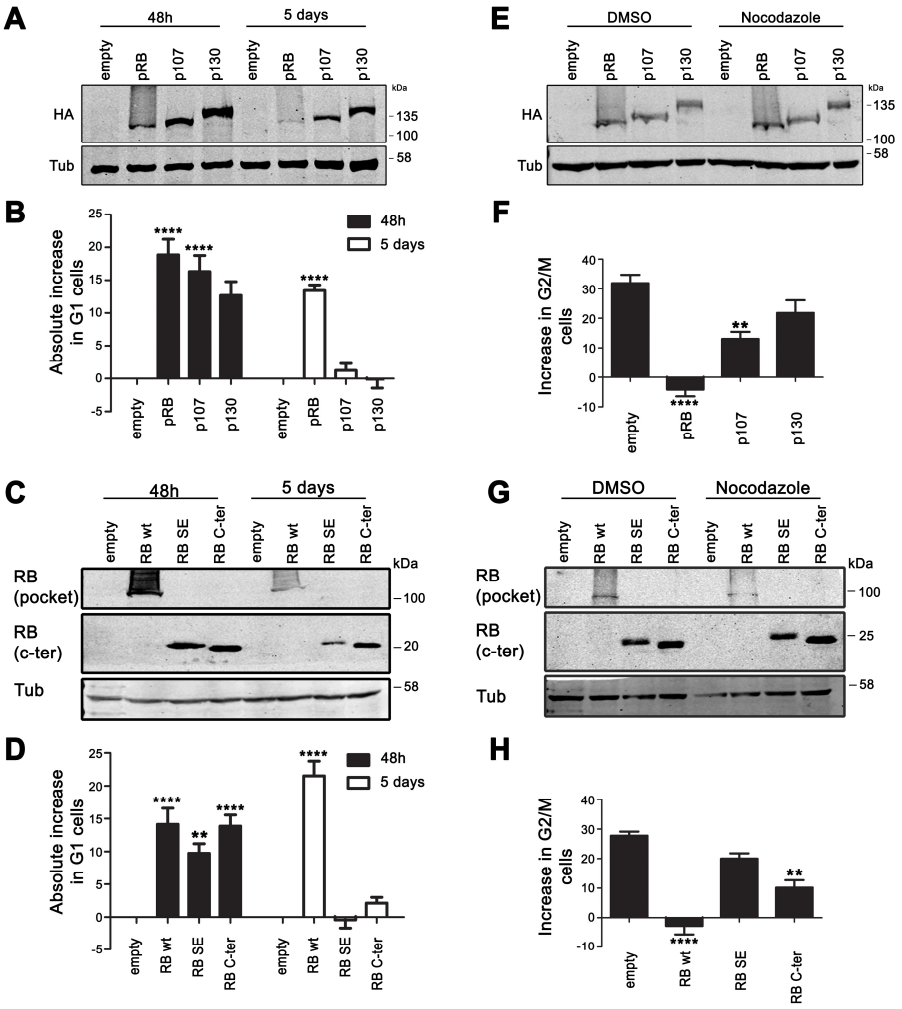

Figure 16. pRB is the only pocket protein with long-term capacity to arrest cells in G1. Saos2 cells were co-transfected with pBB14 and plasmids encoding the indicated proteins. **(A-D)** Two days and 5 days after transfection, protein extracts were prepared and the cell cycle was analyzed. **(A, C)** Representative anti-HA and anti-RB immunoblots (n=3) of these cells, Tubulin (Tub) was used as loading control. **(B, D)** Cell cycle analysis, represented as absolute increase in G1 cells relative to empty vector, evaluated 48 hours (filled bars) and 5 days (empty bars) after transfection (mean ± SEM; n=3). **(E-H)** Two days after transfection, Saos2 cells were treated with 25 ng/ml nocodazole (or DMSO) for 24 hours. **(E, G)** Representative anti-HA and anti-RB immunoblots (n=3) of these cells. **(F, H)** Cell cycle analysis, represented as increase in G2/M cells relative to DMSO-treated cells (mean ± SEM; n=3). **p<0.01, ***p<0.001, ****p<0.0001 vs empty; by two-way ANOVA with Tukey's multiple comparisons test (B, D) or one-way ANOVA with Bonferroni's multiple comparisons test (F, H).

5.2.1. pRB is the only pocket protein that inhibits E2F activity in the long-term

The capacity of the pocket proteins to inhibit cell cycle progression has been linked to their capacity to inhibit E2F activity (Dyson 1998). As pRB is the only pocket protein that inhibits cell cycle progression in the long-term, we investigated the long-term capacity of pRB family members, RB 1-792, and RB C-ter to inhibit E2F activity using luciferase reporter vectors driven by various E2F-responsive promoters. We transiently co-transfected Saos2 cells with plasmids encoding these proteins and reporter vectors driven by the promoters of *E2F1*, *CDC6*, tumor protein p73 (*TP73*), or the *E2F1* promoter with an inactive E2F site (E2F1 mut promoter).

We found that the wt *E2F1* promoter was sharply inhibited by all pocket proteins and by RB C-ter 48h after transfection and that pRB was the only of these proteins that inhibited the activity of this promoter in the long-term as efficiently as in the short-term (Fig. 17A). The overexpression of p107 modestly inhibited its activity, whereas forced expression of p130, RB 1-792, or RB C-ter did not substantially affect the activity of this promoter 5 days after transfection (Fig. 17A). None of these proteins affected the activity of the *E2F1* mut promoter (Fig. 17B). Similarly, pRB was the only of these proteins that inhibited the activity of the *TP73* or the *CDC6* promoter in the long-term as efficiently as in the short-term (Fig. 17C, 17D). Overexpressed p107 and p130 inhibited the activity of these promoters in the short-term as markedly as pRB, but negligibly affected their activity 5 days after transfection (Fig. 17C, 17D).

We conclude that pRB is the only pocket protein able to permanently inhibit E2F activity, and propose that this feature is linked to its unique capacity to arrest the cell cycle in the long-term and to inhibit cell death.

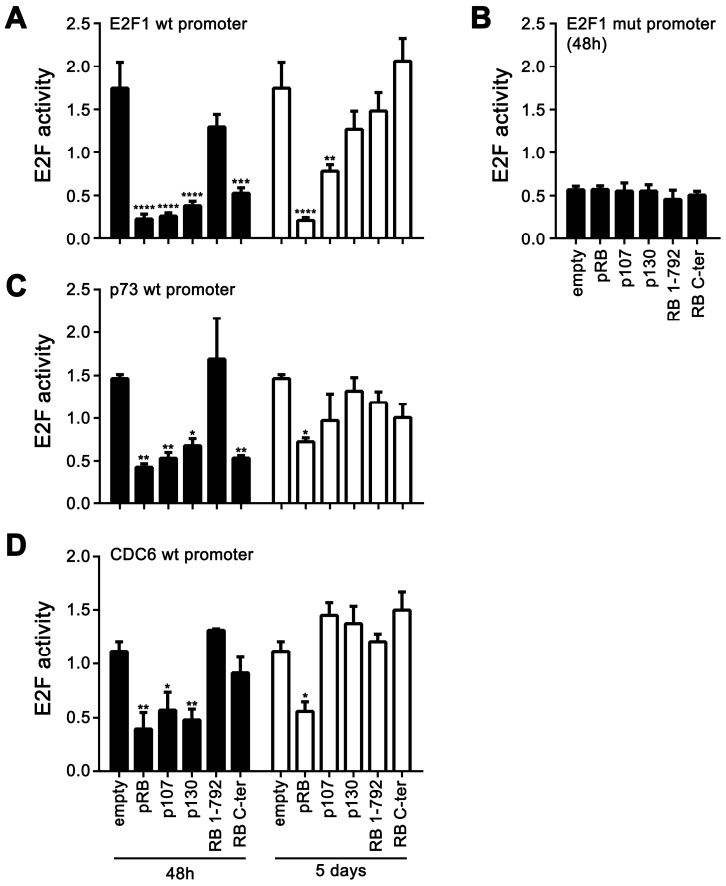

Figure 17. Short-term and long-term regulation of E2F activity by pRB family members and mutant derivatives. Luciferase dual-reporter assay of Saos2 cells co-transfected with plasmids encoding the indicated proteins, a renilla expression plasmid and the Firefly luciferase reporter plasmids driven by **(A)** the wt E2F1 promoter, **(B)**; an E2F1 promoter with mutant E2F-binding sites; **(C)** the wt TP73 promoter; and **(D)** the wt CDC6 promoter. Values of luciferase emission were analyzed 48 hours and 5 days after transfection and normalized with renilla and with the media of each experiment. Data are shown as mean ± SEM (n=3). *p<0.05, **p<0.01, **p<0.001, ****p<0.0001 vs empty; by two-way ANOVA with Bonferroni's multiple comparisons test.

5.3. The CIP/KIP inhibitors p21 and p27 inhibit cell cycle progression only transiently and do not prevent IR-induced cell death

Inhibition of Cyclin/CDK complexes by the CIP/KIP family members p21, p27, and p57 can block cell cycle progression independently of pRB (Vidal and Koff 2000). We therefore investigated whether these proteins arrest the cell cycle in the long-term and inhibit IR-induced cell death. Saos2 cells were transiently transfected with plasmids encoding HA-tagged versions of p21, p27, pRB or RB 1-792 and their expression was confirmed by immunoblotting 48h and 5 days later (Fig.18A). While pRB, p21, and p27 sharply inhibited cell proliferation and arrested cells in G1 48h after transfection, pRB was the only of these proteins that maintained its inhibitory activity 5 days after transfection (Fig. 18B, 18C).

Accordingly, pRB was the only of these proteins that markedly inhibited IR-induced cell death (Fig. 18D).

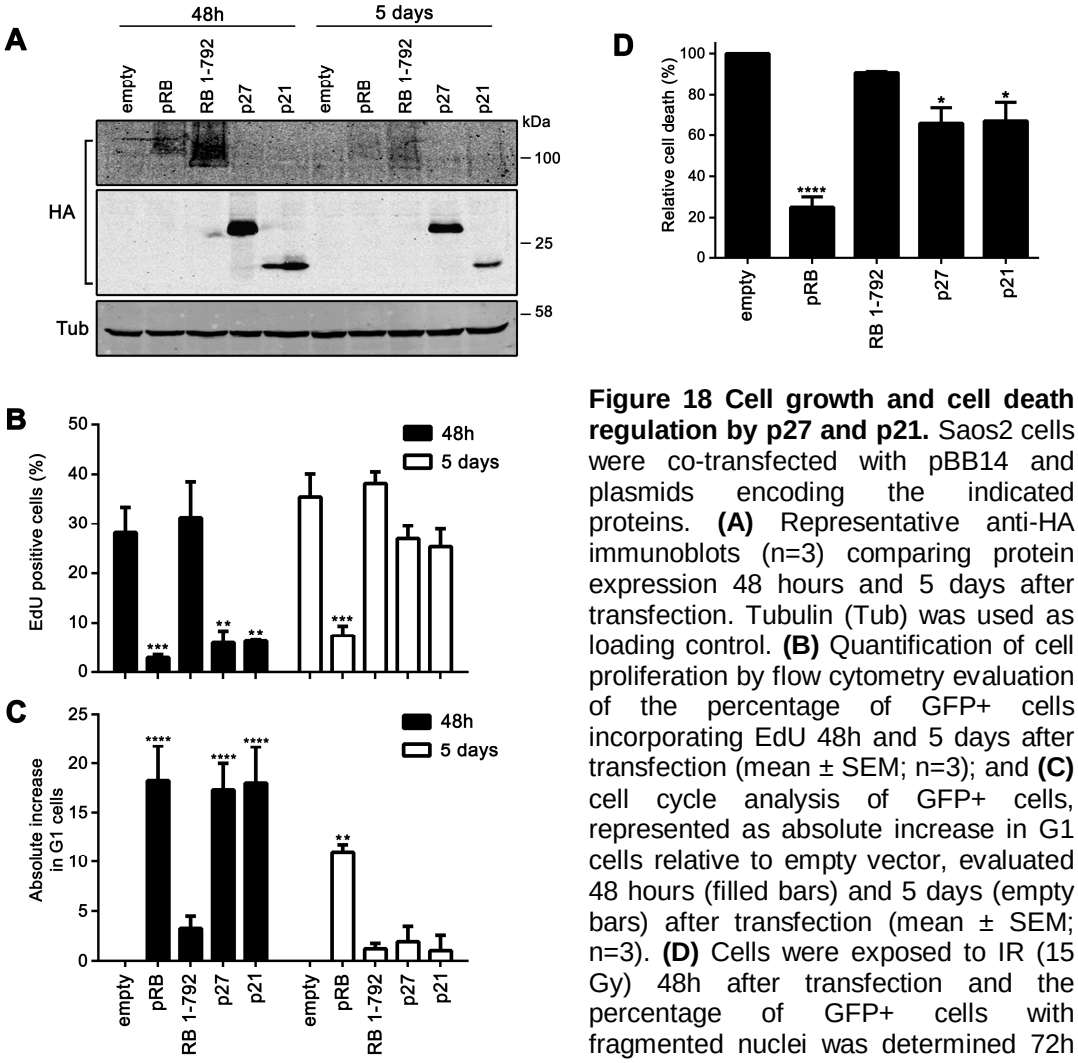

Figure 18 Cell growth and cell death regulation by p27 and p21. Saos2 cells were co-transfected with pBB14 and plasmids encoding the indicated proteins. **(A)** Representative anti-HA immunoblots (n=3) comparing protein expression 48 hours and 5 days after transfection. Tubulin (Tub) was used as loading control. **(B)** Quantification of cell proliferation by flow cytometry evaluation of the percentage of GFP+ cells incorporating EdU 48h and 5 days after transfection (mean ± SEM; n=3); and **(C)** cell cycle analysis of GFP+ cells, represented as absolute increase in G1 cells relative to empty vector, evaluated 48 hours (filled bars) and 5 days (empty bars) after transfection (mean ± SEM; n=3). **(D)** Cells were exposed to IR (15 Gy) 48h after transfection and the percentage of GFP+ cells with fragmented nuclei was determined 72h after IR exposure. Data are shown relative to empty vector as mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs empty; by (B) two-way ANOVA with Bonferroni's multiple comparisons test or (C)Tukey's multiple comparisons test, and by (D) one-way ANOVA with Tukey's multiple comparisons test.

6. The pRB small pocket and carboxyl terminus are structurally integrated

6.1. Biochemical evidence of the structural integration of the small pocket and carboxyl terminus of pRB.

Since the small pocket and the carboxyl terminal domain of pRB are both required for cell death inhibition, we considered several possible models to explain their involvement: 1) the presence of both domains is required for the correct formation of a binding site for a protein partner X required for cell death inhibition (Fig. 19A, top left); 2) stable binding to partner X requires simultaneous interaction with small pocket and carboxyl terminal domains (Fig.19A, top right); 3) the simultaneous and independent interaction of pRB with two partners, X and Y, through the small pocket and the carboxyl terminus, respectively, is required for cell death inhibition (Fig. 19A, bottom left); and 4) the interaction between pRB partners X and Y is required for cell death inhibition and pRB, interacting with them through its small pocket and its carboxyl terminus, acts as an scaffold to facilitate their interaction (Fig.19A, bottom right).

If the small pocket and the carboxyl terminus were required for the interaction with partner X (model 2) or to bring together partners X and Y (model 4), the simultaneous expression of wt pRB with either RB 1-792 or RB carboxyl terminal proteins would compete for the interaction with partner X (model 2) or partners X and Y (model 4) and would therefore inhibit cell death. To challenge these two models, we transiently co-transfected Saos2 cells with RB wt and an excess of RB 1-792, RB SE or RB C-ter and confirmed their expression in the cells by immunoblotting (Fig. 19B). We found that forced expression of RB 1-792 or the carboxyl terminal pRB fragments did not block the capacity of wt pRB to inhibit cell cycle progression (Fig.19C) or IR-induced cell death (Fig. 19D), therefore suggesting that models 2 and 4 are not valid. Although pRB carboxyl terminal fragments modestly inhibited IR-induced cell death in the absence of wt pRB in this particular set of experiments, they showed no substantial effect in previous experiments (compare with data in Fig. 5).

If model 3 was correct, the expression of the small pocket and the carboxyl terminus of pRB as independent fragments should enable their interaction with partners X and Y and inhibit cell death. To test this hypothesis, we co-transfected Saos2 cells with RB 1-792 plus RB SE or RB C-ter and confirmed their expression (Fig.19E). Co-expression of pRB carboxyl terminal fragments (RB SE or RB C-ter) with RB 1-792 accumulated cells in G1 (Fig.19F) as efficiently as in the absence of RB 1-792 (compare with Fig. 19C), but failed to substantially inhibit IR-cell death (Fig. 19G). Together, these data strongly suggest that models 2, 3, and 4 are not

valid and therefore support the notion that the small pocket and the carboxyl terminus are structurally integrated to create a specific domain required for cell death inhibition.

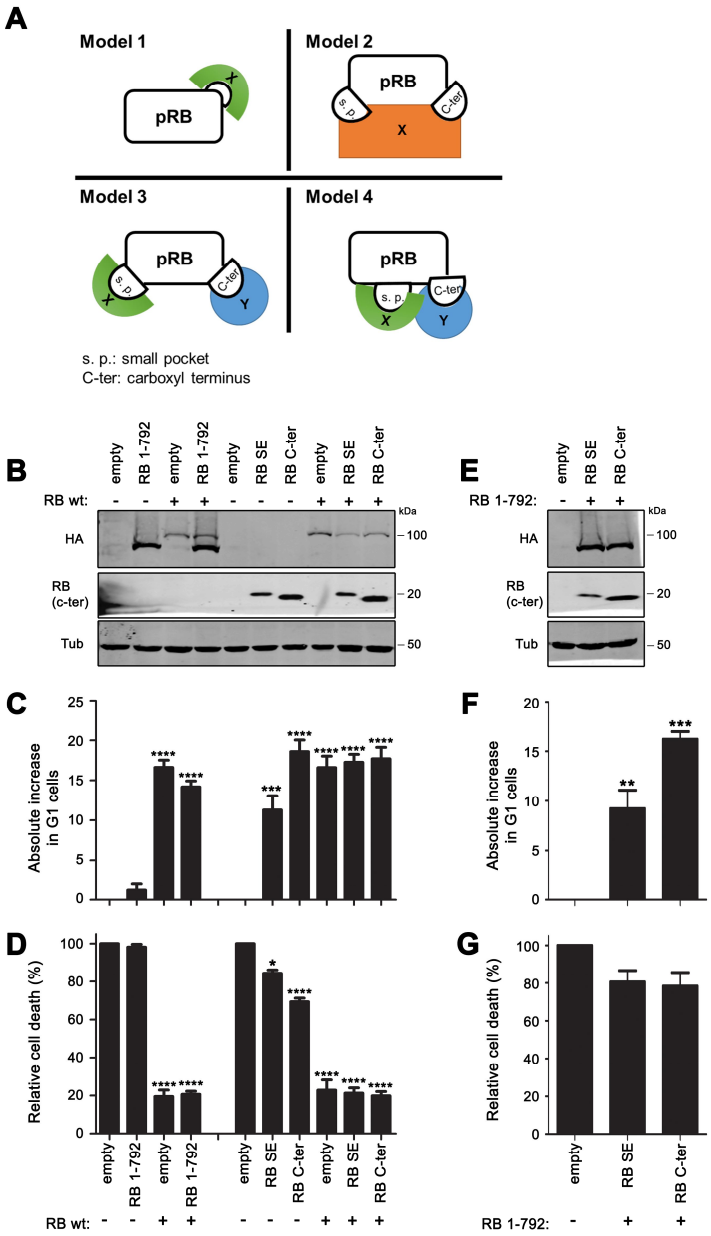

Figure 19. Biochemical evidence of the structural integration of the small pocket and carboxyl terminus of pRB. (A) Models depicting the interaction of pRB with potential protein partners required for cell death inhibition. **(B-G)** Saos2 cells were co-transfected with pBB14 and plasmids encoding the indicated proteins. **(B, E)** Representative anti-HA and anti-RB (c-ter) immunoblots (n=3) of these cells. Tubulin (Tub) was used as loading control. **(C, F)** Cell cycle analysis, represented as absolute increase in G1 cells relative to empty vector, evaluated 48 hours after transfection (mean ± SEM; n=3). **(D, G)** Cells were exposed to IR (15 Gy) 48h after transfection and the percentage of GFP+ cells with fragmented nuclei was determined 72h after IR exposure. Data are shown relative to empty vector as mean ± SEM (n=3). *p<0,05, **p<0.01, ***p<0.001, ****p<0.0001 vs empty; by one-way ANOVA with Tukey's multiple comparisons test.

6.2. *In-silico* model of human Retinoblastoma protein

To gain insight into the structural relationship between the small pocket and the carboxyl terminus of pRB in the absence of a crystallographic structure of the whole protein, we have performed an *in-silico* three-dimensional (3D) model of the protein based on the available crystallographic structure of small domains of pRB.

To obtain the *in-silico* 3D model of human pRB, we submitted its Fasta sequence (Uniprot ID: P06400, residues 2-928) to a local implementation of I-Tasser software suite v4.4 (Yang et al. 2015) for threading modeling with homology and selected the best model, with minimal energy and best structural alignment to the published (Burke et al. 2012) crystal structure of the pocket domain deposited in the Protein Data Bank under the accession code pdb ID: 4eljA, RB-prerefine (Fig. 20A). In this model, the terminal end of the carboxyl domain has a short tail that does not fold with the protein core and projects to the exterior. This uncommon folding suggested that it is a disordered region and, to confirm this hypothesis, we analyzed this sequence with Disopred v3.16 program, a software that predicts secondary structures, disordered regions and protein binding profiles of proteins (Jones and Cozzetto 2015). This analysis identified four disordered regions corresponding to the four domains of the protein: amino terminus (N-term), A and B domains, and the carboxyl terminus (Fig. 20B). In the RB-prerefine model, the folding of A and B domains is restricted by the spacer region, producing a stable structure, the pocket region (Xiao et al. 2003; Balog et al. 2011), whereas the core of the amino terminus folds well with the pocket in a stable structure that leaves the extreme amino-terminal floppy tail, corresponding to the disordered region, being moved around different conformational states (Fig. 20A). This model is the most stable conformation state for the amino terminus and the pocket region. However, the C-tail in the RB-prerefine model might not be accurate and represents only the unfolded centroid state with minimal clashes with the protein core. This model may not be accurate because I-Tasser software lacked the computational capacity to precisely model the disordered c-terminal big tail in the context of the full-length protein.

To do a correct folding of the carboxyl terminus, we submitted the Fasta sequence corresponding to the B domain and the carboxyl terminus of pRB (Uniprot ID P06400, residues 640-928) to the local implementation of I-Tasser suite v4.4 for modeling with threading/homology to generate more than 20.000 models. The model with the best structural alignment with the structure of the B domain, less clashes with the backbone and minor energy was selected, the B-Ctail (Fig. 20C).

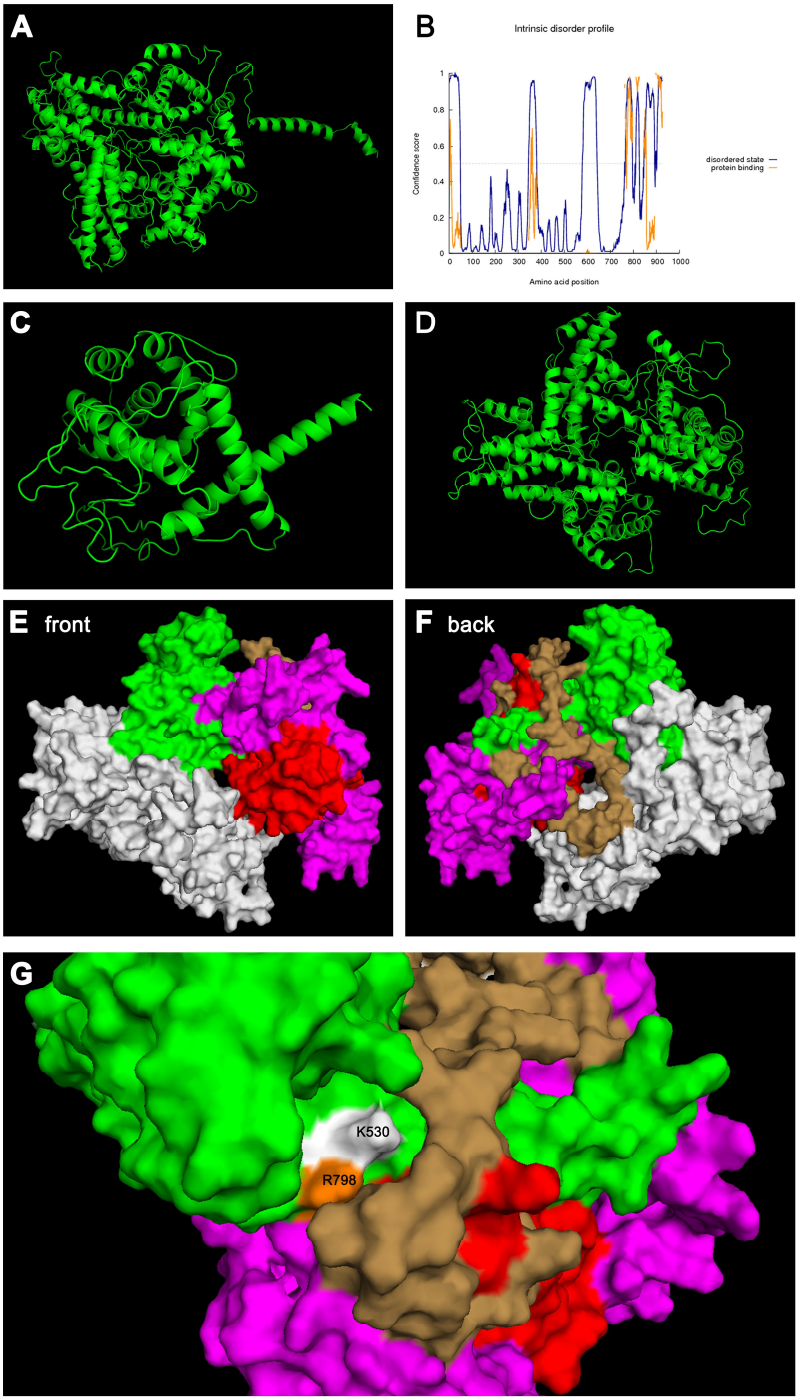

Figure 20. In silico model of human Retinoblastoma protein. (A) 3D view (cartoon representation) of the RB-prerefine model. **(B)** Disopred output plot showing the predicted disordered state (blue) and protein binding profile (orange) for amino acid positions of human pRB. Dashed line shows the score threshold. **(C)** 3D view (cartoon representation) of the model of the B-Ctail. **(D)** Cartoon representation and **(E, front view; F, back view)** surface representation of the refined in-silico 3D model of human pRB. **(G)** Zoom view of the surface representation of the pRB pocket. K530 and R798 are colored in white and orange, respectively. (E-G) Colors show domains: amino terminus, grey; A-domain, green; spacer, yellow sand; B-domain, red; carboxyl terminus, magenta.

We replaced B and C domains of the RB-prerefine model with the B-Ctail to generate a model with the N and A domains of RB-prerefine and the B and C domains of RB-B-Ctail. We used this new model as template and gaps were closed using the loopmodel tool (Mandell et al. 2009; Stein and Kortemme 2013) of Rosetta suite v3.5 release 2015.38.58158 (www.rosettacommons.org). The model with less score, but high energy yet, was selected as final template for refinement using the relax tool (Nivon et al. 2013; Conway et al. 2014) of Rosetta suite v3.5 release 2015.38.58158 (www.rosettacommons.org).

We finally selected the model with minor energy and correct global topology: correct alignment with the reference pocket structure (pdb ID: 4eljA), compatible with known post translational modifications and binding sites (exposed residues) for human pRB-associated proteins previously shown in bibliography (Fig. 20D-20F). Since the amino and the carboxyl terminus are disordered regions, this model represents the most stable conformational state of the protein. A close inspection of this model revealed the presence of an amino-acid from the carboxyl terminus, R798, at the bottom surface of the pocket in close proximity to K530 (Fig. 20G). This observation might therefore represent an important evidence of the structural integration of the small pocket and the carboxyl terminus to form the large pocket.

6.3. How a point mutation could affect the protein conformation: study of two human patients' mutants, RB 661W and RB 567L

Since RB R661W and RB S567L were partially or completely inactive in cell growth and cell death inhibition (Fig. 12), we hypothesized that the structure of these mutants may be different from that of wt pRB. We therefore generated a 3D model of these mutants by submitting Fasta sequences of human pRB protein with the mutated residues (R661W or S567L) to a local implementation of I-Tasser software suite v5.0 (Yang et al. 2015) for modeling with homology to the previously modeled wt pRB. For each one, the best model with minimal energy and correct folding (best structural alignment to template) was selected as final template for refinement using the relax tool of Rosetta suite v3.6 to obtain the final models, pRB_R661W.pdb and pRB_S567L.pdb (Fig. 21).

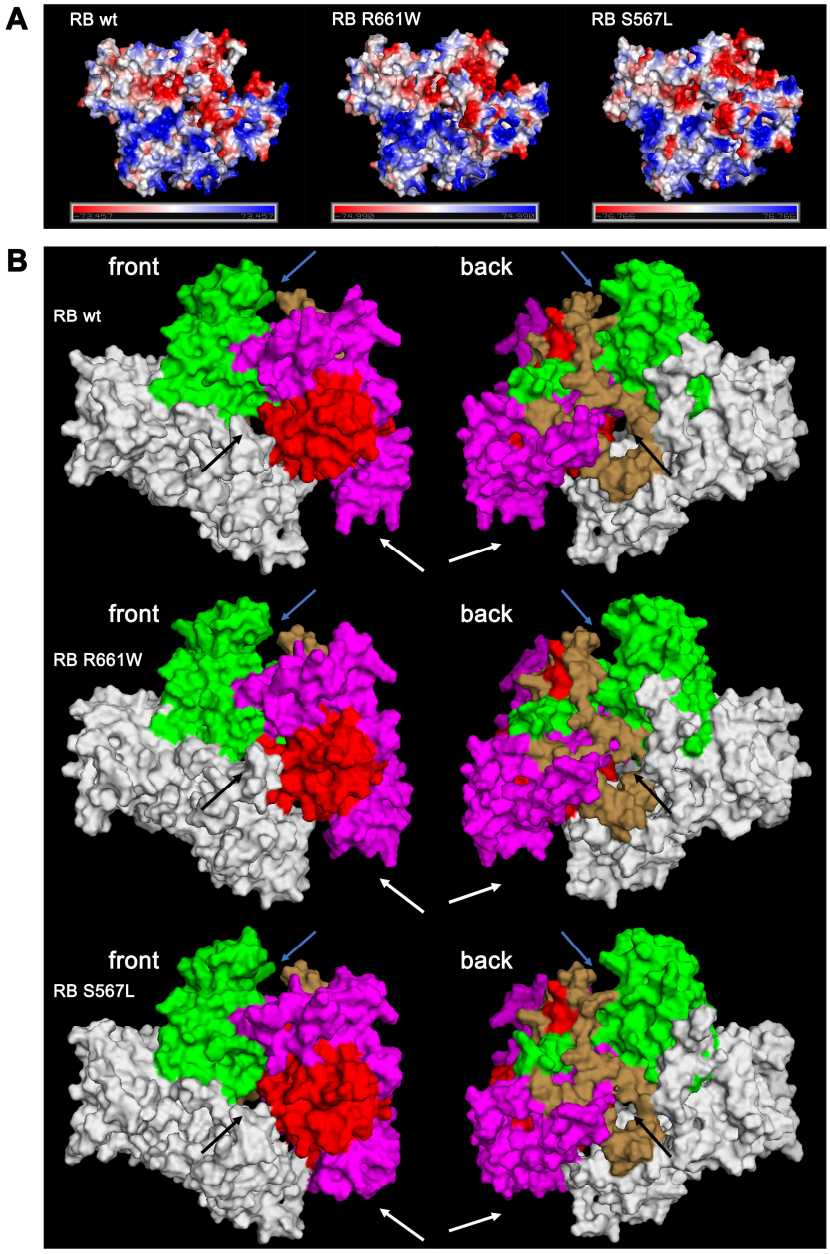

Figure 21. Mutants RB R661W and RB S567L show major structural alterations of the carboxyl terminus and only minor changes in the pocket. (A) Vacuum electrostatic surface representation and **(B)** surface representation of the front and back views of 3D in-silico model of human wt pRB and mutants RB R661W and RB S567L. (B) Colors show domains: amino terminus, grey; A-domain, green; spacer, yellow sand; B-domain, red; carboxyl terminus, magenta. Black arrows indicate the pore; blue arrows indicate the pocket; white arrows indicate the cleft between the amino terminus and the carboxyl terminus.

We found that the global topology of both mutants was similar to that of wt pRB, showing a global Root-mean-square deviation of atomic positions (RMSD) of 1.27Å for R661W and 1,29Å for S567L relative to wt pRB. Both mutants showed a destabilizing effect, that was higher in R661W (ddG of -1,15 kcal/mol) than in S567L (-0,22 kcal/mol), as calculated with the STRUM pipeline (Mandell et al.

2009), likely because the bigger impact of the amino-acid change (from polar charged positively to big hydrophobic aromatic in R661W vs small polar to small hydrophobic in S567L). These mutations produce a conformational change in the side chains accompanied by a modest reorganization of the surface electrostatic potential in the pocket domain and slightly more substantial around the pore and the carboxyl terminus (Fig. 21A). While the pore and the cleft between the amino terminus and the carboxyl terminus are more closed in both mutants, the pocket shows only minor structural changes in both mutants relative to wt pRB (Fig. 21B). However, we observed substantial conformational changes in the carboxyl terminus of RB S567L relative to wt pRB and only a modest modification in RB R661W (Fig. 21B). Together these data show that inactivating mutations in the A or in the B domain of the pocket affect only minimally the pocket conformation but disturb substantially the conformation of the carboxyl terminus. These data further support the notion that the small pocket and the carboxyl terminus are structurally interconnected.

Discussion

The Retinoblastoma protein can be considered as a prototype of tumor suppressor protein, both for the recessive nature of the mutations in the *RB1* gene and for the high frequency of pRB inactivation found in different types of human cancers.

pRB is a key regulator of G1-to-S transition of the cell cycle, it is involved in transcription regulation, in chromatin remodeling and it takes part in G1 checkpoint regulation. pRB acts in different cellular processes, like differentiation, senescence, DNA replication and cell death (Dyson 1998; Classon and Harlow 2002; Chau and Wang 2003; Cobrinik 2005; Giacinti and Giordano 2006; Goodrich 2006; Henley and Dick 2012; Dick and Rubin 2013; Indovina et al. 2015; Dyson 2016). However, it was (and it is) difficult to describe a precise molecular mechanism by which pRB operates.

Retinoblastoma protein was classified by Hasan and colleagues as a “party hub” protein: a protein that can act in different cellular processes and interact simultaneously with different partners at the same time and space (Hasan et al. 2013). Not by chance, according to current interaction databases (European Bioinformatics Institute [EBI]-IntAct, Molecular Interaction [MINT], Interologous Interaction Database [I2D], and String) (Morris and Dyson 2001; Dyson 2016), it was suggested that pRB can interact with more than 300 different proteins. Indeed, since 1998 Lee and coworkers suggested that pRB can simultaneously bind to more than one target, but they also hinted that an optimal conformation of the protein is necessary for the optimal performance of its different cellular processes (Lee et al. 1998).

However, one of the most critical issues in the field of Retinoblastoma protein’s research is that *“different groups have focused on different partners of pRB, and very few of the reported interactions have been confirmed by independent studies”*, as reported by Nicholas J. Dyson on its recent review (Dyson 2016).

The lack of an exact molecular mechanism, the difficulties of a proteomic/interactomic study and the consequent observation that it is easier to describe pRB functions in general terms rather than in specific details, prompted us to start a global and systematic study of this protein, focusing on cell growth and cell death regulation.

1. RB pathway in cancer therapy

The heterogeneity of human cancer makes nearly impossible the development of a general therapy. In the last years, the cell cycle machinery and particularly the RB pathway have become important targets for the design of specific/personalized molecular cancer therapies (Knudsen and Wang 2010). However, the difficulty in understanding the specific characteristics and alterations of each cancer case, have

caused that cytotoxic chemotherapeutic agents and ionizing radiation are still the most common therapeutic approaches in cancer.

The RB pathway has been found highly compromised in a great number of different human cancers. Loss of the *RB1* gene, epigenetic modification that cause the loss of p16INK4 expression and the consequent gain of function of Cyclin D-dependent CDKs and pRB hyperphosphorylation/inactivation, are only some of the alterations of the RB pathway found in human cancer. Very promising results were obtained using specific cell cycle inhibitors that act directly on this pathway. These specific inhibitors (some of them in clinical trials) recover a correct cell cycle control and increase the susceptibility of cancer cells to apoptosis, over all if combined with conventional therapy (Senderowicz 2002; Schwartz and Shah 2005; Dickson and Schwartz 2009; Diaz-Moralli et al. 2013).

However, even if a specific cancer therapy could be very effective against a specific cancer, if a single cancer cell is able to arrest its cell cycle and activate survival programs, the process of cancer formation might restart. This is a critical issue in the context of cancer relapse that requires additional research to better understand this process and to improve cancer therapy.

2. The importance of the "context specificity" in the inhibition of cell death mediated by Retinoblastoma protein

The role of Retinoblastoma protein in the inhibition of cell death is still a controversial issue (Indovina et al. 2015). The first reports about the function of pRB in this cellular process showed an anti-apoptotic function of the protein. The huge extension of apoptosis detected in *Rb1*$^{-/-}$ mice during embryogenesis (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Chau and Wang 2003) or the anti-apoptotic effect of the exogenous expression of pRB in irradiated cells (Haas-Kogan et al. 1995), were a convincing indication of a protective role of Retinoblastoma protein. In contrast, it was suggested a function of pRB in the induction of apoptosis (Hsieh et al. 1999; De Leon et al. 2008; Ianari et al. 2009; Hilgendorf et al. 2013; Antonucci et al. 2014) and, to complicate more the situation, it was also proposed that pRB could perform both an anti- and a pro-apoptotic function, depending on the cellular context and the stimuli to which cells are subjected (Bosco and Knudsen 2005; Masselli and Wang 2006).

The context specificity could be also the explanation of why different pRB-interacting proteins or molecular mechanisms proposed to demonstrate pRB role in cell death regulation, have not been confirmed by independent studies (Dyson 2016). Indeed, we have found that RB ΔS, described as a mutant unable to inhibit the specific pro-apoptotic function of E2F1 (Julian et al. 2008), inhibited cell death induced by

ionizing radiation as efficiently as the wild-type protein, suggesting that pRB inhibition of IR-induced cell death is independent of its binding to E2F1 through the carboxyl terminus of pRB. In the reports that described the E2F1-specific binding site at the carboxyl terminus of pRB (Dick and Dyson 2003; Julian et al. 2008), C33A cells were used, instead of Saos2 cells, and cell death was induced over-expressing E2F1, the only E2F family member with pro-apoptotic function (DeGregori et al. 1997), instead of ionizing radiation. The specific interaction between pRB and E2F1 is regulated by DNA damage-dependent acetylation of pRB in K873/874 (Markham et al. 2006), suggesting the possibility that this post-translational modification is not active in irradiated Saos2 cells.

According to Janicke and colleagues, the mutant RB ΔI should be highly prone to degradation in a situation of DNA damage, because the caspase-specific cleavage of the last 42 amino acids (corresponding to the amino acids loss in mutant RB ΔI) was described as the first step for the full degradation of pRB (Janicke et al. 1996). It was suggested that the degradation of pRB caused the loss of its interaction with HDM2, HMD2 proteolysis and, consequently, the increase of p53 levels (Janicke et al. 1996). Accordingly, pRB full degradation was considered as a necessary step to avoid the activation of its anti-apoptotic function. In marked contrast, pRB was shown to form a trimeric complex with HDM2 and p53 to inhibit HDM2 activity and stabilize p53, suggesting a pro-apoptotic p53-dependent function of the Retinoblastoma protein (Hsieh et al. 1999). Finally, our data suggest that HDM2 interaction is not relevant for cell death inhibition in irradiated cells because the exogenous expression of RB ΔI in irradiated Saos2 cells protected them from nuclei fragmentation as efficiently as the wild-type protein. Furthermore, previous data of the laboratory could not confirm the interaction between ectopically expressed pRB and endogenous HDM2 in Saos2 cells (data not show).

Another point of debate is the possible interaction between pRB and the oncoprotein c-Abl, an interaction that has been reported only by the group of Jean Wang (Welch and Wang 1993; Welch and Wang 1995b; Welch and Wang 1995a; Wang 2000). They suggested that this interaction inhibited c-Abl kinase activity and therefore its capacity to induce proliferation and/or apoptosis. However, previous experiments in our laboratory could not detect the interaction of over-expressed pRB with endogenous c-Abl in Saos2 cells (data not show), and pRB mutant RB 13S, described as a mutant that loss the capacity to interact with c-Abl and therefore to inhibit its pro-apoptotic capacity (Whitaker et al. 1998), inhibited IR-induced cell death as efficiently as wt pRB.

The Retinoblastoma protein has also been shown to form a ternary complex with BRCA1 and type II topoisomerases β (TOPO2), in Mouse Embryonic Fibroblasts, to repair Etoposide-induced DNA damage (Xiao and Goodrich 2005). However, we could not detect the association of ectopically expressed pRB with endogenous BRCA1 or TOPO2 in control- or in IR-treated Saos2 cells (data not show).

It is also possible that the interaction reported between pRB and BRG1 (Khavari et al. 1993) is not necessary for the induction of cell cycle arrest or cell death inhibition in Saos2 cells. It is important to note, however, that we have not confirmed the dominant negative effect of the mutant BRG1-DN in Saos2 cells.

Hilgendorf and co-workers, using MEFs, have proposed recently that pRB induces apoptosis directly at the mitochondria, in a Bax-dependent way (Hilgendorf et al. 2013). The phosphorylation of pRB Serine 807 is required for this interaction (Antonucci et al. 2014), but in the phospho-defective mutant of pRB (RB NPC), this Serine is one of the residues substituted by Alanine. Since RB NPC inhibited cell death induced by ionizing radiation in Saos2 cells, it seems that pRB does not induce Bax-dependent apoptosis at the mitochondria in this specific setting.

Finally, several reports have proposed molecular mechanisms of how pRB regulates cell death depending on its phosphorylation state (Adegbola and Pasternack 2005; De Leon et al. 2008; Lentine et al. 2012; Antonucci et al. 2014). However, most of the phospho residues identified in these studies have been substituted by Alanine in the phospho-defective mutant RB NPC. For this reason, we propose that the inhibition of cell death by pRB is not regulated by CDK-mediated phosphorylation.

In conclusion, the context specificity and/or the different types of DNA insults received by cells may influence the different mechanisms of action by which Retinoblastoma protein regulates cell death. Accordingly, it is possible that pRB does not use a general mechanism, but, depending on the cellular context and/or the type of DNA damage, it can interact with different partners and activate/inhibit different pathways, with the final goal to properly regulate cell death in a positive or in a negative manner.

3. A new perspective of cell death inhibition induced by Retinoblastoma protein: what if pRB does not have a direct role in the regulation of cell death?

An important finding that we introduce with this work concerns the unique capacity of pRB, relative to the other two members of the Retinoblastoma family, to inhibit both cell growth and cell death. It is particularly interesting that p107, p130 and, above all, the carboxyl terminal fragments of pRB (RB SE and RB C-ter) inhibit cell

proliferation, accumulate cells in G1, and inhibit E2F transcriptional activity, but fail to inhibit IR-induced cell death. Furthermore, the capacity of the carboxyl terminal domain of pRB to inhibit cell growth but not cell death, suggests that pRB may use different mechanisms of action in these two cellular processes.

We have highlighted above the difficulties to identify a specific molecular mechanism by which pRB could regulate cell death. We have emphasized the relevance of the cellular context and the type of DNA damage, and we proposed a model that focused the attention on the appropriate folding of the large pocket to form a domain that allows pRB to perform its cellular functions.

But, what if the situation was “easier” than we anticipated?

When we analyzed the cell cycle profiles of non-irradiated cells 5 days after transfection, we observed that pRB maintained the inhibition of cell cycle progression, whereas p107, p130, RB SE and RB C-ter failed to maintain the accumulation of cells in G1. A possible explanation of these results could be that pRB may be the only of these proteins able to permanently inhibit E2F activity, and that the capacity to permanently arrest the cell cycle in G0/G1 is a specific feature of pRB, and not of other proteins with a role in regulation of cell cycle progression, including p27 or p21.

Therefore, what if the function of pRB in a context of cell death is “only” to sustainably arrest the cell cycle and, consequently, to allow the correct activation of the DNA damage response? Under this perspective, pRB would not use different molecular mechanisms to induce growth suppression and to inhibit cell death, and the failure of RB SE, RB C-ter, p107 and p130 to inhibit cell death would be the consequence of their failure to induce a sustained arrest of the cell cycle. The short-term effects on cell cycle progression observed with these proteins could be a temporary effect due to their overexpression or to the capacity of these proteins to delay cell cycle progression, but not to arrest it (Whitaker et al. 1998; Chung et al. 2002).

Although great efforts have been made to identify the molecular mechanisms of cell death inhibition by pRB, it might be possible that this protein has the fundamental function of long-term inhibition of the cell cycle, until the DNA injury is solved. We cannot anticipate if pRB could have a direct role in DDR activation or if the long-term G1 arrest is sufficient to allow DNA repair. At least in IR-treated Saos2 cells, it seems that pRB inhibits cell death by the induction of a long-term arrest of the cell cycle in G0/G1. Accordingly, a permanent G0/G1 cell cycle arrest induced by treatment with Mimosine, HU or CHX inhibited IR-induced cell death. Obviously, these treatments are toxic by themselves and increase cell death independently of ionizing radiation, but the cells that survived these treatments were permanently arrested in G0/G1 and resistant

to IR-induced cell death. Together, these data strongly suggest that a long-term G0/G1 arrest of the cell cycle is a necessary and sufficient condition to resist cell death induced by IR. In fact, during DNA damage response (DDR), the activation of checkpoints avoids the progression to the next phase of the cell cycle, allowing the action of the DNA repair system and therefore preventing DNA mutation, which could lead to cellular transformation (Weinert 1998a; Weinert 1998b; Nyberg et al. 2002; Sancar et al. 2004).

4. Importance of the integrity of the pRB large pocket in cell death inhibition

Retinoblastoma protein is a member of a family of three proteins. Together with p107 and p130, they represent the most important proteins involved in the regulation of cell cycle progression (Cobrinik 2005; Henley and Dick 2012). Although these proteins share cellular functions and, in some cases, overlapping roles (Cobrinik et al. 1996; Lee et al. 1996; Classon et al. 2000; Sage et al. 2000), it is interesting that pRB is the only family member able to inhibit both cell cycle progression and cell death. Even if p107 and p130 inhibited proliferation, accumulated cells in the G1 phase of the cell cycle and inhibited E2F transcriptional activity, they could not avoid nuclei fragmentation following ionizing radiation. This is of particular interest because the three members of the Retinoblastoma family show a high structural homology.

Once confirmed that cell death's inhibition mediated by pRB was not regulated by Cyclin/CDKs phosphorylation in Saos2 cells, we performed a global and systematic study of the different domains of the protein, assessing the role of the amino terminus, the small pocket, the carboxyl terminus and the large pocket in growth suppression and cell death inhibition, and investigating if there is a correlation or a separation of these functions of pRB.

According to Xu and colleagues (Xu et al. 1994), we observed that an amino-terminal truncated mutant of pRB (RB 379-928) acts as the wild-type protein both in cell growth suppression and in cell death inhibition following ionizing radiation, suggesting that the amino terminus is not necessary for pRB functions in these cellular processes.

On the other hand, a mutant lacking the carboxyl terminus of pRB (RB 1-792) was a non-functional protein, at least regarding the functions that we analyzed. Since a bipartite nuclear localization signal (NLS) was described in the carboxyl terminus of Retinoblastoma protein (Zacksenhaus et al. 1993), we tried to enrich the presence of this mutant in the nuclear fraction, cloning the NLS of SV40 large tumor antigen (Kalderon et al. 1984) in its amino terminus. However, we did not observe any substantial effect in cell growth suppression or in cell death inhibition, suggesting that

the loss of the ability of pRB to translocate to the nucleus is not the cause of pRB inactivation in the absence of its carboxyl terminus.

These observations led us to investigate the effects of the overexpression of fragments corresponding to the carboxyl terminal domain of pRB (RB SE and RB C-ter). Although RB SE and RB C-ter induced cell growth suppression, these mutants did not inhibit cell death following ionizing radiation. Therefore, we concluded that the carboxyl terminus of pRB is necessary and sufficient to inhibit cell cycle progression in the short-term; and necessary, but not sufficient, to inhibit cell death following ionizing radiation.

The results commentated above could be a consequence of the different capacity of these mutants to interact with some important protein/s that allow cell growth suppression and/or cell death inhibition. The carboxyl terminal domain of pRB, might be fundamental to achieve the correct conformation of the large pocket, the minimum region of pRB able to both inhibit cell growth and cell death. Since the carboxyl terminus of pRB presents significant differences with respect to p107 and p130 (Hasan et al. 2013), we substituted the carboxyl terminus of p130 with the one of pRB and vice versa, to study if there is a gain or a loss of function, respectively, in the context of cell death inhibition. Although we could demonstrate the loss of function of the chimera pRB/p130, the chimera p130/pRB did not inhibit cell death. These data suggested that the carboxyl terminus of pRB has a specific structure sufficient to perform the function in growth suppression, but only its correct integration into the large pocket, and its consequent correct interaction with specific interactor/s, allows the protein to inhibit cell death.

In fact, we have shown that perturbation of the small pocket affects the regulation of E2F activity by pRB, supporting the notion that the small pocket is critical for pRB functions. The substitution of residues in pRB that are highly conserved in p107 and p130 with the flexible linker's sequence NAAIRS, allowed us to study different mutants and to identify three groups of them, according to their effect relative to wt pRB. RB Δ503, RB Δ529 and RB Δ685 acted as the wild-type protein, whereas RB Δ657 and RB Δ678 lost functions of cell growth suppression and cell death inhibition. Interestingly, we identified a third group of mutants that share functions with wt pRB but could not reach its levels of activity (RB Δ535, RB Δ542 and RB Δ651). Differences in activity of these mutants could be explained by substantial modification of their conformation as a consequence of the substitution of six amino acids with the flexible linker (NAAIRS); the different ability of these mutants to interact with specific interactor/s, allowing pRB to perform its function as growth suppressor protein and/or as a protein with anti-cell death activity, could be attributed to these conformational

modifications. Together, our data emphasize the importance of the integrity of the large pocket for pRB biological functions.

5. An appropriate conformation of the large pocket is necessary for cell death inhibition mediated by the Retinoblastoma protein

Another difficulty in the study of Retinoblastoma protein is the absence of the full-length structure of the protein: the crystal structures available correspond to single domains of the protein interacting with specific partners (Xiao et al. 2003; Rubin et al. 2005). Our *in-silico* analysis of the full-length sequence of pRB revealed a highly disorder protein that may recruit the different interacting proteins, required for the specific function that pRB has to perform. The carboxyl terminus might be the “binder”, but pRB could perform its function of “activator/inhibitor” only when its large pocket is folded in a precise conformation.

Thus, in a hypothetical mechanism of action, pRB interacts with a specific partner/s through its carboxyl terminal domain. Once established the interaction, the partner/s is/are brought into the pocket, which can accommodate proteins of different sizes due to its plasticity; in this way, pRB could activate or inactivate this/these protein/s and perform properly its biological functions.

This hypothesis could explain why the loss of the carboxyl terminus, the presence of only this domain, or the mutation of the small pocket, all failed to inhibit cell death. In fact, the *in-silico* model of two mutants found in patients, RB 661W and RB 567L, showed important changes in the conformation of the protein that affected more severely the carboxyl terminus than the pocket.

We proposed several models by which pRB may carry out its role in cell death regulation. In the first model, pRB needs a specific conformation of its large pocket for the correct interaction with partner X. In the second model, this protein X interacts with pRB through both the small pocket and the carboxyl terminus. In both models, this interaction could be necessary to activate the anti-cell growth or anti-cell death activity of this protein X or, alternatively, to inhibit its pro-cell growth or pro-cell death activity.

Models 3 and 4 contemplated the possibility that pRB interacts with two different proteins wither in an independent way (protein X interacts through the small pocket and protein Y through the carboxyl terminus) or in a dependent way, acting as a scaffold for the interaction between protein X and protein Y, to form a specific ternary complex. Once again, these interactions could be necessary to activate the anti-cell growth or anti-cell death function of protein X and protein Y or those of the ternary complex. Alternatively, these interactions could inhibit the pro-cell growth or pro-cell death activity of them.

The overexpression of an excess of mutant RB 1-792 or of fragments corresponding to the carboxyl terminus of pRB (RB SE or RB C-ter) did not interfere with the capacity of the wild-type protein to inhibit cell growth and cell death. These results suggested that none of these fragments competed with full-length pRB for the interaction with partner/s (protein X and/or protein Y), excluding models 2 and 4.

We also performed complementation experiments to investigate if the small pocket and the carboxyl terminal domain are independent units of the protein or if the integrity of the large pocket is necessary for pRB biological functions. Thus, the presence in the cells of both domains separated did not inhibit cell death following irradiation, excluding model 3. Together, these data strongly suggest that the small pocket and the carboxyl terminus of pRB have to be physically linked, as they are in the large pocket, to adequately expose a domain of interaction with its specific partner/s and to operate in cell growth suppression and in cell death inhibition.

Conclusions

The conclusions obtained with this thesis were the following:

1- pRB is the only pocket family member that possesses the capacity to inhibit cell death in a situation of DNA damage.

2- The inhibition of cell death by pRB is not regulated by CDK-mediated phosphorylation.

3- pRB function in cell growth suppression and cell death inhibition is not mediated by BRG1.

4- While the amino terminus of pRB is not required for the regulation of cell cycle progression and cell death inhibition, the carboxyl terminus is sufficient to block E2F activity and to inhibit cell cycle progression. Moreover, the carboxyl terminus of pRB is necessary, but not sufficient, to inhibit IR-induced cell death.

5- pRB carboxyl terminus plays a critical role in cell growth and cell death inhibition regardless its contribution to the preferential location of pRB in the nucleus.

6- pRB cell growth and cell death inhibitory activities are mediated by a very precise spatial configuration of the small pocket and the carboxyl terminus.

7- The association of pRB C-terminal with E2F1, c-Abl, or HDM2 does not mediate cell death inhibition and cell growth inhibition capacities of pRB.

8- The small pocket of pRB is required for cell death inhibition.

9- A permanent arrest in the G1 phase of the cell cycle is necessary and sufficient to inhibit IR-induced cell death and only pRB arrests cell cycle in G0/G1 in a long-term manner, probably due to its ability to permanently inhibit E2F activity. The capacity to permanently arrest the cell cycle in G0/G1 is a specific feature of pRB, and not of other proteins with a role in regulation of cell cycle progression, including p27 or p21.

10- The small pocket and the carboxyl terminus of pRB are structurally integrated to create a specific domain required for cell death inhibition.

Las conclusiones que se obtuvieron en esta tesis son:

1- pRB es el único miembro de la familia Retinoblastoma que posee la capacidad de inhibir la muerte celular en una situación de daño al ADN.

2- La inhibición de la muerte celular por pRB no está regulada por la fosforilación mediada por las quinasas dependientes de ciclinas.

3- La función de pRB en la supresión del crecimiento celular y la inhibición de la muerte celular no está mediada por BRG1.

4- El dominio amino terminal de pRB no es necesario para inhibir la progresión del ciclo celular y la muerte celular, mientras que el extremo carboxilo es suficiente para bloquear la actividad de E2F e inhibir la progresión del ciclo celular. Además, el carboxilo terminal de pRB es necesario, pero no suficiente, para inhibir la muerte celular inducida por radiación ionizante.

5- El dominio carboxilo terminal de pRB es esencial para inhibir el crecimiento celular y la muerte celular independientemente de su contribución a la ubicación preferencial de pRB en el núcleo.

6- La asociación del dominio carboxilo terminal de pRB con E2F1, c-Abl, o HDM2 no media la capacidad de pRB de inhibir el crecimiento y la muerte celular.

7- El *bolsillo pequeño* de pRB es necesario para inhibir la muerte celular.

8- La inhibición de la progresión del ciclo celular y de la muerte celular están mediadas por una configuración espacial muy precisa del *bolsillo pequeño* y del extremo carboxilo de pRB.

9- Una detención sostenida en la fase G1 del ciclo celular es necesaria y suficiente para inhibir la muerte celular inducida por radiación ionizante y sólo pRB detiene el ciclo celular en G0/G1 a largo plazo, probablemente debido a su capacidad de inhibir sostenidamente la actividad de E2F. La capacidad de detener sostenidamente el ciclo celular en G0/G1 es una característica específica de pRB, y no de otras proteínas con un papel en la regulación de la progresión del ciclo celular, como p27 o p21.

10- El *bolsillo pequeño* y el extremo carboxilo de pRB se integran estructuralmente para crear un dominio específico requerido para la inhibición de la muerte celular.

Bibliography

Adams PD, Sellers WR, Sharma SK, Wu AD, Nalin CM, Kaelin WG, Jr. 1996. Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. *Molecular and cellular biology* **16**: 6623-6633.

Adegbola O, Pasternack GR. 2005. Phosphorylated retinoblastoma protein complexes with pp32 and inhibits pp32-mediated apoptosis. *The Journal of biological chemistry* **280**: 15497-15502.

Alexander K, Hinds PW. 2001. Requirement for p27(KIP1) in retinoblastoma protein-mediated senescence. *Molecular and cellular biology* **21**: 3616-3631.

Almasan A, Yin Y, Kelly RE, Lee EY, Bradley A, Li W, Bertino JR, Wahl GM. 1995. Deficiency of retinoblastoma protein leads to inappropriate S-phase entry, activation of E2F-responsive genes, and apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 5436-5440.

Antonucci LA, Egger JV, Krucher NA. 2014. Phosphorylation of the Retinoblastoma protein (Rb) on serine-807 is required for association with Bax. *Cell Cycle* **13**: 3611-3617.

Avni D, Yang H, Martelli F, Hofmann F, ElShamy WM, Ganesan S, Scully R, Livingston DM. 2003. Active localization of the retinoblastoma protein in chromatin and its response to S phase DNA damage. *Molecular cell* **12**: 735-746.

Baldi A, Boccia V, Claudio PP, De Luca A, Giordano A. 1996. Genomic structure of the human retinoblastoma-related Rb2/p130 gene. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 4629-4632.

Balog ER, Burke JR, Hura GL, Rubin SM. 2011. Crystal structure of the unliganded retinoblastoma protein pocket domain. *Proteins* **79**: 2010-2014.

Barbie DA, Kudlow BA, Frock R, Zhao J, Johnson BR, Dyson N, Harlow E, Kennedy BK. 2004. Nuclear reorganization of mammalian DNA synthesis prior to cell cycle exit. *Molecular and cellular biology* **24**: 595-607.

Bartek J, Lukas C, Lukas J. 2004. Checking on DNA damage in S phase. *Nature reviews Molecular cell biology* **5**: 792-804.

Bartlett C, Orvis TJ, Rosson GS, Weissman BE. 2011. BRG1 mutations found in human cancer cell lines inactivate Rb-mediated cell-cycle arrest. *Journal of cellular physiology* **226**: 1989-1997.

Bellan C, De Falco G, Tosi GM, Lazzi S, Ferrari F, Morbini G, Bartolomei S, Toti P, Mangiavacchi P, Cevenini G et al. 2002. Missing expression of pRb2/p130 in human retinoblastomas is associated with reduced apoptosis and lesser differentiation. *Investigative ophthalmology & visual science* **43**: 3602-3608.

Besson A, Dowdy SF, Roberts JM. 2008. CDK inhibitors: cell cycle regulators and beyond. *Developmental cell* **14**: 159-169.

Binne UK, Classon MK, Dick FA, Wei W, Rape M, Kaelin WG, Jr., Naar AM, Dyson NJ. 2007. Retinoblastoma protein and anaphase-promoting complex physically interact and functionally cooperate during cell-cycle exit. *Nature cell biology* **9**: 225-232.

Blow JJ, Hodgson B. 2002. Replication licensing--defining the proliferative state? *Trends in cell biology* **12**: 72-78.

Bosco EE, Knudsen ES. 2005. Differential role of RB in response to UV and IR damage. *Nucleic acids research* **33**: 1581-1592.

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

Broceno C, Wilkie S, Mittnacht S. 2002. RB activation defect in tumor cell lines. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 14200-14205.

Brugarolas J, Moberg K, Boyd SD, Taya Y, Jacks T, Lees JA. 1999. Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated

G1 arrest after gamma-irradiation. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 1002-1007.

Burke JR, Hura GL, Rubin SM. 2012. Structures of inactive retinoblastoma protein reveal multiple mechanisms for cell cycle control. *Genes & development* **26**: 1156-1166.

Campanero MR, Armstrong MI, Flemington EK. 2000. CpG methylation as a mechanism for the regulation of E2F activity. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 6481-6486.

Campisi J. 2001. Cellular senescence as a tumor-suppressor mechanism. *Trends in cell biology* **11**: S27-31.

Campisi J, d'Adda di Fagagna F. 2007. Cellular senescence: when bad things happen to good cells. *Nature reviews Molecular cell biology* **8**: 729-740.

Cappell SD, Chung M, Jaimovich A, Spencer SL, Meyer T. 2016. Irreversible APC(Cdh1) Inactivation Underlies the Point of No Return for Cell-Cycle Entry. *Cell* **166**: 167-180.

Cavenee WK, Dryja TP, Phillips RA, Benedict WF, Godbout R, Gallie BL, Murphree AL, Strong LC, White RL. 1983. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature* **305**: 779-784.

Cecchini MJ, Dick FA. 2011. The biochemical basis of CDK phosphorylation-independent regulation of E2F1 by the retinoblastoma protein. *The Biochemical journal* **434**: 297-308.

Cerqueira A, Santamaria D, Martinez-Pastor B, Cuadrado M, Fernandez-Capetillo O, Barbacid M. 2009. Overall Cdk activity modulates the DNA damage response in mammalian cells. *The Journal of cell biology* **187**: 773-780.

Chau BN, Wang JY. 2003. Coordinated regulation of life and death by RB. *Nature reviews Cancer* **3**: 130-138.

Chellappan S, Kraus VB, Kroger B, Munger K, Howley PM, Phelps WC, Nevins JR. 1992. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 4549-4553.

Chellappan SP, Hiebert S, Mudryj M, Horowitz JM, Nevins JR. 1991. The E2F transcription factor is a cellular target for the RB protein. *Cell* **65**: 1053-1061.

Chew YP, Ellis M, Wilkie S, Mittnacht S. 1998. pRB phosphorylation mutants reveal role of pRB in regulating S phase completion by a mechanism independent of E2F. *Oncogene* **17**: 2177-2186.

Chung J, Cho JW, Baek WK, Suh SI, Kwon TK, Park JW, Suh MH. 2002. Expression of RB C pocket fragments in HSF induces delayed cell cycle progression and sensitizes to apoptosis upon cellular stresses. *Cell proliferation* **35**: 247-256.

Clarke AR, Maandag ER, van Roon M, van der Lugt NM, van der Valk M, Hooper ML, Berns A, te Riele H. 1992. Requirement for a functional Rb-1 gene in murine development. *Nature* **359**: 328-330.

Classon M, Dyson N. 2001. p107 and p130: versatile proteins with interesting pockets. *Experimental cell research* **264**: 135-147.

Classon M, Harlow E. 2002. The retinoblastoma tumour suppressor in development and cancer. *Nature reviews Cancer* **2**: 910-917.

Classon M, Salama S, Gorka C, Mulloy R, Braun P, Harlow E. 2000. Combinatorial roles for pRB, p107, and p130 in E2F-mediated cell cycle control. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 10820-10825.

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

Cobrinik D, Lee MH, Hannon G, Mulligan G, Bronson RT, Dyson N, Harlow E, Beach D, Weinberg RA, Jacks T. 1996. Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes & development* **10**: 1633-1644.

Conway P, Tyka MD, DiMaio F, Konerding DE, Baker D. 2014. Relaxation of backbone bond geometry improves protein energy landscape modeling. *Protein science : a publication of the Protein Society* **23**: 47-55.

Dahiya A, Gavin MR, Luo RX, Dean DC. 2000. Role of the LXCXE binding site in Rb function. *Molecular and cellular biology* **20**: 6799-6805.

Dannenberg JH, Schuijff L, Dekker M, van der Valk M, te Riele H. 2004. Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. *Genes & development* **18**: 2952-2962.

De Duve C. 1963. The lysosome. *Scientific American* **208**: 64-72.

De Leon G, Sherry TC, Krucher NA. 2008. Reduced expression of PNUTS leads to activation of Rb-phosphatase and caspase-mediated apoptosis. *Cancer biology & therapy* **7**: 833-841.

De Luca A, MacLachlan TK, Bagella L, Dean C, Howard CM, Claudio PP, Baldi A, Khalili K, Giordano A. 1997. A unique domain of pRb2/p130 acts as an inhibitor of Cdk2 kinase activity. *The Journal of biological chemistry* **272**: 20971-20974.

DeCaprio JA, Ludlow JW, Figge J, Shew JY, Huang CM, Lee WH, Marsilio E, Paucha E, Livingston DM. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**: 275-283.

DeCaprio JA, Ludlow JW, Lynch D, Furukawa Y, Griffin J, Piwnica-Worms H, Huang CM, Livingston DM. 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. *Cell* **58**: 1085-1095.

DeGregori J, Leone G, Miron A, Jakoi L, Nevins JR. 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 7245-7250.

Derenzini M, Donati G, Mazzini G, Montanaro L, Vici M, Ceccarelli C, Santini D, Taffurelli M, Trere D. 2008. Loss of retinoblastoma tumor suppressor protein makes human breast cancer cells more sensitive to antimetabolite exposure. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14**: 2199-2209.

Diaz-Moralli S, Tarrado-Castellarnau M, Miranda A, Cascante M. 2013. Targeting cell cycle regulation in cancer therapy. *Pharmacology & therapeutics* **138**: 255-271.

Dick FA, Dyson N. 2003. pRB contains an E2F1-specific binding domain that allows E2F1-induced apoptosis to be regulated separately from other E2F activities. *Molecular cell* **12**: 639-649.

Dick FA, Rubin SM. 2013. Molecular mechanisms underlying RB protein function. *Nature reviews Molecular cell biology* **14**: 297-306.

Dickson MA, Schwartz GK. 2009. Development of cell-cycle inhibitors for cancer therapy. *Current oncology (Toronto, Ont)* **16**: 36-43.

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

Dou QP, An B, Will PL. 1995. Induction of a retinoblastoma phosphatase activity by anticancer drugs accompanies p53-independent G1 arrest and apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 9019-9023.

Dowdy SF, Hinds PW, Louie K, Reed SI, Arnold A, Weinberg RA. 1993. Physical interaction of the retinoblastoma protein with human D cyclins. *Cell* **73**: 499-511.

Dunaief JL, King A, Esumi N, Eagen M, Dentchev T, Sung CH, Chen S, Zack DJ. 2002. Protein Phosphatase 1 binds strongly to the retinoblastoma protein but not to p107 or p130 in vitro and in vivo. *Current eye research* **24**: 392-396.

Dunaief JL, Strober BE, Guha S, Khavari PA, Alin K, Luban J, Begemann M, Crabtree GR, Goff SP. 1994. The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell* **79**: 119-130.

Dunn JM, Phillips RA, Becker AJ, Gallie BL. 1988. Identification of germline and somatic mutations affecting the retinoblastoma gene. *Science (New York, NY)* **241**: 1797-1800.

Dyson N. 1998. The regulation of E2F by pRB-family proteins. *Genes & development* **12**: 2245-2262.

Dyson N, Howley PM, Munger K, Harlow E. 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science (New York, NY)* **243**: 934-937.

Dyson NJ. 2016. RB1: a prototype tumor suppressor and an enigma. *Genes & development* **30**: 1492-1502.

Ewen ME, Sluss HK, Sherr CJ, Matsushime H, Kato J, Livingston DM. 1993. Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**: 487-497.

Ewen ME, Xing YG, Lawrence JB, Livingston DM. 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. *Cell* **66**: 1155-1164.

Fang W, Mori T, Cobrinik D. 2002. Regulation of PML-dependent transcriptional repression by pRB and low penetrance pRB mutants. *Oncogene* **21**: 5557-5565.

Fiorentino FP, Symonds CE, Macaluso M, Giordano A. 2009. Senescence and p130/Rbl2: a new beginning to the end. *Cell research* **19**: 1044-1051.

Flemington EK, Speck SH, Kaelin WG, Jr. 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proceedings of the National Academy of Sciences of the United States of America* **90**: 6914-6918.

Fogh J, Fogh JM, Orfeo T. 1977. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *Journal of the National Cancer Institute* **59**: 221-226.

Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323**: 643-646.

Fuchs Y, Steller H. 2015. Live to die another way: modes of programmed cell death and the signals emanating from dying cells. *Nature reviews Molecular cell biology* **16**: 329-344.

Fung YK, Murphree AL, T'Ang A, Qian J, Hinrichs SH, Benedict WF. 1987. Structural evidence for the authenticity of the human retinoblastoma gene. *Science (New York, NY)* **236**: 1657-1661.

Galanos P, Vougas K, Walter D. 2016. Chronic p53-independent p21 expression causes genomic instability by deregulating replication licensing. **18**: 777-789.

Giacinti C, Giordano A. 2006. RB and cell cycle progression. *Oncogene* **25**: 5220-5227.

Gonzalo S, Garcia-Cao M, Fraga MF, Schotta G, Peters AH, Cotter SE, Eguia R, Dean DC, Esteller M, Jenuwein T et al. 2005. Role of the RB1 family in stabilizing histone methylation at constitutive heterochromatin. *Nature cell biology* **7**: 420-428.

Goodrich DW. 2006. The retinoblastoma tumor-suppressor gene, the exception that proves the rule. *Oncogene* **25**: 5233-5243.

Goodrich DW, Wang NP, Qian YW, Lee EY, Lee WH. 1991. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. *Cell* **67**: 293-302.

Haas-Kogan DA, Kogan SC, Levi D, Dazin P, T'Ang A, Fung YK, Israel MA. 1995. Inhibition of apoptosis by the retinoblastoma gene product. *The EMBO journal* **14**: 461-472.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* **100**: 57-70.

Hannan KM, Hannan RD, Smith SD, Jefferson LS, Lun M, Rothblum LI. 2000. Rb and p130 regulate RNA polymerase I transcription: Rb disrupts the interaction between UBF and SL-1. *Oncogene* **19**: 4988-4999.

Harley CB, Futcher AB, Greider CW. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature* **345**: 458-460.

Harrington EA, Bruce JL, Harlow E, Dyson N. 1998. pRB plays an essential role in cell cycle arrest induced by DNA damage. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 11945-11950.

Hasan MM, Brocca S, Sacco E, Spinelli M, Papaleo E, Lambrughi M, Alberghina L, Vanoni M. 2013. A comparative study of Whi5 and retinoblastoma proteins: from sequence and structure analysis to intracellular networks. *Frontiers in physiology* **4**: 315.

Hayflick L. 1965. THE LIMITED IN VITRO LIFETIME OF HUMAN DIPLOID CELL STRAINS. *Experimental cell research* **37**: 614-636.

Heichman KA, Roberts JM. 1994. Rules to replicate by. *Cell* **79**: 557-562.

Helin K, Harlow E, Fattaey A. 1993. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Molecular and cellular biology* **13**: 6501-6508.

Helmbold H, Deppert W, Bohn W. 2006. Regulation of cellular senescence by Rb2/p130. *Oncogene* **25**: 5257-5262.

Henley SA, Dick FA. 2012. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. *Cell division* **7**: 10.

Hernando E, Nahle Z, Juan G, Diaz-Rodriguez E, Alaminos M, Hemann M, Michel L, Mittal V, Gerald W, Benezra R et al. 2004. Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. *Nature* **430**: 797-802.

Hiebert SW. 1993. Regions of the retinoblastoma gene product required for its interaction with the E2F transcription factor are necessary for E2 promoter repression and pRb-mediated growth suppression. *Molecular and cellular biology* **13**: 3384-3391.

Hiebert SW, Chellappan SP, Horowitz JM, Nevins JR. 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes & development* **6**: 177-185.

Hilgendorf KI, Leshchiner ES, Nedelcu S, Maynard MA, Calo E, Ianari A, Walensky LD, Lees JA. 2013. The retinoblastoma protein induces apoptosis directly at the mitochondria. *Genes & development* **27**: 1003-1015.

Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI, Weinberg RA. 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70**: 993-1006.

Hsieh JK, Chan FS, O'Connor DJ, Mittnacht S, Zhong S, Lu X. 1999. RB regulates the stability and the apoptotic function of p53 via MDM2. *Molecular cell* **3**: 181-193.

Hu QJ, Dyson N, Harlow E. 1990. The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. *The EMBO journal* **9**: 1147-1155.

Huang HJ, Yee JK, Shew JY, Chen PL, Bookstein R, Friedmann T, Lee EY, Lee WH. 1988. Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science (New York, NY)* **242**: 1563-1566.

Huang S, Wang NP, Tseng BY, Lee WH, Lee EH. 1990. Two distinct and frequently mutated regions of retinoblastoma protein are required for binding to SV40 T antigen. *The EMBO journal* **9**: 1815-1822.

Hung DT, Jamison TF, Schreiber SL. 1996. Understanding and controlling the cell cycle with natural products. *Chemistry & biology* **3**: 623-639.

Hurford RK, Jr., Cobrinik D, Lee MH, Dyson N. 1997. pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. *Genes & development* **11**: 1447-1463.

Ianari A, Natale T, Calo E, Ferretti E, Alesse E, Screpanti I, Haigis K, Gulino A, Lees JA. 2009. Proapoptotic function of the retinoblastoma tumor suppressor protein. *Cancer cell* **15**: 184-194.

Indovina P, Pentimalli F, Casini N, Vocca I, Giordano A. 2015. RB1 dual role in proliferation and apoptosis: cell fate control and implications for cancer therapy. *Oncotarget*.

Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA. 1992. Effects of an Rb mutation in the mouse. *Nature* **359**: 295-300.

Jackson MW, Agarwal MK, Yang J, Bruss P, Uchiumi T, Agarwal ML, Stark GR, Taylor WR. 2005. p130/p107/p105Rb-dependent transcriptional repression during DNA-damage-induced cell-cycle exit at G2. *Journal of cell science* **118**: 1821-1832.

Janicke RU, Walker PA, Lin XY, Porter AG. 1996. Specific cleavage of the retinoblastoma protein by an ICE-like protease in apoptosis. *The EMBO journal* **15**: 6969-6978.

Ji P, Jiang H, Rekhtman K, Bloom J, Ichetovkin M, Pagano M, Zhu L. 2004. An Rb-Skp2-p27 pathway mediates acute cell cycle inhibition by Rb and is retained in a partial-penetrance Rb mutant. *Molecular cell* **16**: 47-58.

Jones DT, Cozzetto D. 2015. DISOPRED3: precise disordered region predictions with annotated protein-binding activity. *Bioinformatics* **31**: 857-863.

Julian LM, Palander O, Seifried LA, Foster JE, Dick FA. 2008. Characterization of an E2F1-specific binding domain in pRB and its implications for apoptotic regulation. *Oncogene* **27**: 1572-1579.

Kaelin WG, Jr. 1997. Alterations in G1/S cell-cycle control contributing to carcinogenesis. *Annals of the New York Academy of Sciences* **833**: 29-33.

Kaelin WG, Jr., Ewen ME, Livingston DM. 1990. Definition of the minimal simian virus 40 large T antigen- and adenovirus E1A-binding domain in the retinoblastoma gene product. *Molecular and cellular biology* **10**: 3761-3769.

Kalderon D, Roberts BL, Richardson WD, Smith AE. 1984. A short amino acid sequence able to specify nuclear location. *Cell* **39**: 499-509.

Kerr JF, Wyllie AH, Currie AR. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer* **26**: 239-257.

Khavari PA, Peterson CL, Tamkun JW, Mendel DB, Crabtree GR. 1993. BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. *Nature* **366**: 170-174.

Knudsen ES, Wang JY. 2010. Targeting the RB-pathway in cancer therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**: 1094-1099.

Knudsen KE, Booth D, Naderi S, Sever-Chroneos Z, Fribourg AF, Hunton IC, Feramisco JR, Wang JY, Knudsen ES. 2000. RB-dependent S-phase response to DNA damage. *Molecular and cellular biology* **20**: 7751-7763.

Knudsen KE, Weber E, Arden KC, Cavenee WK, Feramisco JR, Knudsen ES. 1999. The retinoblastoma tumor suppressor inhibits cellular proliferation through two distinct mechanisms: inhibition of cell cycle progression and induction of cell death. *Oncogene* **18**: 5239-5245.

Knudson AG, Jr. 1971. Mutation and cancer: statistical study of retinoblastoma. *Proceedings of the National Academy of Sciences of the United States of America* **68**: 820-823.

Knudson AG, Jr., Hethcote HW, Brown BW. 1975. Mutation and childhood cancer: a probabilistic model for the incidence of retinoblastoma. *Proceedings of the National Academy of Sciences of the United States of America* **72**: 5116-5120.

Lacy S, Whyte P. 1997. Identification of a p130 domain mediating interactions with cyclin A/cdk 2 and cyclin E/cdk 2 complexes. *Oncogene* **14**: 2395-2406.

Lara MF, Santos M, Ruiz S, Segrelles C, Moral M, Martinez-Cruz AB, Hernandez P, Martinez-Palacio J, Lorz C, Garcia-Escudero R et al. 2008. p107 acts as a tumor suppressor in pRb-deficient epidermis. *Molecular carcinogenesis* **47**: 105-113.

Lazzerini Denchi E, Helin K. 2005. E2F1 is crucial for E2F-dependent apoptosis. *EMBO reports* **6**: 661-668.

Lee EY, Cam H, Ziebold U, Rayman JB, Lees JA, Dynlacht BD. 2002. E2F4 loss suppresses tumorigenesis in Rb mutant mice. *Cancer cell* **2**: 463-472.

Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A. 1992. Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359**: 288-294.

Lee JO, Russo AA, Pavletich NP. 1998. Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. *Nature* **391**: 859-865.

Lee KY, Ladha MH, McMahon C, Ewen ME. 1999. The retinoblastoma protein is linked to the activation of Ras. *Molecular and cellular biology* **19**: 7724-7732.

Lee MH, Williams BO, Mulligan G, Mukai S, Bronson RT, Dyson N, Harlow E, Jacks T. 1996. Targeted disruption of p107: functional overlap between p107 and Rb. *Genes & development* **10**: 1621-1632.

Lee WH, Bookstein R, Hong F, Young LJ, Shew JY, Lee EY. 1987. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science (New York, NY)* **235**: 1394-1399.

Lehmann BD, Brooks AM, Paine MS, Chappell WH, McCubrey JA, Terrian DM. 2008. Distinct roles for p107 and p130 in Rb-independent cellular senescence. *Cell Cycle* **7**: 1262-1268.

Lentine B, Antonucci L, Hunce R, Edwards J, Marallano V, Krucher NA. 2012. Dephosphorylation of threonine-821 of the retinoblastoma tumor suppressor protein (Rb) is required for apoptosis induced by UV and Cdk inhibition. *Cell Cycle* **11**: 3324-3330.

Litovchick L, Chestukhin A, DeCaprio JA. 2004. Glycogen synthase kinase 3 phosphorylates RBL2/p130 during quiescence. *Molecular and cellular biology* **24**: 8970-8980.

Liu X, Yang JM, Zhang SS, Liu XY, Liu DX. 2010. Induction of cell cycle arrest at G1 and S phases and cAMP-dependent differentiation in C6 glioma by low concentration of cycloheximide. *BMC cancer* **10**: 684.

Lohmann DR, Brandt B, Hopping W, Passarge E, Horsthemke B. 1994. Distinct RB1 gene mutations with low penetrance in hereditary retinoblastoma. *Human genetics* **94**: 349-354.

Ludlow JW, Glendening CL, Livingston DM, DeCarprio JA. 1993. Specific enzymatic dephosphorylation of the retinoblastoma protein. *Molecular and cellular biology* **13**: 367-372.

Ludlow JW, Skuse GR. 1995. Viral oncoprotein binding to pRB, p107, p130, and p300. *Virus research* **35**: 113-121.

Lundberg AS, Weinberg RA. 1998. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Molecular and cellular biology* **18**: 753-761.

Ma D, Zhou P, Harbour JW. 2003. Distinct mechanisms for regulating the tumor suppressor and antiapoptotic functions of Rb. *The Journal of biological chemistry* **278**: 19358-19366.

Macdonald JI, Dick FA. 2012. Posttranslational modifications of the retinoblastoma tumor suppressor protein as determinants of function. *Genes & cancer* **3**: 619-633.

Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B, Jacks T. 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes & development* **9**: 935-944.

Magnaghi-Jaulin L, Groisman R, Naguibneva I, Robin P, Lorain S, Le Villain JP, Troalen F, Trouche D, Harel-Bellan A. 1998. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391**: 601-605.

Malumbres M, Barbacid M. 2001. To cycle or not to cycle: a critical decision in cancer. *Nature reviews Cancer* **1**: 222-231.

-. 2005. Mammalian cyclin-dependent kinases. *Trends in biochemical sciences* **30**: 630-641.

Mandell DJ, Coutsias EA, Kortemme T. 2009. Sub-angstrom accuracy in protein loop reconstruction by robotics-inspired conformational sampling. *Nature methods* **6**: 551-552.

Manning AL, Longworth MS, Dyson NJ. 2010. Loss of pRB causes centromere dysfunction and chromosomal instability. *Genes & development* **24**: 1364-1376.

Markham D, Munro S, Soloway J, O'Connor DP, La Thangue NB. 2006. DNA-damage-responsive acetylation of pRb regulates binding to E2F-1. *EMBO reports* **7**: 192-198.

Masselli A, Wang JY. 2006. Phosphorylation site mutated RB exerts contrasting effects on apoptotic response to different stimuli. *Oncogene* **25**: 1290-1298.

Moberg K, Starz MA, Lees JA. 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Molecular and cellular biology* **16**: 1436-1449.

Morimoto H, Morimoto Y, Ohba T, Kido H, Kobayashi S, Haneji T. 1999. Inhibitors of protein synthesis and RNA synthesis protect against okadaic acid-induced apoptosis in human osteosarcoma cell line MG63 cells but not in Saos-2 cells. *Journal of bone and mineral metabolism* **17**: 266-273.

Morris EJ, Dyson NJ. 2001. Retinoblastoma protein partners. *Advances in cancer research* **82**: 1-54.

Munger K, Werness BA, Dyson N, Phelps WC, Harlow E, Howley PM. 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *The EMBO journal* **8**: 4099-4105.

Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW. 2003. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113**: 703-716.

Nivon LG, Moretti R, Baker D. 2013. A Pareto-optimal refinement method for protein design scaffolds. *PloS one* **8**: e59004.

Nyberg KA, Michelson RJ, Putnam CW, Weinert TA. 2002. Toward maintaining the genome: DNA damage and replication checkpoints. *Annual review of genetics* **36**: 617-656.

Onadim Z, Hogg A, Baird PN, Cowell JK. 1992. Oncogenic point mutations in exon 20 of the RB1 gene in families showing incomplete penetrance and mild expression of the retinoblastoma phenotype. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 6177-6181.

Pucci B, Claudio PP, Masciullo V, Bellincampi L, Terrinoni A, Khalili K, Melino G, Giordano A. 2002. pRb2/p130 promotes radiation-induced cell death in the glioblastoma cell line HJC12 by p73 upregulation and Bcl-2 downregulation. *Oncogene* **21**: 5897-5905.

Qian Y, Luckey C, Horton L, Esser M, Templeton DJ. 1992. Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. *Molecular and cellular biology* **12**: 5363-5372.

Qin XQ, Chittenden T, Livingston DM, Kaelin WG, Jr. 1992. Identification of a growth suppression domain within the retinoblastoma gene product. *Genes & development* **6**: 953-964.

Qin XQ, Livingston DM, Kaelin WG, Jr., Adams PD. 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 10918-10922.

Rubin SM. 2013. Deciphering the retinoblastoma protein phosphorylation code. *Trends in biochemical sciences* **38**: 12-19.

Rubin SM, Gall AL, Zheng N, Pavletich NP. 2005. Structure of the Rb C-terminal domain bound to E2F1-DP1: a mechanism for phosphorylation-induced E2F release. *Cell* **123**: 1093-1106.

Saavedra HI, Wu L, de Bruin A, Timmers C, Rosol TJ, Weinstein M, Robinson ML, Leone G. 2002. Specificity of E2F1, E2F2, and E2F3 in mediating phenotypes induced by loss of Rb. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **13**: 215-225.

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

Sage J, Mulligan GJ, Attardi LD, Miller A, Chen S, Williams B, Theodorou E, Jacks T. 2000. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes & development* **14**: 3037-3050.

Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annual review of biochemistry* **73**: 39-85.

Schwartz GK, Shah MA. 2005. Targeting the cell cycle: a new approach to cancer therapy. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **23**: 9408-9421.

Sellers WR, Novitch BG, Miyake S, Heith A, Otterson GA, Kaye FJ, Lassar AB, Kaelin WG, Jr. 1998. Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. *Genes & development* **12**: 95-106.

Sellers WR, Rodgers JW, Kaelin WG, Jr. 1995. A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 11544-11548.

Senderowicz AM. 2002. The cell cycle as a target for cancer therapy: basic and clinical findings with the small molecule inhibitors flavopiridol and UCN-01. *The oncologist* **7 Suppl 3**: 12-19.

Shan B, Durfee T, Lee WH. 1996. Disruption of RB/E2F-1 interaction by single point mutations in E2F-1 enhances S-phase entry and apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 679-684.

Shan B, Lee WH. 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Molecular and cellular biology* **14**: 8166-8173.

Sherr CJ. 1996. Cancer cell cycles. *Science (New York, NY)* **274**: 1672-1677.

Sherr CJ, Roberts JM. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & development* **13**: 1501-1512.

Shew JY, Lin BT, Chen PL, Tseng BY, Yang-Feng TL, Lee WH. 1990. C-terminal truncation of the retinoblastoma gene product leads to functional inactivation. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 6-10.

Sotillo R, Hernando E, Diaz-Rodriguez E, Teruya-Feldstein J, Cordon-Cardo C, Lowe SW, Benezra R. 2007. Mad2 overexpression promotes aneuploidy and tumorigenesis in mice. *Cancer cell* **11**: 9-23.

Stein A, Kortemme T. 2013. Improvements to robotics-inspired conformational sampling in rosetta. *PloS one* **8**: e63090.

Strobeck MW, Knudsen KE, Fribourg AF, DeCristofaro MF, Weissman BE, Imbalzano AN, Knudsen ES. 2000. BRG-1 is required for RB-mediated cell cycle arrest. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 7748-7753.

Strober BE, Dunaief JL, Guha, Goff SP. 1996. Functional interactions between the hBRM/hBRG1 transcriptional activators and the pRB family of proteins. *Molecular and cellular biology* **16**: 1576-1583.

Sun H, Chang Y, Schweers B, Dyer MA, Zhang X, Hayward SW, Goodrich DW. 2006. An E2F binding-deficient Rb1 protein partially rescues developmental defects associated with Rb1 nullizygosity. *Molecular and cellular biology* **26**: 1527-1537.

Takahashi Y, Rayman JB, Dynlacht BD. 2000. Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. *Genes & development* **14**: 804-816.

Tan X, Martin SJ, Green DR, Wang JY. 1997. Degradation of retinoblastoma protein in tumor necrosis factor- and CD95-induced cell death. *The Journal of biological chemistry* **272**: 9613-9616.

Templeton DJ, Park SH, Lanier L, Weinberg RA. 1991. Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 3033-3037.

Theis S, Roemer K. 1998. c-Abl tyrosine kinase can mediate tumor cell apoptosis independently of the Rb and p53 tumor suppressors. *Oncogene* **17**: 557-564.

Thomas DM, Carty SA, Piscopo DM, Lee JS, Wang WF, Forrester WC, Hinds PW. 2001. The retinoblastoma protein acts as a transcriptional coactivator required for osteogenic differentiation. *Molecular cell* **8**: 303-316.

Thomas DM, Johnson SA, Sims NA, Trivett MK, Slavin JL, Rubin BP, Waring P, McArthur GA, Walkley CR, Holloway AJ et al. 2004. Terminal osteoblast differentiation, mediated by runx2 and p27KIP1, is disrupted in osteosarcoma. *The Journal of cell biology* **167**: 925-934.

Tonini T, Gabellini C, Bagella L, D'Andrilli G, Masciullo V, Romano G, Scambia G, Zupi G, Giordano A. 2004. pRb2/p130 decreases sensitivity to apoptosis induced by camptothecin and doxorubicin but not by taxol. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10**: 8085-8093.

Tosi GM, Trimarchi C, Macaluso M, La Sala D, Ciccodicola A, Lazzi S, Massaro-Giordano M, Caporossi A, Giordano A, Cinti C. 2005. Genetic and epigenetic alterations of RB2/p130 tumor suppressor gene in human sporadic retinoblastoma: implications for pathogenesis and therapeutic approach. *Oncogene* **24**: 5827-5836.

Trouche D, Le Chalony C, Muchardt C, Yaniv M, Kouzarides T. 1997. RB and hbrm cooperate to repress the activation functions of E2F1. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 11268-11273.

Tsujimoto Y, Shimizu S. 2005. Another way to die: autophagic programmed cell death. *Cell death and differentiation* **12 Suppl 2**: 1528-1534.

Vairo G, Livingston DM, Ginsberg D. 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. *Genes & development* **9**: 869-881.

Vidal A, Koff A. 2000. Cell-cycle inhibitors: three families united by a common cause. *Gene* **247**: 1-15.

Vogelstein B, Kinzler KW. 1993. The multistep nature of cancer. *Trends in genetics : TIG* **9**: 138-141.

Wang JY. 2000. Regulation of cell death by the Abl tyrosine kinase. *Oncogene* **19**: 5643-5650.

Weaver BA, Silk AD, Montagna C, Verdier-Pinard P, Cleveland DW. 2007. Aneuploidy acts both oncogenically and as a tumor suppressor. *Cancer cell* **11**: 25-36.

Weinert T. 1998a. DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. *Cell* **94**: 555-558.

-. 1998b. DNA damage checkpoints update: getting molecular. *Current opinion in genetics & development* **8**: 185-193.

Welch PJ, Wang JY. 1993. A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. *Cell* **75**: 779-790.

-. 1995a. Abrogation of retinoblastoma protein function by c-Abl through tyrosine kinase-dependent and -independent mechanisms. *Molecular and cellular biology* **15**: 5542-5551.

-. 1995b. Disruption of retinoblastoma protein function by coexpression of its C pocket fragment. *Genes & development* **9**: 31-46.

Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ. 2000. Target gene specificity of E2F and pocket protein family members in living cells. *Molecular and cellular biology* **20**: 5797-5807.

Whitaker LL, Su H, Baskaran R, Knudsen ES, Wang JY. 1998. Growth suppression by an E2F-binding-defective retinoblastoma protein (RB): contribution from the RB C pocket. *Molecular and cellular biology* **18**: 4032-4042.

Whyte P, Buchkovich KJ, Horowitz JM, Friend SH, Raybuck M, Weinberg RA, Harlow E. 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334**: 124-129.

Wilson IA, Haft DH, Getzoff ED, Tainer JA, Lerner RA, Brenner S. 1985. Identical short peptide sequences in unrelated proteins can have different conformations: a testing ground for theories of immune recognition. *Proceedings of the National Academy of Sciences of the United States of America* **82**: 5255-5259.

Woo MS, Sanchez I, Dynlacht BD. 1997. p130 and p107 use a conserved domain to inhibit cellular cyclin-dependent kinase activity. *Molecular and cellular biology* **17**: 3566-3579.

Wu X, Levine AJ. 1994. p53 and E2F-1 cooperate to mediate apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 3602-3606.

Wuarin J, Nurse P. 1996. Regulating S phase: CDKs, licensing and proteolysis. *Cell* **85**: 785-787.

Xiao B, Spencer J, Clements A, Ali-Khan N, Mittnacht S, Broceno C, Burghammer M, Perrakis A, Marmorstein R, Gamblin SJ. 2003. Crystal structure of the retinoblastoma tumor suppressor protein bound to E2F and the molecular basis of its regulation. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 2363-2368.

Xiao H, Goodrich DW. 2005. The retinoblastoma tumor suppressor protein is required for efficient processing and repair of trapped topoisomerase II-DNA-cleavable complexes. *Oncogene* **24**: 8105-8113.

Xiao ZX, Chen J, Levine AJ, Modjtahedi N, Xing J, Sellers WR, Livingston DM. 1995. Interaction between the retinoblastoma protein and the oncoprotein MDM2. *Nature* **375**: 694-698.

Xu HJ, Xu K, Zhou Y, Li J, Benedict WF, Hu SX. 1994. Enhanced tumor cell growth suppression by an N-terminal truncated retinoblastoma protein. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 9837-9841.

Xu HJ, Zhou Y, Ji W, Perng GS, Kruzelock R, Kong CT, Bast RC, Mills GB, Li J, Hu SX. 1997. Reexpression of the retinoblastoma protein in tumor cells induces senescence and telomerase inhibition. *Oncogene* **15**: 2589-2596.

Yandell DW, Campbell TA, Dayton SH, Petersen R, Walton D, Little JB, McConkie-Rosell A, Buckley EG, Dryja TP. 1989. Oncogenic point mutations in the human retinoblastoma gene: their application to genetic counseling. *The New England journal of medicine* **321**: 1689-1695.

Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. 2015. The I-TASSER Suite: protein structure and function prediction. *Nature methods* **12**: 7-8.

Yao G, Lee TJ, Mori S, Nevins JR, You L. 2008. A bistable Rb-E2F switch underlies the restriction point. *Nature cell biology* **10**: 476-482.

Yata K, Esashi F. 2009. Dual role of CDKs in DNA repair: to be, or not to be. *DNA repair* **8**: 6-18.

Zacksenhaus E, Bremner R, Phillips RA, Gallie BL. 1993. A bipartite nuclear localization signal in the retinoblastoma gene product and its importance for biological activity. *Molecular and cellular biology* **13**: 4588-4599.

Zhang HS, Dean DC. 2001. Rb-mediated chromatin structure regulation and transcriptional repression. *Oncogene* **20**: 3134-3138.

Zhu L, Enders G, Lees JA, Beijersbergen RL, Bernards R, Harlow E. 1995. The pRB-related protein p107 contains two growth suppression domains: independent interactions with E2F and cyclin/cdk complexes. *The EMBO journal* **14**: 1904-1913.

Zhu L, Lu Z, Zhao H. 2015. Antitumor mechanisms when pRb and p53 are genetically inactivated. *Oncogene* **34**: 4547-4557.

Zhu L, van den Heuvel S, Helin K, Fattaey A, Ewen M, Livingston D, Dyson N, Harlow E. 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes & development* **7**: 1111-1125.

Zhu Y, Alvarez C, Doll R, Kurata H, Schebye XM, Parry D, Lees E. 2004. Intra-S-phase checkpoint activation by direct CDK2 inhibition. *Molecular and cellular biology* **24**: 6268-6277.

Appendix

List of Publications

Publications not directly related with this thesis:

E2F4 plays a key role in Burkitt lymphoma tumorigenesis.

Molina-Privado I, Jiménez-P R, Montes-Moreno S, **Chiodo Y**, Rodríguez-Martínez M, Sánchez-Verde L, Iglesias T, Piris MA, Campanero MR.

Leukemia. 2012 Oct;26(10):2277-85. doi: 10.1038/leu.2012.99. Epub 2012 Apr 5. PMID: 22475873