University of Siena

Ph.D in Oncology and Genetics

PIN1, the cell cycle control and cancer: a new player in the RB pathway

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INTRODUCTION

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INTRODUCTION

Aims of the thesis

The focus of my thesis is to investigate the mechanisms that regulate cell cycle control, the deregulation of which can lead to the development of cancer. The laboratory of Dr. Giordano has dedicated many years to analyzing the mechanisms of cell cycle regulation. The first discovery was done twenty years ago when Dr. Giordano discovered for the first time that an identical protein species occurs in complexes within both a virus and with the cell cycle regulatory kinase cdc2 [1]. Later, this protein species was identified as cyclin A. In the early 1990s, Giordano's laboratory cloned a new member of the Retinoblastoma family, Rb2/p130 [2], and in the years that followed have clarified its tumor suppressor role in different tissue such as lung, liver, ovary, breast and prostate.

The majority of somatic cells are quiescent. Since pRb, Rb2/p130 and p107, members of the Retinoblastoma gene family, play a pivotal role in cell cycle entry, progression, and exit, they are fundamental in the tumorigenesis process. The activity of pRb is regulated by phosphorylation. Many serine and threonine residues are specifically phosphorylated at different stages of cell cycle progression. PIN1 is a member of the evolutionarily conserved peptidyl-prolyl isomerase (PPIase) family of proteins that catalyzes the conformational switch from cis to trans of target proteins, which is especially important because Pro-directed kinases and phosphatases are conformation-specific and act only on the trans isoform [3, 4]. The specific hypothesis behind the

proposed research thesis is that the PIN1 protein interacts with pRb and it is a key regulator of pRb function during the cell cycle. That hypothesis is based on the following observations. First, mouse embryonic fibroblast deficient in the PIN1 gene showed a defect in cell cycle entry and progression [5-7]. The PIN1 knockout (KO) cells had a reduced proliferation rate compared to wild type cells. Second, in PIN1-/- MEF cells, the pRb protein is hypo-phosphorylated. Third, PIN1 protein is upregulated in many tumor types [8] and is a prognostic factor in prostate cancer [9]. Based on these observations, the experimental focus of this thesis is based on the regulation of Retinoblastoma protein through the PIN1 protein. The specific aims were designed to provide a comprehensive assessment of pRb regulation during the cell cycle when PIN1 is altered.

1. Characterize the functional interaction between PIN1 and pRb during the cell cycle. Cyclin (D)/CDK4(6) and cyclin E (A)/CDK2 are involved in pRb phosphorylation during the G0 and G1/S cell cycle transition. Both proteins are controlled by PIN1 at transcriptional (cyclin D1) [5-7] and protein levels (Cyclin D1 and E) [10]. In particular, down-regulation of cyclin D1 and pRb hypophosphorylation is demonstrated in PIN1 knockdown (kd) cells [5-7]. Since re-expression of cyclin D1 doesn't allow phosphorylation of pRB, a different mechanism could be involved. pRB protein is composed of many ser/thr-pro motifs that are potential PIN1 recognition sites and a physical interaction between PIN1 and pRb could be hypothesized. This hypothesis has been tested by different approaches:

A. To test the activity of the cyclin/CDK complex on pRb, we performed a western blot analysis in wild type and PIN1 knock down cells (Fig. 4b) and a kinase assay (Fig. 4d)

B. The level of the pRb protein's phosphorylation were tested by western blot in PIN1 knock down cells (Fig. 5a)

C. The interaction between PIN1 and pRb were done by GST pull-down approach (Fig. 6b) and co-immunoprecipitation of endogenous proteins in T98G cells (Fig. 7a).

D. To assess if the interaction is phosphorylation dependent, the T98G cells were treated with shrimp alkaline phosphates followed by GST pulldown (Fig. 6c).

2. Identify the role of PIN1 in cell cycle control through RB pathways. Many papers showed a direct link between PIN1 and the cell cycle. As first discovered, PIN1 was identified as a player in G2/M and a protein that regulate mitosis. After that, a plethora of molecules were identified, such as cdc25, cdc27, TOPIIa. More recently other molecules that regulate G1/S phases have been identified, among them Cyclin D1, cyclin E, Ki67 and c-Myc [11]. These results suggest that different target proteins could be involved at different stages of the cell cycle. Since pRb is the major player of the G0 and G1/S phases, we want test if it is an essential PIN1 target to control proliferation. The role of PIN1 and pRb interaction in the cell cycle were tested in PIN1 knockdown cells by cell proliferation assays XTT (Fig. 2b)

and FACS analysis (Fig. 2c). Double knockdown cells pRb/PIN1 were prepared and tested by XTT (Fig. 8b) and colony forming assay (Fig. 8c).

Since we believed that an alteration in cell cycle control is at the basis of almost all tumors, we are dedicating our time to clarifying the fine mechanism underlying this mechanism with the final goal being the development of new effective drugs to treat cancer patients. My goal in the PhD experience was to acquire the research skills in Cancer Molecular Genetics, Cellular and Molecular Biology and Molecular Mechanisms of Disease. The training program has covered all the fundamental areas of genetics, cell biology and molecular biology that are essential for understanding Cancer Biology and Molecular Medicine.

At the end of the program, my research thesis has contributed to the discovery of new uncharacterized interactions between two key players of tumorigenesis and I have acquired the necessary skills to become an expert in cancer biology. I am very convinced that this training puts me at the forefront of cancer biology research and will permit me to become a productive scientist.

RB pathway

Normal cells became tumor cells through deregulation of multiple pathways. Evidences suggest that each type of tumor involves different proteins so that each type of cancer cell is different from the others. However, there are some pathways that are altered in almost all tumors. Recently, genome-wide studies have added new information to this finding. For example, in lung carcinoma, genetic alterations occur more frequently in the MAPK, p53, Wnt, RB and mTOR pathways [12]. In glioblastoma, RAS/PI3K, p53 and RB pathways are the major causes of cancer [13]. This data suggests that some pathways have a pivotal role in different tumors. Among them, pRb controls cell cycle entry, progression, and exit. Since the majority of the cells are quiescent, deregulation of cell cycle control and hence pRb protein, it is the first step to transforming normal cells into immortal cells (see Article 1).

Article 1

RB Gene Family: Genome-Wide ChIP Approaches Could Open Undiscovered Roads

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RB Gene Family: Genome-Wide ChIP Approaches Could Open Undiscovered Roads

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ABSTRACT

Many in vitro and reporter assays have helped to clarify how transcription factors regulate gene transcription. Today, it is important to decode the map of all transcription factor binding sites in the genome context. Chromatin immunoprecipitation followed by genome-wide analyses have tremendously opened new ways to analyze the mechanisms of action of DNA binding factors, cofactors and epigenetic modifications. It is now possible to correlate these regulatory mechanisms with genomic features such as the promoter, enhancer, silencer, intragenic, and intergenic DNA sequences. These approaches help to clarify the complex rules that govern many biological processes. In this review we discuss the genome-wide approaches applied to the retinoblastoma gene family (RBF), the central player of cell cycle control. There are also new, possible directions that are suggested within the review that can be followed to further explore the role of each pRb members in the transcriptional networks of the cell. J. Cell. Biochem. 109: 839–843, 2010.

KEY WORDS: RBF; CELL CYCLE; ChIP GENOME-WIDE

pRb is the key gene in a rare pediatric eye neoplasm (sporadic and hereditary) arising from retinal cells that harbor either a deletion or mutational inactivation of both pRb alleles [Knudson, 1971; Dunn et al., 1988; Paggi and Giordano, 2001; Cobrinik, 2005] pRb is a bona fide tumor suppressor gene, and its mutation or deletion is shared by several malignancies [Paggi et al., 1996]. For these reasons, pRb is considered one of the hallmarks of human malignancies [Hannon et al., 1993; Mayol et al., 1993; Zhu et al., 1993].

The pRb gene is considered as the founder of the RB family since two other genes have been identified, both of which are structurally and functionally related. These genes are named p107 [Ewen et al., 1991; Zhu et al., 1993] and Rb2/p130 [Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993]. Cytogenetically, Rb2/p130 maps to the 16q12.2–13, a genomic region repeatedly altered in human cancers [Goodrich et al., 1991; Hannon et al., 1993; Li et al., 1993]. We have demonstrated that, Rb2/p130 has tumor-suppressor properties in JC virus-induced hamster brain tumor cells [Howard et al., 1998], and the genetic alteration of the Rb2/p130 genes have often been detected in human cancers. Specifically, Rb2/p130 is altered in breast, ovarian, prostate, small-cell lung cancers and many other tumor types [Paggi and Giordano, 2001]. p107 maps to the human chromosome region 20q11.2, a locus not frequently found involved in human neoplasms [Ewen et al., 1991; Ichimura et al., 2000]. It should be noted, however, that p107 suppresses the development of Retinoblastoma in pRb-deficient mice [Robanus-Maandag et al., 1998].

In the recent past, genome-wide approaches have elucidated the mechanism of action of RBF on target genes and yielded some unexpected results. In this review, we summarize the recent findings, give a critical point of view on what has been done up to this point, and finally, highlight the anticipated steps to be taken in the near future.

RBF AND E2F PROTEINS IN CELL CYCLE CONTROL

The first data, identifying pRb in the cell cycle regulation, emerged more than 10 years ago. pRb controls the cell cycle through the interaction with E2F transcription factors [DeGregori et al., 1997; Attwooll et al., 2004; DeGregori and Johnson, 2006]. These interactions are regulated during cell cycle by a phosphorylation

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mechanism. In the early and mid-G1stages, D type cyclins bind CDK4 or CDK6 proteins, and in late G1, cyclin E (or A), along with CDK2 proteins, gradually phosporylate pRb. Hyper-phosphorylated pRb releases E2F transcription factors and allows the expression of genes that mediate S phase entry [Flemington et al., 1993; Helin and Ed, 1993].

The interaction between RBF members and E2F proteins brings about a repressive function that is mediated by two different mechanisms. The first general mechanism relies on the finding that the E2F transactivation domain and the pRb binding domain physically overlap at the E2F-C terminal [Flemington et al., 1993; Helin and Ed, 1993]. This interaction suggests a competitive model between pRb and the promoters of E2F target genes for activate transcription. The effects of this simple mechanism are not enough to explain why pRb, alone, can reduce E2F luciferase reporter activity in the absence of E2F [Weintraub et al., 1992] or why artificially pRb fused to E2F binding domain could act as a repressor on a basic promoter [Sellers et al., 1995]. The second general mechanism is based on the interaction between pRb and different chromatin modifier enzymes. pRb is able to interact with HDAC1,2,3 histone deacetylases, SUV39H methylases, and Brg1 and Brm chromatin-remodeling enzymes on the promoters of target genes [Cobrinik, 2005]. Further evidence of pRb's repressive function can be derived from site-direct mutagenesis of E2F binding elements on B-Myb, Cdc2, cyclin E and E2F1 target genes, which result in increased gene expression in quiescent and G1 cells. Genomic footprinting also supports these results because E2F complexes are bound to the B-Myb, cyclin A, and Cdc2 genes in quiescent cells and during early G1 when these genes are repressed [Liu et al., 1996]. Collectively, these data support the hypothesis that the RBF/E2F complex can bind the promoters of target genes and repress their expression.

RBF UNIQUE AND OVERLAPPING FUNCTIONS

Due to structural similarities, pRb, Rb2/p130, and p107 have many overlapping functions. All three proteins can repress gene transcription, cause an arrest of the cell cycle in the G1 phase, interact with viral oncoproteins, and share many protein partners [Mulligan and Jacks, 1998; Morris and Dyson, 2001]. Although RBF members possess many sequence similarities, they have additionally unique functions. Examples of differences include their expression pattern, E2F family member interaction (p107 and Rb2/p130 interact with E2F4/5 (repressing E2Fs) and pRb interacts with E2F1-3 (activating E2Fs)), cyclin/cdk complexes [Nevins, 1998; Classon and Dyson, 2001; Classon and Harlow, 2002; Cobrinik, 2005] and sets of target genes. Rb2/p130 is highly expressed in quiescent and differentiated cells while p107 is most often expressed in proliferating cells. pRb is ubiquitously expressed and can be detected in proliferating, quiescent and differentiated cells [Cobrinik, 2005].

An important distinction among the pocket proteins is observed during development. pRb nullizygous mice die during mid-gestation with defects in the nervous system, hematopoietic system and lens. In contrast, p107 and Rb2/p130 nullizygous mice having the same genetic background develop normally. Mice nullizygous for both Rb2/p130;p107 die at birth with abnormalities in endochondral bone formation and epidermal development. Embryos nullizygous for p107 or Rb2/p130, together with pRb loss, die approximately 2 days earlier than pRb null embryos and show more severe defects in the nervous and hematopoietic systems [Wikenheiser-Brokamp, 2006]. The RBF confirmed overlapping functions as well in development. Rb2/p130 is able to compensate for pRb deficiency in cardiac muscle development [MacLellan et al., 2005] and p107 can compensate for the loss of pRb function in the epidermal tissue [Ruiz et al., 2004]. These results support the observation that E2F transcription factors that normally bind pRb (i.e., E2F1, E2F2, E2F3) may bind p107 in pRb-deficient cells [Lee et al., 2002].

Pocket proteins have unique and overlapping functions in tumorigenesis as well in development. pRb heterozygous mice are prone to developing tumors of the pituitary, thyroid, and adrenal glands. p107 and Rb2/p130 ablation, alone or in combination, does not predispose to tumor formation. However, p107 and Rb2/p130 can function to suppress tumorigenesis in the context of pRb deficiency. Mice nullizygous for pRb do not develop retinoblastoma as is seen in humans. However, loss of p107 or Rb2/p130 in combination with pRb results in retinoblastoma [Wikenheiser-Brokamp, 2006]. Additionally, pRb ablation in astrocytes [Marino et al., 2000], mammary [Robinson et al., 2001] and prostate epithelial cells results in no phenotypic abnormalities, whereas loss of total pocket protein function by expression of a truncated form of SV40 large T antigen leads to tumor formation [Xiao et al., 2002; Simin et al., 2004]. Furthermore, chimeric pRb;p107 and pRb;Rb2/p130 null mice develop tumors in addition to those seen with pRb ablation alone [Dannenberg et al., 2004]. The tumor spectra in pRb, pRb;p107 and pRb;Rb2/p130 deficient mice do not totally overlap, providing evidence that the pocket proteins have unique as well as overlapping functions in tumor suppression.

GENOME-WIDE APPROACHES APPLIED TO RBF PROTEINS

In the last few years, ChIP genome-wide approaches have opened new roads to the analyses of transcription factors and chromatin modifications. These new methodologies are becoming important to identify basic players of different biological processes, such as gene expression, DNA replication and repair. RB family members play a key role in many gene regulatory networks that govern the cellular response to anti-mitogenic signals and whose deregulation constitutes one of the hallmarks of cancer. With the advent of ChIP technology, many important questions can now be addressed. Are the target genes among the RB family shared? Which are the main targets of each member? What are the relations between each RBF members and chromatin modifications on single target genes?

ChIP-on-chip, gene expression microarray and proteomic approaches have allowed different groups to "de-convolute" the specific roles of each pRb members. Many articles have demonstrated that at the genomic level, p107 and Rb2/p130 are the central pocket proteins that bind the E2F responsive promoters during G0 and early G1, and most of them are genes that regulate cell cycle

progression [Cam et al., 2004; Balciunaite et al., 2005; Litovchick et al., 2007; Farnham, 2009]. In particular, ChIP-on-chip in the T98G glioblastoma cell lines have shown Rb2/p130 and E2F4 cooperating to repress a common set of genes under different growth arrest conditions; however p107 or pRb do not function in doing this. The repression involves a set of genes not only involved in the cell cycle but also in mitochondrial biogenesis and metabolism with the NRF1 protein (nuclear respiratory factor-1) as a co-regulator of a number of E2F target genes [Cam et al., 2004]. In early G1 cycling cells, the same investigators reported three new functional categories of target genes uniquely bound to p107 and/or E2F4 (stress response, signal transduction, and immune response) and a distinct set of genes. In addition, specific combination of RBF and E2F4 proteins correspond to a distinct code of histone acetylation and Sin3B corepressor recruitment, highlighting a complex relation between RBF and chromating remodeling [Balciunaite et al., 2005].

In a more recent work, proteomic, ChIP-promoter array, gene expression array and bioinformatics analysis have allowed the discovery of a Rb2/p130-associated protein complex that contributes to repress cell cycle-dependent genes during quiescence [Litovchick et al., 2007]. Combined protein immunoprecipitation with multidimensional protein identification technology (MudPIT), Litovchick et al., identified 12 Rb2/p130 interacting proteins, 9 of which are homologous to Drosophila dREAM complex. In Drosophila this complex was determinated to be essential for the silencing of developmentally regulated genes. Interestingly, the human complex assembles in two different ways during the cell cycle: in G0, Rb2/p130, E2F4/5, and DP1/2 interact with LIN9, LIN37, LIN52, LIN54, and RBBP4 to repress transcription. During S phase LIN9, LIN37, LIN52, LIN54 dissociate from Rb2/p130 and interact with the B-MYB protein. Promoter chip assays revealed that the G0 complex bound and cooperated to specifically repress E2F target genes. Because pRb is not found in this complex, the authors postulated that Rb2/p130, not pRb, serves as the functional ortholog of pRb from fly and worm to human.

The rather surprising result that emerged from ChIP experiments was the difficulty to detect the presence of pRb on the promoters of many well-established E2F target genes. The only exception is the cyclin E gene that is also deregulated in pRb deficient mouse embryonic fibroblasts. It was speculated that pRb forms the repressor complex in the cytoplasm instead of on chromatin [Stevaux and Dyson, 2002; Iaquinta and Lees, 2007]. Another possible explanation was that pRb can bind regulatory regions other than promoters by a direct E2F mediated mechanism (this is improbable because E2F proteins bind preferentially the promoter region of genes) or by a different mechanism involving other factors [Markey et al., 2002]. With the limits of past microarray technology, we could not analyze the regions outside the promoter. We know, from the β-globin locus control region [Misteli, 2007] and genome-wide studies [Farnham, 2009] that, DNA elements apart several kilobases from the gene are able to enhance gene transcription. The mapping of the ERα binding site is one of the best examples [Carroll et al., 2005, 2006; Laganiere et al., 2005]. The group of Myles Brown analyzed the complete non-repetitive sequence of human chromosome 21 and 22 [Carroll et al., 2005]. They established that most of the ERα binding sites mapped outside the promoter in many sequences with enhancer functions, as demonstrated by chromosome capture and luciferase assays. Subsequently, taking advantage of ChIP-on-chip on all the non-repetitive sequence of the human genome, the same group mapped ERα and RNA Pol II proteins binding in MCF7 breast cancer cells. Only 4% of estrogen binding sites mapped within 1 kb promoter. By combining transcriptional profiling arrays and chip-on-chip data, the authors demonstrated a positive correlation between binding sites within 50 kb of transcription start site and gene expression activation [Carroll et al., 2006]. Although the mechanism of action of ERα protein is cell type specific, these results correlate with data obtained on c-Myc, p53 and Sp1 binding on chromosome 21 and 22, suggesting the necessity of genome-wide studies in opposition to promoter analysis [Cawley et al., 2004].

At least two different articles suggest that the problems encountering in ChIP experiments carried out on the pRb protein could be ascribed to the antibodies [Takahashi et al., 2000; Stengel et al., 2009]. In a pioneering experiment, Takahashi et al., analyzed all three members of pRb and found that the repression of each promoter in T98G glioblastoma quiescent cells is associated with recruitment of E2F-4 and Rb2/p130. The authors tested eight different pRb antibodies without obtaining enrichment on background of E2F target genes [Takahashi et al., 2000]. After this report, the Farnham laboratory analyzed the in vivo binding sites of pRb in Raji cells utilizing chip-on-chip CpG array. Different pRb binding sites were detected in G0/G1 and during S phase. Surprisingly the number of hits was low compared to the other pRb family members [Wells et al., 2003; Balciunaite et al., 2005]. The differences in results found in literature could be ascribed to the cell lines utilized; however, very recently, an independent group reported that a number of pRb antibodies are not able to immunoprecipitate the crosslinked chromatin in SOS-2 cells. To overcome these problems, the authors prepared a GFP-pRb fusion protein as well Rb2/p130 or p107 and ChIP analyses were carried out with anti-GFP antibody. Positive results were obtained on *plk-1* and *dhfr* E2F target promoters. The binding of pRb, Rb2/p130, and p107 on chromatin were also confirmed by Fluorescence Recovery After Photobleaching analyses [Stengel et al., 2009].

These results were supported by another laboratory where it was confirmed that pRb could be detected and associated with chromatin on *cdc6* and *dhfr* E2F target promoters only when special chromatin fixation protocols (dimethyl adipimidate followed by 1% formaldehyde) were applied [Vandromme et al., 2008]. These data strongly suggest that ChiP-grade antibodies are necessary to analyze the pRb-binding site on chromatin to discriminate which unique and overlapping functions have the pRb family of proteins.

CONCLUSIONS AND FUTURE DIRECTIONS

One key point of the post-genomic era is to clarify how the cell machinery utilizes genomic information in normal and anomalous cells. Transcription factors, cofactors, histone modifications, and histone variants participate at different levels to regulate gene transcription in diverse processes including cell growth, proliferation, differentiation, and death [Kouzarides, 2007]. The pRb pathway

is central to regulating cell proliferation, one of the first steps in tumorigenesis. A lot of studies have shown that many chromatin modifier enzymes work in concert with pRb. It is time to investigate through the use of new approaches (e.g., RNA interference) how the RBF members can influence DNA and chromatin modifications and integrate these data with others ''omics'' approaches.

Until now, gene expression profiling has been the principal topic compared with various issues such as disease recurrence, invasive potential, treatment response, and molecular subtype. But at an upper level, integrating expression with protein-protein and protein/DNA interaction can help us better understand the pathways relevant to human pathological diseases such as cancer. Now, there are many integrative analysis platforms that can help discern between important functional connections and to identify relationships among transcriptional programs, protein complexes, disease subpopulations and drug treatments. Several tools (DAVID, Gene set Enrichment analysis, System biology, L2L, Connectivity MAP, etc.) are able to interrogate data from public repositories and display all the information in a network data system (Cytoscape, Osprey, PIANA, GenMAPP, GRAPHVIZ, etc.). Most of the current tools analyze single target signatures across a set of reference signatures. More sophisticated programs are necessary to integrate different types of data, which yields the emergence of all-versus-all, comparing approaches, such as ''Molecular concept map'' in Oncomine.

REFERENCES

Attwooll C, Lazzerini Denchi E, Helin K. 2004. The E2F family: Specific functions and overlapping interests. EMBO J 23:4709–4716.

Balciunaite E, Spektor A, Lents NH, Cam H, Te Riele H, Scime A, Rudnicki MA, Young R, Dynlacht BD. 2005. Pocket protein complexes are recruited to distinct targets in quiescent and proliferating cells. Mol Cell Biol 25:8166–8178.

Cam H, Balciunaite E, Blais A, Spektor A, Scarpulla RC, Young R, Kluger Y, Dynlacht BD. 2004. A common set of gene regulatory networks links metabolism and growth inhibition. Mol Cell 16:399–411.

Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M. 2005. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell 122: 33–43.

Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, Wang Q, Bekiranov S, Sementchenko V, Fox EA, Silver PA, Gingeras TR, Liu XS, Brown M. 2006. Genome-wide analysis of estrogen receptor binding sites. Nat Genet 38:1289–1297.

Cawley S, Bekiranov S, Ng HH, Kapranov P, Sekinger EA, Kampa D, Piccolboni A, Sementchenko V, Cheng J, Williams AJ, Wheeler R, Wong B, Drenkow J, Yamanaka M, Patel S, Brubaker S, Tammana H, Helt G, Struhl K, Gingeras TR. 2004. Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell 116:499–509.

Classon M, Dyson N. 2001. p107 and p130: Versatile proteins with interesting pockets. Exp Cell Res 264:135–147.

Classon M, Harlow E. 2002. The retinoblastoma tumour suppressor in development and cancer. Nat Rev Cancer 2:910–917.

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

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 Dev 18:2952–2962.

DeGregori J, Johnson DG. 2006. Distinct and overlapping roles for E2F family members in transcription, proliferation and apoptosis. Curr Mol Med 6:739–748.

DeGregori J, Leone G, Miron A, Jakoi L, Nevins JR. 1997. Distinct roles for E2F proteins in cell growth control and apoptosis. Proc Natl Acad Sci USA 94:7245–7250.

Dunn JM, Phillips RA, Becker AJ, Gallie BL. 1988. Identification of germline and somatic mutations affecting the retinoblastoma gene. Science 241:1797–1800.

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.

Farnham PJ. 2009. Insights from genomic profiling of transcription factors. Nat Rev Genet 10:605–616.

Flemington EK, Speck SH, Kaelin WG, Jr. 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc Natl Acad Sci USA 90:6914–6918.

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.

Hannon GJ, Demetrick D, Beach D. 1993. Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. Genes Dev 7:2378–2391.

Helin K, Ed H. 1993. The retinoblastoma protein as a transcriptional repressor. Trends Cell Biol 3:43–46.

Howard CM, Claudio PP, Gallia GL, Gordon J, Giordano GG, Hauck WW, Khalili K, Giordano A. 1998. Retinoblastoma-related protein pRb2/p130 and suppression of tumor growth in vivo. J Natl Cancer Inst 90:1451–1460.

Iaquinta PJ, Lees JA. 2007. Life and death decisions by the E2F transcription factors. Curr Opin Cell Biol 19:649–657.

Ichimura K, Hanafusa H, Takimoto H, Ohgama Y, Akagi T, Shimizu K. 2000. Structure of the human retinoblastoma-related p107 gene and its intragenic deletion in a B-cell lymphoma cell line. Gene 251:37–43.

Knudson AG, Jr. 1971. Mutation and cancer: Statistical study of retinoblastoma. Proc Natl Acad Sci USA 68:820–823.

Kouzarides T. 2007. Chromatin modifications and their function. Cell 128:693–705.

Laganiere J, Deblois G, Giguere V. 2005. Functional genomics identifies a mechanism for estrogen activation of the retinoic acid receptor alpha1 gene in breast cancer cells. Mol Endocrinol 19:1584–1592.

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.

Li Y, Graham C, Lacy S, Duncan AM, Whyte P. 1993. The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes Dev 7:2366–2377.

Litovchick L, Sadasivam S, Florens L, Zhu X, Swanson SK, Velmurugan S, Chen R, Washburn MP, Liu XS, DeCaprio JA. 2007. Evolutionarily conserved multisubunit RBL2/p130 and E2F4 protein complex represses human cell cycle-dependent genes in quiescence. Mol Cell 26:539–551.

Liu N, Lucibello FC, Zwicker J, Engeland K, Muller R. 1996. Cell cycle-regulated repression of B-myb transcription: Cooperation of an E2F site with a contiguous corepressor element. Nucleic Acids Res 24:2905–2910.

MacLellan WR, Garcia A, Oh H, Frenkel P, Jordan MC, Roos KP, Schneider MD. 2005. Overlapping roles of pocket proteins in the myocardium are unmasked by germ line deletion of p130 plus heart-specific deletion of Rb. Mol Cell Biol 25:2486–2497.

Marino S, Vooijs M, van Der Gulden H, Jonkers J, Berns A. 2000. Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev 14:994–1004.

Markey MP, Angus SP, Strobeck MW, Williams SL, Gunawardena RW, Aronow BJ, Knudsen ES. 2002. Unbiased analysis of RB-mediated transcriptional repression identifies novel targets and distinctions from E2F action. Cancer Res 62:6587–6597.

Mayol X, Grana X, Baldi A, Sang N, Hu Q, Giordano A. 1993. Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. Oncogene 8:2561–2566.

Misteli T. 2007. Beyond the sequence: Cellular organization of genome function. Cell 128:787–800.

Morris EJ, Dyson NJ. 2001. Retinoblastoma protein partners. Adv Cancer Res 82:1–54.

Mulligan G, Jacks T. 1998. The retinoblastoma gene family: Cousins with overlapping interests. Trends Genet 14:223–229.

Nevins JR. 1998. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ 9:585–593.

Paggi MG, Giordano A. 2001. Who is the boss in the retinoblastoma family? The point of view of Rb2/p130, the little brother. Cancer Res 61:4651–4654.

Paggi MG, Baldi A, Bonetto F, Giordano A. 1996. Retinoblastoma protein family in cell cycle and cancer: A review. J Cell Biochem 62:418–430.

Robanus-Maandag E, Dekker M, van der Valk M, Carrozza ML, Jeanny JC, Dannenberg JH, Berns A, te Riele H. 1998. p107 is a suppressor of retinoblastoma development in pRb-deficient mice. Genes Dev 12:1599–1609.

Robinson GW, Wagner KU, Hennighausen L. 2001. Functional mammary gland development and oncogene-induced tumor formation are not affected by the absence of the retinoblastoma gene. Oncogene 20:7115–7119.

Ruiz S, Santos M, Segrelles C, Leis H, Jorcano JL, Berns A, Paramio JM, Vooijs M. 2004. Unique and overlapping functions of pRb and p107 in the control of proliferation and differentiation in epidermis. Development 131:2737–2748.

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. Proc Natl Acad Sci USA 92:11544–11548.

Simin K, Wu H, Lu L, Pinkel D, Albertson D, Cardiff RD, Van Dyke T. 2004. pRb inactivation in mammary cells reveals common mechanisms for tumor initiation and progression in divergent epithelia. PLoS Biol 2:E22.

Stengel KR, Thangavel C, Solomon DA, Angus SP, Zheng Y, Knudsen ES. 2009. Retinoblastoma/p107/p130 pocket proteins: Protein dynamics and interactions with target gene promoters. J Biol Chem 284:19265–19271.

Stevaux O, Dyson NJ. 2002. A revised picture of the E2F transcriptional network and RB function. Curr Opin Cell Biol 14:684–691.

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 Dev 14:804–816.

Vandromme M, Chailleux C, Escaffit F, Trouche D. 2008. Binding of the retinoblastoma protein is not the determinant for stable repression of some E2F-regulated promoters in muscle cells. Mol Cancer Res 6:418–425.

Weintraub SJ, Prater CA, Dean DC. 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature 358:259–261.

Wells J, Yan PS, Cechvala M, Huang T, Farnham PJ. 2003. Identification of novel pRb binding sites using CpG microarrays suggests that E2F recruits pRb to specific genomic sites during S phase. Oncogene 22:1445–1460.

Wikenheiser-Brokamp KA. 2006. Retinoblastoma family proteins: Insights gained through genetic manipulation of mice. Cell Mol Life Sci 63:767–780.

Xiao A, Wu H, Pandolfi PP, Louis DN, Van Dyke T. 2002. Astrocyte inactivation of the pRb pathway predisposes mice to malignant astrocytoma development that is accelerated by PTEN mutation. Cancer Cell 1:157–168.

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 Dev 7:1111–1125.

CDK/cyclin complex

A key step for pRb protein activity is its phosphorylation. Among different protein kinase families, CDKs play a central role. CDKs are a well-conserved family of serine/threonine protein kinases whose activity is regulated by different proteins. Their activation requires association with a cyclin partner and phosphorylation by CDK activating kinase (CAK). Other residues such as conserved threonine and tyrosine must be dephosphorylated for CDK activity, which involves the Cdc25 phosphatase family of proteins. In the majority of tumors, alteration of CDK activity is associated with an inactivation of the pRb pathway followed by an increase in the cell proliferation rate. In the last ten years a number of laboratories (including our lab) have focused their attention to developing new small molecules to inhibit CDK function (see article 2).

Article 2

CDK Inhibitors: From the Bench to Clinical Trials

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CDK Inhibitors: From the Bench to Clinical Trials

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Abstract: Cell cycle deregulation is one of the first steps that transform normal cells into tumor cells. CDKs are a family of proteins devoted to controlling cell cycle entry, progression and exit. Studies from animal models show a tissue-specific essentiality of the single CDKs. In cancer cells, mis-regulation of CDK function is a common event. For this reason the pioneer compound Flavopiridol was developed and many new drugs are currently under development. ATP and the last generation of non-ATP competitive inhibitors are now emerging as one of the most potentially powerful target therapies. Many clinical trials are ongoing, as either a single agent or in combination with the classical cytotoxic agents. In this review, we discuss new strategies and methods to design more potent, selective and specific CDK inhibitors, starting from evidence emerging from animal and cancer cell models.

Keywords: CDK, kinase inhibitors, CDK clinical trials.

INTRODUCTION

Since the first discovery from Boveri and von Hansemann in which bizarre chromosomes in cancer cells were observed under the microscope [1, 2], the translocation between chromosomes 9 and 22 (Philadelphia translocation) in chronic myeloid leukemia [3], and the first naturally occurring mutation in the HRAS gene [4, 5], the number of new gene mutations discovered in cancer has dramatically increased. Among them, driver mutations are causally implicated in oncogenesis and confer growth advantages to cancer cells. Ninety percent of known somatically mutated cancer genes have a gain-of-function mutation and are defined as oncogenes. Systematic sequencing of the cancer genome showed that among oncogenes, a high number are kinases and a wave of drug discoveries started to identify new molecules with an anti-cancer inhibitory function [6].

The alteration of mechanisms that regulate cell cycle control is a common feature of most cancer types. Since the majority of somatic cells are quiescent, fine controls of cell cycle re-entry, progression and control are fundamental for the normal life of the cells [7, 8]. Fundamental to maintaining the quiescent state is what is termed the "restriction point" (R). The original definition was given by Arthur Pardee [9], who described it as an event after which cells can proliferate in the absence of a mitogenic signal. Thirty years after it was first described, the term restriction point is now known as the point that divides the early and late G1 cell cycle phases [10]. The pools of proteins that control this mechanism are still not well-defined. However, one of the major players is the RB gene family (pocket proteins) since ablation of these proteins eliminates R [11, 12]. Once the cells have left the G_0 state, they enter the cell cycle. The two basal steps of the cell cycle involve copying the genetic material (S) and dividing the entire cellular component into two identical daughter cells (M). The two other phases are called the gap period (G1 and G2), in which the cells are prepared for the S and M phases [13]. To ensure that daughter cells acquire all the characteristics of their parental cells, different checkpoints are in place, controlling each step of cell cycle. CDKs are a family of proteins that control many aspects of the cell cycle and their alterations are at the core of different cancer types [10, 14].

Many reviews published in recent years focus on drugs that target kinases in cancer [10, 15-18] and some of them focus on CDK inhibitors. Among CDK inhibitors, the ATP competitive inhibitors are the most discussed because clinical trials are already in progress. More recently, new CDK inhibitors that are non-ATP competitive inhibitors are being reported and discussed. The majority of reviews are written either from a biological or pharmacological point of view, with the former focusing on *in vitro* and *in vivo* models that are useful in understanding the role of CDKs in normal and/or cancerous cells and the latter discussing the mechanism of action and selectivity of the drugs on the target kinases.

The present review highlights the most important and newly identified inhibitors of CDKs, starting from the animal models with a discussion on the mechanism of action of various drugs and ending with clinical trials.

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CDK-CYCLIN FUNCTION

Most of the attention in cell cycle research is now focused on CDKs since this protein family controls most of the steps of the cell cycle, either directly or indirectly [19, 20]. Progression of the eukaryotic cell throughout the four phases of the cell cycle is mediated by sequential activation and inhibition of CDKs. CDKs are a well-conserved family of serine/threonine protein kinases whose activity is regulated by different proteins. Their activation requires association with a cyclin partner and phosphorylation by CDK-activating kinase (CAK). Other residues such as conserved threonine and tyrosine must be dephosphorylated for CDK activity, which involves the Cdc25 phosphatase family of proteins [15, 16].

Cyclins are small proteins whose expression changes during the cell cycle. Upon mitogenic stimulation, cyclin D1 associates with CDK4 and CDK6 during early G1 to phosphorylate the pRb family of proteins. The cyclin D-CDK complexes are crucial to coupling extracellular signals to the cell cycle and for cell progression from G_0 to G_1. pRb phosphorylation and its partial inactivation permits the transcription of genes necessary for S-phase [21]. In late G_1, after passing the restriction point (R-point), cyclin E activates CDK2 during the G1-S transition phase. The CDK2-cyclin E complex phosphorylates the RB family and irreversibly inactivates it, a step that is necessary in order to pass the restriction point. In fact, mouse embryonic fibroblast (MEF) triple knockouts of pocket proteins loose the restriction point and proliferate in the absence of mitogenic stimuli [11, 12]. CDK2-cyclin E is fundamental for DNA replication and acts by recruiting MCM proteins to the replication origins. During S phase, cyclin E is degraded by proteasomes and CDK2 binds cyclin A to phosphorylate many proteins necessary to complete and exit from S phase. Cyclin A is able to bind CDK2 and CDK1 in the G2-M transition phase. The two complexes share many protein targets but the specific role of each complex on common targets during the G2-to-M-phase transition is not well understood. After cyclin A degradation, CDK1 binds B-type cyclins to trigger mitosis. More than 70 proteins are phosphorylated by this complex, all of which participate in different steps of mitosis from entry to progression and finally to exit [22, 23].

CDK activity inhibition is mediated by the CDK-inhibitory subunits (CKIs). In mammalian cells, two classes of CKIs, the Cip/Kip and the Ink4 families, provide a tissue-specific mechanism by which cell cycle progression can be restrained in response to extracellular and intracellular signals. The Cip/Kip family includes p21Cip1, p27Kip1 and p57Kip2 and predominantly inhibits the CDKs of the G1-to-S-phase transition [24]. The Ink4 (inhibitors of CDK4) family contains four members, p15Ink4b, p16Ink4, p18Ink4c and p19Ink4d, several of which are mutated or deleted in certain types of human cancers.

In addition to interphase CDKs (CDK2, CDK4 and CDK6) and the mitotic CDK1, there are other CDKs that are related to cell cycle control and cancer. Among them, CDK8 and CDK10 have recently been shown to contribute to colon cancer by a mechanism involving gene amplification [25] and resistance to endocrine therapy in breast cancer, respectively [26]. Moreover, antisense and dominant negative CDK10 mutants are able to arrest the cell in G2-M cell cycle phase. Very recently, CDK9 and cyclin T1 complex (pTEFb) has been shown to control the expression of G1-specific genes such as cyclin D1 and early transcription factors such as c-Myc and JunB through interaction with BRD4 protein. During the M-G1 transition, the pTEFb complex associates with chromosomes to control the expression of specific cell cycle regulatory genes, illustrating a new role for CDK9 in cell cycle control [27].

IN VIVO LESSON

The unique role of each CDK complex has been derived from cell culture studies. In mice, Cdk proteins have shown some unexpected results [28]. The only Cdk that is essential for the cell cycle is Cdk1 [29]. Cdk1 knockout (KO) mice die at the two-cell embryo stage. Triple knockout Cdk2, Cdk4 and Cdk6 are able to develop until the mid-gestation stage due to hematopoietic defects, suggesting that they are not essential for basic cell cycle function [29]. More importantly, the analysis of single Cdk knockouts highlights the specificity of each Cdk in different cell types. For instance, Cdk4 is important for proliferation of pituitary lactotrophs and pancreatic β-cells. Cdk4 homozygous mice result in growth retardation defects, most probably a consequence of deficiency in hormone production. MEF-/- cells proliferate normally and show a delay in cell cycle entry. These data highlight the importance of Cdk4 in the proliferation of specific types of endocrine cells [30-33]. Cdk6 KO mice have defects in the erythroid lineage with a decreased number of red blood cells [34]. Double KO mice die at mid-gestation due to a defect in the erythroid lineage that leads to a severe anemia. But overall, cell proliferation and organogenesis are comparable to single knockout mice [34].

Surprisingly, Cdk2 is not important for mitotic cells but has a fundamental role during meiotic division. In particular, Cdk2 plays a role in chromosome pairing during meiotic prophase [35, 36]. Double Cdk2/Cdk6 knockouts are viable and have defects as single mutants [29]. These data suggest that each Cdk cannot completely compensate each other. In line with this evidence, elegant knock-in experiments in which Cdk2 was inserted in the Cdk1 locus, confirm that Cdk1 has a unique role in cell proliferation and compensation is not a common mechanism [37]. Despite the evolutionary difference between human and yeast cells, these data emphasize the fact that the basic aspects of the cell cycle are not substantially different and only one Cdk (Cdk1) is indispensable for the cell cycle.

Ablation of cyclins in mice, the Cdk activators, has underlined the role of this protein family in cell cycle control. Single cyclin D knockouts are viable and have specific developmental defects. Cyclin D1 null mice have reduced body size, hypoplastic retinas and neurological defects. In addition, these mice failed to have mammary gland proliferation during pregnancy. Cyclin D2 knockout mice females are infertile. Oocyte maturation is unaffected whereas granulosa cells do not proliferate after FSH stimulation. Males are fertile but have hypoplastic testes and a reduced sperm count. In the somatic tissues, the organs affected in Cyclin D2 -/- mice included b-lymphocytes,

pancreatic β-cells and neurogenesis. Cyclin D3 null mice have defects in the development of T-lymphocytes. Triple cyclin knockouts have been generated. These animals show specific defects in myocardial cells and hematopoietic stem cells and die at mid-gestation [38]. These data suggest that cyclin D proteins are not essential for basic cell cycle but only for the development of specific organs.

Cyclin E1 and E2 mutant mice develop normally [39]. Only double knockout mice die at the E11.5 embryonic stage, most likely due to defects in extra-embryonic tissues. Since Cdk2 has no phenotype, these data suggest an extra function for cyclin E not related to its Cdk2 protein partner.

The two cyclin as show a different expression profile. Cyclin A1 is germ cell-specific and knockout mice have defects in spermatogenesis. The meiotic cells are blocked before the first meiotic division. In the female, the ovary is normal [40]. Cyclin A2 knockout mice are embryonically lethal at E5.5 [41]. These results indicate that cyclin A2 is not essential in early development at least until the blastocyst stage. Other proteins, most likely cyclin B3, compensate for the loss of cyclin A2.

The three cyclin Bs are important during mitosis. Cyclin B1 and B2 form protein complexes with Cdk1, whereas cyclin B3 is a partner of Cdk2. Cyclin B1 null mice die at mid-gestation, where cyclin B2 null mice have no obvious defects [42]. The differences can be ascribed to the pattern of localization. Cyclin B1 translocates to the nucleus in G2 phase and is important for mitotic spindle assembly, chromosome condensation and nuclear envelope breakdown. Cyclin B2 is localized in the cytoplasm, where it functions to reorganize the Golgi apparatus during mitosis. No cyclin B3 null mice are available as of yet. From the expression pattern, cyclin B3 could have a role in the testes and fetal ovary.

These data illustrate the necessity to study the basis of cell cycle machinery in a cell-specific context. Single and combined mutant mice suggest that each CDK and cyclin complex act at specific stages in development and in specific cellular contexts. As such, in clinical practice, inhibitory CDK drugs should be administered for specific cancers where CDKs have been demonstrated to have a key role in the oncogenic process. The point is to define whether normal cells have the same requirements as cancer cells.

CDK-CYCLIN COMPLEXES IN CANCER

In most primary tumors, alterations of CDK-cyclin complexes have been reported. One of the more frequently altered is the CDK4-cyclin D1 complex. A CDK4 or cyclin D1 mutation has been reported in breast, lung, pancreatic, gastrointestinal, endometrial, bladder, bone marrow, head and neck, lymphocytic, skin, liver, prostate, gonadal and bone tumors and sarcoma. The first and one of the few discovered point mutations of CDK4, Arg24Cys, was found in melanoma patients and it is a mutation that abolishes the binding of the INK4 inhibitor. CDK6 is mutated in a smaller number of human cancers including, lymphomas, sarcomas and gliomas. Overall, CDK4 is preferentially mutated in epithelial tumors and CDK6 in mesenchymal tumors. Although CDK2 is rarely mutated in cancer, the activator and inhibitor partners are frequently altered in cancer, suggesting an involvement of CDK2 [16].

Different from knockout mice, the loss of specific Cdk-cyclin complexes inhibits the proliferation of human cancer cells [43]. For instance, CDK2 inhibition prevents the proliferation of glioblastoma, non-small cell lung cancer, and melanoma cells. On the other hand, CDK2 does not interfere with several colon cancer cell lines [44, 45]. CDK4-cyclin D1 affects proliferation in most cell types [46-50] and CDK6 appears more important for proliferation of hematopoietic cells. Finally, CDK1 loss interferes with G2-M transition in NCI_HI1299 and U2OS cells and a compensatory mechanism involving CDK2 could be hypothesized [51].

Mice with gain-of-function mutations of CDK or cyclin partners are still lacking. A knock-in CDK4 line that expresses the activating mutation Arg24Cys displays endocrine neoplasia, epithelial hyperplasia (liver, breast and gut) and sarcomas. Unlike in humans, melanoma is induced only after treatments with skin carcinogens [52-54]. In line with the evidence obtained from primary tumors, Cdk/cyclin loss in mouse tumor models inhibits cancer development. Mammary tissue expressing Erbb2 or Hras is resistant to form tumors in CDK4 null background. Over-expression of Erbb2 in cyclin D1 KO mice is resistant to tumor formation. In the skin, tumors induced by Myc are prevented if CDK4 is lost but not cyclin D1 [55-58].

These results suggest that inhibition of CDK-cyclin complexes may have a therapeutic benefit if tissue and molecular alterations in specific tumors are taken into account. At least in mice, and unlike tumor cell lines, normal and tumor cells have specific sensitivity to each type of CDK. This proves to be a key point necessary in designing a therapeutic program.

ATP COMPETITIVE INHIBITORS IN CLINICAL TRIALS

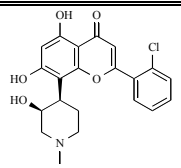
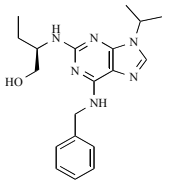
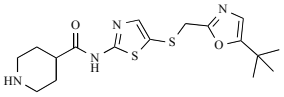
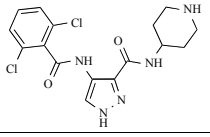
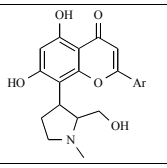
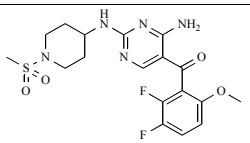
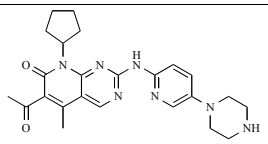
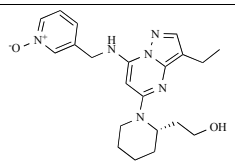
Genetic, epigenetic and gene expression studies have suggested that CDK-cyclin pathways could be useful targets in oncology. Many academic and private companies have started developing drugs that target these pathways. Since CDK activity is dependent on ATP binding molecules, most of the current drugs are based on ATP competitive inhibitors.

At least eleven classes of CDK ATP competitive inhibitors have been developed: Staurosporine, Flavonoid, Purine, Indole, Pyridine, Pyrimidine, Indirubin, Pyrazole, Thiazole, Paullone, and Hymenialdisine derivatives. None of these compounds are commercially available; however, as shown in Table **1**, some of them are currently in phase I and II clinical trials. Among the first discovered CDK inhibitors are Flavopiridol and Roscovitine [59, 60]. Besides Flavopiridol and Roscovitine, new molecules have been designed with the final goal of obtaining more potent, specific and tolerable ligands against specific CDKs. Some of them are already in clinical trials.

Flavopiridol

Flavopiridol (Alvocidib) is a pan-CDK inhibitor that acts on CDK1, 2, 4, 6, 7 and 9 at nanomolar concentrations. Acti-

Table 1. CDK Inhibitors in Clinical Trials

Compound	Structure	Phase
Flavopiridol		II
(*R*)-roscovitine		II
BMS-387032		I
AT7519		I
P276-00		I
R547		I
PD 0332991		I
SCH 727965		II

vity against Epidermal growth factor receptor tyrosine kinase and protein kinase A has also been reported but at micromolar concentrations [61]. Recently, Flavopiridol was co-crystallized and solved with CDK9-cyclin T1 at 2.8 Å resolution [62]. As shown in Fig. (**1A**), Flavopiridol is almost entirely buried in the ATP-binding pocket, with only a small part of its surface area exposed to the solvent. With regard to the main interactions of Flavopiridol in the binding site, there are hydrogen bonds from the flavopiridol O4 oxygen and O5 hydroxyl to the hinge residues Cys106 and Asp104 of CDK9. Furthermore, the protonated N1 atom of the piperidinyl group interacts with Asp167, and the O3 hydroxyl group interacts with Lys48 (see Fig. (**1B**)).

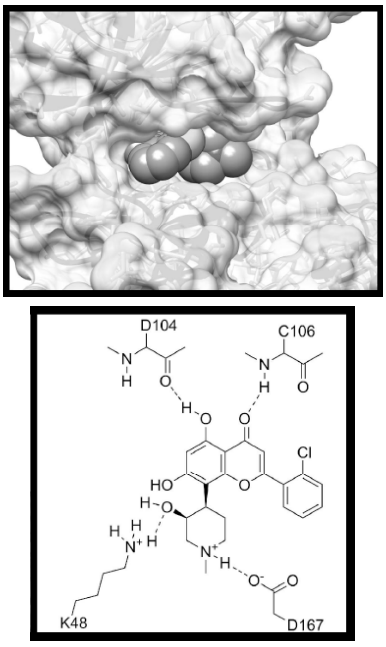


Fig. (1). Flavopiridol (displayed as CPK) in the CDK9 binding site (left) and a schematic representation of the main interactions of the ligand in the binding site.

In 2008 Karaman and co-workers published a remarkable paper in which they presented the interaction maps for 38 kinase inhibitors across a panel of 317 kinases representing >50% of the predicted human protein kinome [63]. Flavopiridol was one of the tested compounds and the binding assays revealed low selectivity for CDKs compared to the others kinases. As shown in Table **2**, Flavopiridol appeared to possess a high nanomolar affinity for all the tested CDKs and also for 25 other kinases. Moreover, beyond these kinases, Flavopiridol possesses a Kd (dissociation constant) affinity comprised between 1000 and 5000 nM for 37 other kinases.

Many phase I and II clinical trials are described [64]. As a single agent, phase II clinical trials were completed for

Table 2. Binding Results (Kd in nM) of Flavopiridol Against 317 Kinases. All Affinity Results with a Kd < 1000 nM are Reported. (Data taken from Ref. [63])

Accession Number	Kinase Target	Kd (nM)	Accession Number	Kinase Target	Kd (nM)
NP_001789.2	CDK2	550	NP_002084.2	GSK3B	730
NP_001249.1	CDK3	410	NP_006192.1	PCTK1	440
NP_004926.1	CDK5	110	NP_002586.2.	PCTK2	480
NP_001790.1	CDK7	23	NP_036527.1	PFTK1	110
NP_001251.1	CDK8	120	NP_002639.1	PIM1	560
NP_001252.1	CDK9	6.4	NP_006866.2	PIM2	770
NP_055891.1	CDK11	57	NP_001001852.1	PIM3	600
NP_004295.2	ALK	670	NP_006245.2	PRKCD	590
NP_115670.1	CAMKK1	79	NP_005391.1	PRKCE	380
NP_006540.3	CAMKK2	430	NP_006246.2	PRKCH	350
NP_009105.1	CIT	110	NP_006248.1	PRKCQ	350
NP_004400.4	DMPK	650	NP_002733.2	PRKD1	520
NP_004705.1	DYRK1B	84	NP_005804.1	PRKD3	170
NP_002740.2	ERK5	620	NP_002944.2	RPS6KA1	720
NP_620590.2	ERK8	330	NP_055311.1	RPS6KA6	800
NP_005246.1	GAK	370	NP_057062.1	TNNI3K	55

multiple myeloma [65], melanoma [66] and endometrial adenocarcinoma [67] with discouraging results. Diarrhea and myelosuppression activity were the major toxicities observed. More activity has been described in hematological malignancies and in combination with others drugs. A phase I study in chronic lymphophocytic leukemia (CLL) showed a partial response in 40% of patients (n=52) with a progression-free survival of 12 months [68]. In acute myelogenous leukemia (AML) phase II clinical trials, Flavopiridol combined with Cytarabine and Mitoxantrone drugs, gave a complete response in 75% of patients [69].

Roscovitine

Roscovitine (Seliciclib) is a purine derivative that inhibits CDK1, 2, 5, 7 and 9 in *in vitro* kinase assays at micromolar concentrations [70]. However, this compound is one of the 38 kinase inhibitors tested by Karaman and co-workers across a panel of 317 kinases [63, 71], with surprising results. As illustrated in Table **3**, with respect to Flavipiridol, Roscovitine shows a higher degree of selectivity as it possesses a fairly good Kd affinity (Kd < 5 μM) for only 11 kinases. However, it was discovered from this analysis that other than primary targets (CDK2, CDK5 and CDK9; CDK1 was not tested), Roscovitine possessed a higher affinity for six off-target proteins and in particular it is about seven-fold more active against delta and epsilon casein kinase 1.

X-ray structures of the complexes between Roscovitine and CDK2 [71, 72] and CDK5, respectively [73], have been reported. In both kinases, the purine ring is engaged in identical interactions with the hinge region, namely Leu83 for CDK2 and Cys83 for CDK5. The benzyl substituent protrudes into a hydrophobic pocket lined by the conserved Ile10 and Phe82 of the two CDKs, whereas the isopropyl group of Roscovitine is inserted in a lipophilic pocket domain mainly delimited by the conserved residues Val64 and Phe80 (see Fig. **2**). The main difference in the interactions of Roscovitine with regards to CDK2 and CDK5 is the contact with the 1-ethyl-2-hydroxy-ethylamino substituent. In the CDK2 binding site, the hydroxyl group does not possess

Table 3. Binding Results (Kd in nM) of Roscovitine Against 317 Kinases. All Affinity Results with a Kd < 5000 nM are Reported. (Data Taken from Ref. [63])

Accession Number	Kinase Target	Kd (nM)
NP_001789.2	CDK2	3400
NP_004926.1	CDK5	1900
NP_001790.1	CDK7	1800
NP_004295.2	ALK	2300
AAA61480.1	CLK1	1200
NP_003984.2	CLK2	700
NP_620693.1	CSNK1D	260
NP_001885.1	CSNK1E	320
NP_004375.2	CSNK1G3	2900
NP_004705.1	DYRK1B	1100
NP_003309.2	TTK	1600

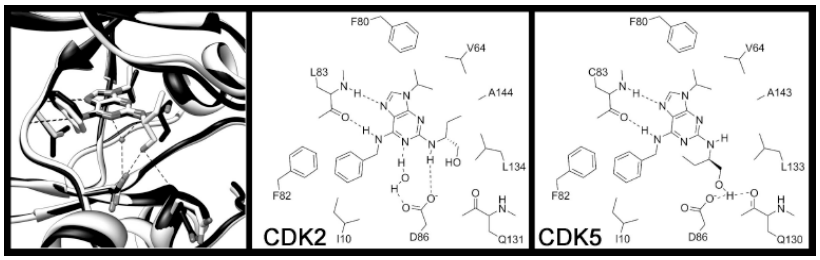


Fig. (2). Superimposition of Roscovitine complexed with CDK2 (black) and CDK5 (white) and schematic representation of the main interactions of Roscovitine in the binding site of CDK2 and CDK5.

important interactions, the ethyl portion interacts with Leu134 and Ala144 and a water molecule mediates the interaction of the amine and heterocyclic nitrogen with Asp86. In CDK5, the 1-ethyl-2-hydroxy-ethylamino substituent is rotated 180° and in this orientation, the hydroxyl group is able to form H-bonds with Asp86 and Gln130 and the ethyl group is engaged in hydrophobic interactions with Ile10 and Val18.

A phase I study of Roscovitine in combination with cisplatin and gemcitabine was performed in 27 patients with non-small cell lung cancer to determine the maximal tolerated dose and the safety thereof. Roscovitine was administered for four days for six cycles. The dose limiting toxicity (DLTs) consisted of Grade 3 elevation of liver enzymes, nausea, vomiting, and transient hypokalemia. The maximum tolerated dose (MTD) was Roscovitine 800 mg twice daily with gemcitabine 1000 mg/m^2 and cisplatin 75 mg/m^2 [74].

A phase I study of Roscovitine in 21 patients with malignant solid tumors has been completed. No significant dose toxicity was observed until the 200 mg dose level was reached. At the maximum tolerated dose (800 mg/m^2 twice daily given for seven days every three weeks), the common side effects were hypokalemia, hyponatremia, hyperglycemia, elevated gamma-glutamyl transpeptidase and skin rash. In another phase I study, vomiting, skin rash and hypokalemia were reported [75]. The DLTs were hypokalemia, rash and fatigue. No objective responses were reported, but disease stabilization occurred in eight patients [76].

A phase II study of Roscovitine as a single agent in non-small cell lung cancer (NSCLC) patients has been closed. No data have yet been reported.

BMS-387032

BMS-387032 (SNS-032) is a thiazole derivative with a reported activity against CDK1, 2, 4, 7, 9 lower than 1000 nM [77]. The lowest IC_{50} is reported with CDK9 (4 nM) and on a panel of 190 human kinases, it has been shown to inhibit only GSK3α and GSK3β with a IC_{50} lower than 1000 nM. However, as shown in Table **4**, the work developed by Karaman and co-workers across the panel of 317 kinases [63] highlights that beyond the above-mentioned kinases, SNS-032 showed a nanomolar binding affinity for 20 other kinases. With respect to the primary targets CDK2, 7 and 9, SNS-032 possesses a high affinity for CDK3 and the PCTAIRE protein kinases 1, 2 and 3.

Table 4. Binding Results (Kd in nM) of SNS-032 Againsts 317 Kinases. All Affinity Results with a Kd < 1000 nM are Reported. (Data Taken from Ref. [63])

Accession Number	Kinase Target	Kd (nM)	Accession Number	Kinase Target	Kd (nM)
NP_277023.1	CDC2L1	98	NP_002739.1	ERK3	500
NP_076916.1	CDC2L2	48	NP_002740.2	ERK5	650
NP_055891.1	CDK11	950	NP_620590.2	ERK8	120
NP_001789.2	CDK2	69	NP_063937.2	GSK3A	28
NP_001249.1	CDK3	56	NP_002084.2	GSK3B	37
NP_004926.1	CDK5	740	NP_149109.1	MYLK2	980
NP_001790.1	CDK7	31	NP_006192.1	PCTK1	7.1
NP_001252.1	CDK9	76	NP_002586.2.	PCTK2	13
AAA61480.1	CLK1	410	NP_002587.2	PCTK3	44
NP_065717.1	CLK4	800	NP_036527.1	PFTK1	690
NP_001885.1	CSNK1E	950	NP_005804.1	PRKD3	750
NP_004751.2	DRAK1	440	NP_056505.1	STK36	260
NP_004705.1	DYRK1B	200			

The three-dimensional X-ray structure of SNS-032 in complex with CDK2 [78] shows that the *t*-butyl oxazole ring is directed toward the ribose pocket without hydrogen bonds between the oxazole ring and the protein. The piperidinyl ring extends toward the external side of the protein whereas both the thiazole nitrogen and exocyclic amide proton form important H-bonds with the backbone of Leu83 (see Fig. **3**).

In agreement with the inhibitory activity against CDKs, the compound inhibits cell cycle progression and transcription. A phase I clinical trial to treat selected advanced solid tumors and advanced B-lymphoid malignancies is ongoing. A phase I study in metastatic refractory solid tumors was discontinued due to a change in portfolio priorities from the sponsor. Partial results showed that the ligand could be well tolerated with the only minor adverse events such as fatigue and nausea. Average oral bioavailability is 19%, suggesting that continuing studies could be useful to test the efficacy of this ligand [77, 79].

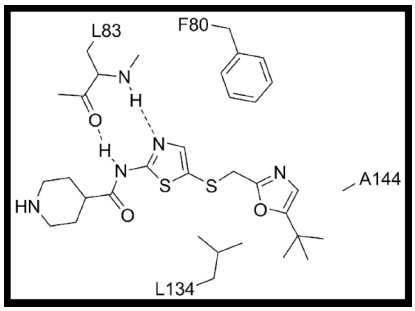


Fig. (3). Schematic representation of the main interactions of BMS-387032 in the binding site of CDK2.

AT7519

AT7519 was derived from a fragment-based X-ray crystallographic screening to detect small molecules that interact with CDK2, followed by lead optimization [80]. The compound is shown to inhibit CDK1, 2, 4, 5, 6 and 9 *in vitro* with a concentration range from 10 to 210 nM (IC_{50}) [81]. The compound showed minor potency on CDK3, 7 and GSK3β. Tests on 23 other kinases showed an IC_{50} greater than 10000 nM (see Table **5**).

Table 5. Binding Results (IC_{50} in nM) of AT7519 Against 32 Kinases. All Affinity Results with a IC_{50} < 1000 nM are Reported. (Data Taken from Ref. [81])

Accession Number	Kinase Target	IC_{50} (nM)
NP_001777.1	CDK1	210
NP_001789.2	CDK2	47
NP_001249.1	CDK3	360
NP_000066	CDK4	100
NP_004926.1	CDK5	13
NP_001138778	CDK6	170
NP_001790.1	CDK7	2400
NP_001252.1	CDK9	<10
NP_002084.2	GSK3B	89

X-ray structure of the CDK2-AT7519 complex reveals that the pyrazole ring and the 6-amide nitrogen forms H-bonds with the backbone of Glu81 and Leu83 and the carbonyl of the 4-benzamide group interacts with the backbone of Asp145. Finally, the piperidinyl ring creates an H-bond with the backbone of His84 (see Fig. **4**).

A phase I clinical trial is currently recruiting patients. The aim is to identify the efficacy of this new compound in advanced or metastatic solid tumors or refractory non-Hodgkin's lymphoma [82].

AT7519 inhibits cellular proliferation in 26 tumor cells from 40 to 920 nM and is p53- and Rb- independent. Interestingly, no activity is reported in non-proliferating fibroblasts. Fluorescent-activated cell sorting (FACS) analysis, tunnel and colony formation assays have shown an induction of apoptosis. The antitumor properties have also been verified in early and late stages of HCT116 colon carcinoma tumor xenograft mouse model. More importantly, compared to other previously published drugs such as Flavopiridol and more newly discovered compounds such as R547 and P276-00, AT7519 is able to cause regression of subcutaneous tumors [81].

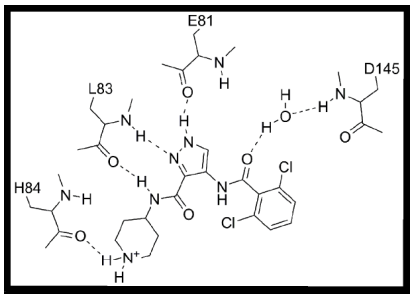


Fig. (4). Schematic representation of the main interactions of AT7519 in the binding site of CDK2.

P276-00

P276-00 was generated from an anticancer drug screening against CDK4 activity [83]. As reported in Table **6**, on a panel of 14 kinases, this compound yielded a nanomolar activity in CDK4-D1, CDK1-B, CDK2-A, CDK6-D3, and CDK9-T1 enzyme assays and a low micromolar activity in the CDK7-H and GSK3β assays.

Table 6. Binding Results (IC_{50} in nM) of P276-00 Against 14 Kinases. All Affinity Results with a IC_{50} < 5000 nM are Reported. (Data Taken from Ref. [83])

Kinase Target	IC_{50} (nM)
CDK1-B	79
CDK2-E	2540
CDK2-A	224
CDK4-D1	63
CDK6-D3	396
CDK7-H	2870
CDK9-T1	20
GSK3B	2771

A phase I clinical trial has been completed in patients with advanced refractory neoplasms. A phase II study has begun for the treatment of recurrent and/or locally advanced head and neck cancers and relapsed and/or refractory Mantle cell lymphoma [83]. *In vitro* studies show that the compound inhibits proliferation of 12 tumor cell line panels between the 300-800 nM range and is inactive on normal fibroblasts. In HL60 cells, P276-00-activated apoptotic signals are measured with caspase 3 activity and DNA fragmentation

experiments. *In vivo* studies of P276-00 have shown a tumor volume decrease by >70% in MM1.S plasmacytomas [84], CA-51, Lewis lung sarcoma, HCT116 and H-460 [83] tumor xenograft mouse models.

R547

R547 is a dyaminopyrimidine derivative that shows a sub-nanomolar inhibitory activity for CDK1, 2, 3, 5 and a nanomolar activity for CDK6 and CDK7. Furthermore, this compound was tested on a panel of 115 other kinases and it proved to be very selective. It has an IC_{50} activity lower than 5 μM only for GSK3α and GSK3β [85]. X-ray structure of the R547-CDK2 complex [86] shows that the diamino-pyrimidine core forms two hydrogen bonds with the back-bone of Glu81 and Leu83. The sulfonamide group gives both direct and water-mediated hydrogen bonds with Asp86, Lys89, and the backbone of His84. The 2,6 difluoro ring is located in a hydrophobic pocket near Phe80 and the alkyl chain of Lys33 (see Fig. **5**).

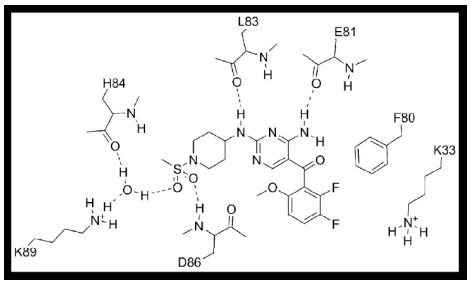


Fig. (5). Schematic representation of the main interactions of R547 in the binding site of CDK2.

This compound is currently in phase I clinical trial in patients with advanced solid tumors [87]. *In vitro* studies demonstrate R547 to be a potent inhibitor of cell proli-feration in 19 cell lines irrespective of p53, pRb and multi-drug resistance status. The compound induces G1 and G2 cell cycle block followed by apoptosis, with the dose concentration ranging from 0.05 to 0.6 μM.

PD 0332991

PD 0332991 is a complete inhibitor of CDK4 and 6 activities with IC_{50} values < 15 nM. Within a panel of enzymes, PD 0332991 exhibits selectivity for CDK4/6 with little or no activity against 36 additional protein kinases including CDK1, 2, and 5 and a variety of tyrosine and serine threonine kinases [88].

The low resolution X-ray structure of the CDK6-PD0332991 complex [89] suggests the presence of three hydrogen bonds between the ligand and the protein. As shown in Fig. (**6**), there are hydrogen bonds between the ligand and the backbone of Val101 of CDK6 that correspond to Leu83 of CDK2. Moreover, there is an additional hydro-gen bond between the acetyl group and the backbone of Asp163. The cyclopentyl substituent is closely bound to what it is supposed binding from the ribose of the natural ligand ATP. The C5 and C6 substituents are positioned in the pocket in front of the gatekeeper residue Phe98 with the piperazinylpyridine substituent pointing out from the binding pocket near Thr107.

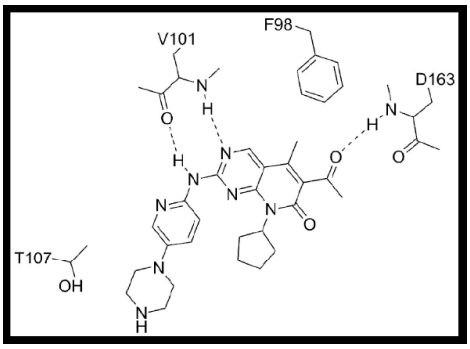


Fig. (6). Schematic representation of the main interactions of PD 0332991 in the binding site of CDK6.

In support of its CDK4/6 activity, the compound shows proliferation inhibition only in pRb- positive cells. The cells are blocked in G1 phase with pRb hypophosphorylated. Oral administration in tumor xenograft mouse models results in tumor regression and no resistance occurs. Several clinical trials are ongoing. A phase I dose escalation trial of PD 0332991 administered daily as an oral single agent was conducted in 57 patients with breast, colorectal, liposarcoma, and melanoma tumors. A dose escalation from 25 mg to 150 mg QD was administered for 21 days in 28-day cycles. Six patients manifested stable disease for 10 cycles and three patients for 20 cycles. The most common adverse events were neutropenia, anemia, fatigue, nausea, constipation, vomiting and diarrhea [90]. Clinical trials on Mantle cell lymphoma and Non-Hodgkin Lymphoma in combination with the proteasome inhibitor, bortemizib, in multiple myeloma is ongoing.

SCH 727965

SCH 727965 is a pyrazolo[1,5-a]pyrimidine derivative that selectively inhibits CDK1, 2, 5 and 9 with an IC_{50} of < 5nM. SCH 727965 induces apoptosis in >100 tumor cell lines and results in tumor growth inhibition or regression in multiple xenograft models. In a phase I clinical trial, 23 subjects with advance malignancies were treated at doses from 1.85 to 58 mg/m^2. SCH 727965 is safe and well-tolerated at dose levels below the maximum administered dose of 58 mg/m^2. The most common adverse events were nausea/vomiting, diarrhea, neutropenia, and fatigue [91]. In another clinical study, 43 patients with advanced malignan-cies showed that SCH727965 was safe and well-tolerated at the recommended phase 2 dose of 12 mg/m^2. No objective responses by Response Evaluation Criteria in Solid Tumors (RECIST) have yet been observed [92]. A phase II clinical trial in breast and lung cancer patients is presently compa-ring the efficacy of SCH727965 with Capacitabine and Erlotinib. A phase II clinical trial in patients with acute mye-logenous leukemia and patients with acute lymphoblastic leukemia (ALL) is currently starting.

NON-ATP COMPETITIVE INHIBITORS

The major problems related to the ATP competitive inhibitors are the high homologies between ATP binding sites among all the protein kinases in the cells. For these reasons, most of these drugs have off-target effects that eventually would limit the dosage and its efficacy. One alternative strategy recently developed is based on non-ATP competitive inhibitors that inhibit kinase substrates and regulatory binding sites. The docking sites and binding interactions are usually different among different protein regulators. In this case, there are many opportunities to develop competitive inhibitors with a high specificity [18, 64]. Starting from this point, our group along with others have developed new small molecules that have a great potential for clinical applications and some have already demonstrated preclinical efficacy. These new compounds can be classified as a) inhibitor derivates from CDK substrates, b) inhibitors of CDK/cyclin complexes, and c) inhibitors of the cyclin binding groove.

Inhibitor Derivates from CDK Substrates

One of the forerunner peptides of this class of new inhibitors was developed in our laboratory [93]. Based on the amino acid sequences that mediate the interaction between pRb2/p130 and CDK2/cyclin A complexes, a peptide of 39 residues was designed (spa310) that is able to inhibit the activity of the CDK2 protein. Since the interaction between pRb2/p130 with cyclinA is mediated by the spacer region, the domain was divided into three domains (Spa A, B and C). Only the Spa A domain, from a.a. 616 to 828, is able to inhibit the activity of CDK2 from interacting with its substrate, histone H1. Subsequent deletion mutants identify a minimal region between a.a. 641 and 679 that retains the ability to inhibit CDK2 activity. Surprisingly, this peptide excludes the canonical RXL motif (a.a. 680-682), suggesting the peptide does not play a key role in cyclin A interaction. Given that, a peptide from a.a. 641 to 673 has no inhibitory effect on CDK2 activity. These results suggest that amino acids from 673 to 679 are important for the stabilization and inhibitory action of spa310. Transfection experiments in NIH3T3 cells have demonstrated a nuclear and cytoplasmatic localization of the peptides and their ability to inhibit endogeneous CDK2 activity on histone H1. More importantly, spa310 is able to reduce the cellular proliferation of NIH3T3 cells by about 60% as demonstrated by colony formation assays. This ability is retained *in vivo* in an A549 xenograft mouse model. Three- to four-fold tumor volume reduction was observed when the spa310 peptide was injected into the mice every four days [93]. Starting from crystallographic data of the CDK2-cyclinA heterodimer complexed with a peptide developed from the p107 protein, a computer-based model was generated for the pRb2/p130 peptide (a.a. 641-649) and CDK2-cyclinA complex. It is important to note that the C-terminal domain of the peptide is likely to interfere with the CDK2 catalytic site and/or CDK2-cyclin A interaction [94].

Another important target of the CDK family, in particular CDK1 and 2, is the tumor suppressor gene p53. A peptide intended to interact with the CDK2 tetramerization docking site has been designed. A 20 mer peptide named CIP is able to inhibit the p53 phosphorylation mediated by CDK2 and induce cell death in A375 melanoma cells [71, 95].

Inhibitors of CDK/Cyclin Complexes

The crystal structure of the CDK2-cyclin A complex has shown that different interactions are necessary to activate the substrate binding site, in particular the PSTAIRE helix of CDK2 associated with the α3 and α5 helix of cyclin A followed by CDK2 T loop exposure and phosphorylation by the CAK complex [96-99]. A α5 peptide (C4) bearing a point mutation E295A that improves solubility has been designed. It is highly specific for CDK2/cyclin A/E (IC_{50}<2μM) and does not affect CDK1-cyclin B, PKA and PKB protein kinases. The peptide is able to interact only with CDK2-cyclin A in a complex as demonstrated by Surface Plasmon Resonance. These results suggest that the peptide is able to block the complex in an inactive form [100, 101].

A different approach starting from the screening of combinatorial libraries led to the discovery of a new hexapeptide, NBI1, that is able to compete for the binding of cyclin A to CDK2. IC_{50} values show a specificity for CDK2-cyclin A, CDK1-cyclin B1 and CDK6-cyclin D3 with a reduced activity on other kinase families. Studies in cell culture have confirmed that a derivative, TAT-NBI1, is able to inhibit HCT116 proliferation of asynchronous cells in S phase and synchronized cells in S and G2/M [102].

Inhibitors of the Cyclin Binding Groove

Many CDK binding proteins such as E2F1, p21 and p27 share a recognition site. A pioneer peptide derived from the E2F1 protein was able to inhibit the phosphorylation of its pRb substrate [103]. This discovery led other groups to investigate how p21 and p27 inhibitors block the functionality of CDK-cyclin complexes. The p21 inhibitor is able to compete with the kinase substrate to the cyclin substrate recruitment site (CRB). An octapeptide that mimics p21 is able to inhibit kinase activity [104-106]. A drug pipeline is now under development based on this mechanism. Starting from the lead octapeptide, a lower molecular weight peptide, Compound 2, was developed. Truncation of the C-terminal along with side-chain optimization, the addition of natural and unnatural amino acids, and rigidification have improved the efficacy of the compound [107].

NEXT STEPS

Since the first discovery of the importance of CDK1 in driving cell cycle in *Xenopous Laevis* and *Saccharomyces cerevisiae*, significant progress has been made to understand the finely controlled process of cell cycle regulation. Starting from analyses using mice tumor models, it is now clear that a major effort should be made to develop more preclinical models based on CDKs or their modulators. Conditional tissue-specific expression should be tested to clarify the contribution of a single CDK in each tumor type [108]. Until now, it has not been clear whether inhibition of CDK activity has had a benefit in cancer therapy and single agent use or in combination with conventional chemotherapy drugs should be tested utilizing the results obtained from animal models.

From a pharmaceutical point of view, ATP competitive inhibitors are the primary choice for use in designing new target drugs. Nevertheless, these compounds have multiple targets and undesirable side effects are to be expected. The ability to selectively target a specific protein kinase implicated in tumorigenesis while not affecting those involved in normal physiological processes remains the key to optimizing CDKs as therapeutic agents. From this point of view, the development of CDK inhibitors that interact in non-ATP binding sites is a promising approach. The described protein–protein interactions involved in kinase regulation and substrate recognition offer the potential for this selectivity and also avoid decreased efficacy as a result of competition with high intracellular ATP concentrations. The reported examples for CDK inhibition are encouraging proofs of the movement toward this trend. However, in our opinion, the development of new non-ATP competitive inhibitors has been delayed by the lack of experimental data on CDKs and their protein partners. We expect that in the coming years, the development of new non-ATP competitive CDK inhibitors will increase significantly with new experimental discoveries that better clarify the interactions of CDKs with kinase substrates and regulatory proteins.

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REFERENCES

[1] von Hansemann D. Ueber asymmetrische Zelltheilung in epithel Krebsen und deren biologische Bedeutung. Virchows Arch Path Anat 1890; 119: 299-326.
[2] Boveri T. Zur Frage der Entstehung Maligner Tumoren. 1914; 1-64.
[3]. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 1973; 243: 290-3.
[4] Tabin CJ, Bradley SM, Bargmann CI, *et al.* Mechanism of activation of a human oncogene. Nature 1982; 300: 143-9.
[5] Reddy EP, Reynolds RK, Santos E, Barbacid M. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature 1982; 300: 149-52.
[6]. Stratton MR, Campbell PJ, Futreal PA. The cancer genome. Nature 2009; 458: 719-24.
[7] Morgan DO. SnapShot: cell-cycle regulators I. Cell 2008; 135: 764e1.
[8] Morgan DO. SnapShot: Cell-cycle regulators II. Cell 2008; 135: 974e1.
[9] Pardee AB. A restriction point for control of normal animal cell proliferation. Proc Natl Acad Sci USA 1974; 71: 1286-90.
[10]. Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 2001; 1: 222-31.
[11] Dannenberg JH, van Rossum A, Schuijff L, te Riele H. Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev 2000; 14: 3051-64.
[12] Sage J, Mulligan GJ, Attardi LD, *et al.* Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev 2000; 14: 3037-50.
[13] Norbury C, Nurse P. Animal cell cycles and their control. Annu Rev Biochem 1992; 61: 441-70.
[14] Cobrinik D. Pocket proteins and cell cycle control. Oncogene 2005; 24: 2796-809.
[15] Lapenna S, Giordano A. Cell cycle kinases as therapeutic targets for cancer. Nat Rev Drug Discov 2009; 8: 547-66.
[16] Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer 2009; 9: 153-66.
[17] Kirkland LO, McInnes C. Non-ATP competitive protein kinase inhibitors as anti-tumor therapeutics. Biochem Pharmacol 2009; 77: 1561-71.
[18] Orzaez M, Gortat A, Mondragon L, Bachs O, Perez-Paya E. ATP-noncompetitive inhibitors of CDK-cyclin complexes. ChemMedChem 2009; 4: 19-24.
[19] Helin K, Harlow E, Fattaey A. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol Cell Biol 1993; 13: 6501-8.
[20] Flemington EK, Speck SH, Kaelin WG Jr. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc Natl Acad Sci USA 1993; 90: 6914-8.
[21] MacLachlan TK, Sang N, Giordano A. Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. Crit Rev Eukaryot Gene Expr 1995; 5: 127-56.
[22]. Pucci B, Giordano A. Cell cycle and cancer. Clin Ter 1999; 150: 135-41.
[23] Malumbres M, Barbacid M. Mammalian cyclin-dependent kinases. Trends Biochem Sci 2005; 30: 630-41.
[24] Harper JW, Elledge SJ. Cdk inhibitors in development and cancer. Curr Opin Genet Dev 1996; 6: 56-64.
[25] Firestein R, Bass AJ, Kim SY, *et al.* CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity. Nature 2008; 455: 547-51.
[26] Iorns E, Turner NC, Elliott R, *et al.* Identification of CDK10 as an important determinant of resistance to endocrine therapy for breast cancer. Cancer Cell 2008; 13: 91-104.
[27] Yang Z, He N, Zhou Q. Brd4 recruits P-TEFb to chromosomes at late mitosis to promote G1 gene expression and cell cycle progression. Mol Cell Biol 2008; 28: 967-76.
[28] Ciemerych MA, Sicinski P. Cell cycle in mouse development. Oncogene 2005; 24: 2877-98.
[29] Santamaria D, Barriere C, Cerqueira A, *et al.* Cdk1 is sufficient to drive the mammalian cell cycle. Nature 2007; 448: 811-5.
[30] Rane SG, Dubus P, Mettus RV, *et al.* Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. Nat Genet 1999; 22: 44-52.
[31] Tsutsui T, Hesabi B, Moons DS, *et al.* Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. Mol Cell Biol 1999; 19: 7011-9.
[32] Martin J, Hunt SL, Dubus P, *et al.* Genetic rescue of Cdk4 null mice restores pancreatic beta-cell proliferation but not homeostatic cell number. Oncogene 2003; 22: 5261-9.
[33] Mettus RV, Rane SG. Characterization of the abnormal pancreatic development, reduced growth and infertility in Cdk4 mutant mice. Oncogene 2003; 22: 8413-21.
[34] Malumbres M, Sotillo R, Santamaria D, *et al.* Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. Cell 2004; 118: 493-504.
[35] Ortega S, Prieto I, Odajima J, *et al.* Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. Nat Genet 2003; 35: 25-31.
[36] Berthet C, Aleem E, Coppola V, Tessarollo L, Kaldis P. Cdk2 knockout mice are viable. Curr Biol 2003; 13: 1775-85.
[37] Satyanarayana A, Berthet C, Lopez-Molina J, Coppola V, Tessarollo L, Kaldis P. Genetic substitution of Cdk1 by Cdk2 leads to embryonic lethality and loss of meiotic function of Cdk2. Development 2008; 135: 3389-400.
[38] Kozar K, Ciemerych MA, Rebel VI, *et al.* Mouse development and cell proliferation in the absence of D-cyclins. Cell 2004; 118: 477-91.
[39] Geng Y, Yu Q, Sicinska E, *et al.* Cyclin E ablation in the mouse. Cell 2003; 114: 431-43.
[40] Liu D, Matzuk MM, Sung WK, Guo Q, Wang P, Wolgemuth DJ. Cyclin A1 is required for meiosis in the male mouse. Nat Genet 1998; 20: 377-80.
[41] Murphy M, Stinnakre MG, Senamaud-Beaufort C, *et al.* Delayed early embryonic lethality following disruption of the murine cyclin A2 gene. Nat Genet 1997; 15: 83-6.
[42] Brandeis M, Rosewell I, Carrington M, *et al.* Cyclin B2-null mice develop normally and are fertile whereas cyclin B1-null mice die in utero. Proc Natl Acad Sci USA 1998; 95: 4344-9.

[43] Berthet C, Kaldis P. Cell-specific responses to loss of cyclin-dependent kinases. Oncogene 2007; 26: 4469-77.

[44] van den Heuvel S, Harlow E. Distinct roles for cyclin-dependent kinases in cell cycle control. Science 1993; 262: 2050-4.

[45] Tetsu O, McCormick F. Proliferation of cancer cells despite CDK2 inhibition. Cancer Cell 2003; 3: 233-45.

[46] Lee RJ, Albanese C, Fu M, *et al.* Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. Mol Cell Biol 2000; 20: 672-83.

[47] Sauter ER, Nesbit M, Litwin S, Klein-Szanto AJ, Cheffetz S, Herlyn M. Antisense cyclin D1 induces apoptosis and tumor shrinkage in human squamous carcinomas. Cancer Res 1999; 59: 4876-81.

[48] Kornmann M, Arber N, Korc M. Inhibition of basal and mitogen-stimulated pancreatic cancer cell growth by cyclin D1 antisense is associated with loss of tumorigenicity and potentiation of cytotoxicity to cisplatinum. J Clin Invest 1998; 101: 344-52.

[49] Arber N, Doki Y, Han EK, *et al.* Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. Cancer Res 1997; 57: 1569-74.

[50] Zhou P, Jiang W, Zhang YJ, *et al.* Antisense to cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer cells. Oncogene 1995; 11: 571-80.

[51] Cai D, Latham VM Jr, Zhang X, Shapiro GI. Combined depletion of cell cycle and transcriptional cyclin-dependent kinase activities induces apoptosis in cancer cells. Cancer Res 2006; 66: 9270-80.

[52] Sotillo R, Renner O, Dubus P, *et al.* Cooperation between Cdk4 and p27kip1 in tumor development: a preclinical model to evaluate cell cycle inhibitors with therapeutic activity. Cancer Res 2005; 65: 3846-52.

[53] Sotillo R, Dubus P, Martin J, *et al*. Wide spectrum of tumors in knock-in mice carrying a Cdk4 protein insensitive to INK4 inhibitors. EMBO J 2001; 20: 6637-47.

[54] Sotillo R, Garcia JF, Ortega S, *et al*. Invasive melanoma in Cdk4-targeted mice. Proc Natl Acad Sci USA 2001; 98: 13312-7.

[55] Yu Q, Sicinska E, Geng Y, *et al*. Requirement for CDK4 kinase function in breast cancer. Cancer Cell 2006; 9: 23-32.

[56] Reddy HK, Mettus RV, Rane SG, Grana X, Litvin J, Reddy EP. Cyclin-dependent kinase 4 expression is essential for neu-induced breast tumorigenesis. Cancer Res 2005; 65: 10174-8.

[57] Landis MW, Pawlyk BS, Li T, Sicinski P, Hinds PW. Cyclin D1-dependent kinase activity in murine development and mammary tumorigenesis. Cancer Cell 2006; 9: 13-22.

[58] Yu Q, Geng Y, Sicinski P. Specific protection against breast cancers by cyclin D1 ablation. Nature 2001; 411: 1017-21.

[59] Dickson MA, Schwartz GK. Development of cell-cycle inhibitors for cancer therapy. Curr Oncol 2009; 16: 36-43.

[60] Sharma PS, Sharma R, Tyagi R. Inhibitors of cyclin dependent kinases: useful targets for cancer treatment. Curr Cancer Drug Targets 2008; 8: 53-75.

[61] Sedlacek HH. Mechanisms of action of flavopiridol. Crit Rev Oncol Hematol 2001; 38: 139-70.

[62] Baumli S, Lolli G, Lowe ED, *et al*. The structure of P-TEFb (CDK9/cyclin T1), its complex with flavopiridol and regulation by phosphorylation. EMBO J 2008; 27: 1907-18.

[63] Karaman MW, Herrgard S, Treiber DK, *et al*. A quantitative analysis of kinase inhibitor selectivity. Nat Biotechnol 2008; 26: 127-32.

[64] McInnes C. Progress in the evaluation of CDK inhibitors as anti-tumor agents. Drug Discov Today 2008; 13: 875-81.

[65] Dispenzieri A, Gertz MA, Lacy MQ, *et al*. Flavopiridol in patients with relapsed or refractory multiple myeloma: a phase 2 trial with clinical and pharmacodynamic end-points. Haematologica 2006; 91: 390-3.

[66] Burdette-Radoux S, Tozer RG, *et al*. Phase II trial of flavopiridol, a cyclin dependent kinase inhibitor, in untreated metastatic malignant melanoma. Invest New Drugs 2004; 22: 315-322.

[67] Grendys EC Jr, Blessing JA, Burger R, Hoffman J. A phase II evaluation of flavopiridol as second-line chemotherapy of endometrial carcinoma: a Gynecologic Oncology Group study. Gynecol Oncol 2005; 98: 249-53.

[68] Phelps MA, Lin TS, Johnson AJ, *et al*. Clinical response and pharmacokinetics from a phase 1 study of an active dosing schedule of flavopiridol in relapsed chronic lymphocytic leukemia. Blood 2009; 113: 2637-45.

[69] Karp JE, Smith BD, Levis MJ, *et al*. Sequential flavopiridol, cytosine arabinoside, and mitoxantrone: a phase II trial in adults with poor-risk acute myelogenous leukemia. Clin Cancer Res 2007; 13: 4467-73.

[70] Meijer L, Kim SH. Chemical inhibitors of cyclin-dependent kinases. Methods Enzymol 1997; 283: 113-28.

[71] De Azevedo WF, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH. Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. Eur J Biochem 1997; 243: 518-26.

[72] Bettayeb K, Oumata N, Echalier A, *et al*. CR8, a potent and selective, roscovitine-derived inhibitor of cyclin-dependent kinases. Oncogene 2008; 27: 5797-807.

[73] Mapelli M, Panjikar S, Tucker PA. The crystal structure of the herpes simplex virus 1 ssDNA-binding protein suggests the structural basis for flexible, cooperative single-stranded DNA binding. J Biol Chem 2005; 280: 2990-7.

[74] Siegel-Lakhai WS, Crul M, Zhang S, *et al*. Phase I and pharmacological study of the farnesyltransferase inhibitor tipifarnib (Zarnestra, R115777) in combination with gemcitabine and cisplatin in patients with advanced solid tumours. Br J Cancer 2005; 93: 1222-9.

[75] Pierga JY, Bonneton C, Magdelenat H, *et al.* Clinical significance of proliferative potential of occult metastatic cells in bone marrow of patients with breast cancer. Br J Cancer 2003; 89: 539-45.

[76] Benson C, White J, De Bono J, *et al*. A phase I trial of the selective oral cyclin-dependent kinase inhibitor seliciclib (CYC202; R-Roscovitine), administered twice daily for 7 days every 21 days. Br J Cancer 2007; 96: 29-37.

[77] Conroy A, Stockett DE, Walker D, *et al*. SNS-032 is a potent and selective CDK 2, 7 and 9 inhibitor that drives target modulation in patient samples. Cancer Chemother Pharmacol 2009; 64: 723-32.

[78] Misra RN, Xiao HY, Kim KS, *et al*. N-(cycloalkylamino)acyl-2-aminothiazole inhibitors of cyclin-dependent kinase 2. N-[5-[[[5-(1,1-dimethylethyl)-2-oxazolyl]methyl]thio]-2-thiazolyl]-4-piperidinecarboxamide (BMS-387032), a highly efficacious and selective antitumor agent. J Med Chem 2004; 47: 1719-28.

[79] Heath EI, Bible K, Martell RE, Adelman DC, Lorusso PM. A phase 1 study of SNS-032 (formerly BMS-387032), a potent inhibitor of cyclin-dependent kinases 2, 7 and 9 administered as a single oral dose and weekly infusion in patients with metastatic refractory solid tumors. Invest New Drugs 2008; 26: 59-65.

[80] Wyatt PG, Woodhead AJ, Berdini V, *et al*. Identification of N-(4-piperidinyl)-4-(2,6-dichlorobenzoylamino)-1H-pyrazole-3-carboxamide (AT7519), a novel cyclin dependent kinase inhibitor using fragment-based X-ray crystallography and structure based drug design. J Med Chem 2008; 51: 4986-99.

[81] Squires MS, Feltell RE, Wallis NG, *et al*. Biological characterization of AT7519, a small-molecule inhibitor of cyclin-dependent kinases, in human tumor cell lines. Mol Cancer Ther 2009; 8: 324-32.

[82] Mahadevan D, Plummer R, Squires MS, *et al.* A dose escalation, pharmacokinetic, and pharmacodynamic study of AT7519, a cyclin-dependent kinase inhibitor in patients with refractory solid tumors. J Clin Oncol 2008; 26(Suppl 20): abstr 3533.

[83] Joshi KS, Rathos MJ, Joshi RD, *et al. In vitro* antitumor properties of a novel cyclin-dependent kinase inhibitor, P276-00. Mol Cancer Ther 2007; 6: 918-25.

[84] Raje N, Hideshima T, Mukherjee S, *et al*. Preclinical activity of P276-00, a novel small-molecule cyclin-dependent kinase inhibitor in the therapy of multiple myeloma. Leukemia 2009; 23: 961-70.

[85] DePinto W, Chu XJ, Yin X, *et al*. *In vitro* and *in vivo* activity of R547: a potent and selective cyclin-dependent kinase inhibitor currently in phase I clinical trials. Mol Cancer Ther 2006; 5: 2644-58.

[86] Chu XJ, DePinto W, Bartkovitz D, *et al*. Discovery of [4-Amino-2-(1-methanesulfonylpiperidin-4-ylamino)pyrimidin-5-yl](2,3-difluoro-6- methoxyphenyl)methanone (R547), a potent and selective cyclin-dependent kinase inhibitor with significant *in vivo* antitumor activity. J Med Chem 2006; 49: 6549-60.

[87] Diab S, Eckhardt S, Tan A, *et al*. A phase I study of R547, a novel, selective inhibitor of cell cycle and transcriptional cyclin dependent kinases (CDKs). J Clin Oncol 2007; 25(suppl 20): abstr 3528.

[88] Fry DW, Harvey PJ, Keller PR, *et al*. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor

activity in human tumor xenografts. Mol Cancer Ther 2004; 3: 1427-38.
[89] Lu H, Schulze-Gahmen U. Toward understanding the structural basis of cyclin-dependent kinase 6 specific inhibition. J Med Chem 2006; 49: 3826-31.
[90] Chen-Kiang S, Di Liberto M, Louie T, *et al*. Targeting Cdk4/6 in combination therapy of chemoresistant multiple myeloma. J Clin Oncol 2008; 26(suppl 20): abstr 8503.
[91] Shapiro GI, Bannerji R, Small K, *et al.* A phase I dose-escalation study of the safety, pharmacokinetics (PK) and pharmacodynamics (PD) of the novel cyclin-dependent kinase inhibitor SCH 727965 administered every 3 weeks in subjects with advanced malignancies. J Clin Oncol 2008; 26: May 20 suppl; abstr 3532.
[92] Nemunaitis J, Saltzman M, Rosenberg MA, *et al*. A phase I dose-escalation study of the safety, pharmacokinetics (PK), and pharmacodynamics (PD) of SCH 727965, a novel cyclin-dependent kinase inhibitor, administered weekly in subjects with advanced malignancies. J Clin Oncol 2009; 27: 15s, suppl; abstr 3535.
[93] Bagella L, Sun A, Tonini T, *et al*. A small molecule based on the pRb2/p130 spacer domain leads to inhibition of cdk2 activity, cell cycle arrest and tumor growth reduction in vivo. Oncogene 2007; 26: 1829-39.
[94] Giordano A, Bellacchio E, Bagella L, Paggi MG. Interaction between the Cdk2/cyclin A complex and a small molecule derived from the pRb2/p130 spacer domain: a theoretical model. Cell Cycle 2007; 6: 2591-3.
[95] Arris CE, Boyle FT, Calvert AH, *et al.* Identification of novel purine and pyrimidine cyclin-dependent kinase inhibitors with distinct molecular interactions and tumor cell growth inhibition profiles. J Med Chem 2000; 43: 2797-804.
[96] Chen YN, Sharma SK, Ramsey TM, *et al*. Selective killing of transformed cells by cyclin/cyclin-dependent kinase 2 antagonists. Proc Natl Acad Sci USA 1999; 96: 4325-9.
[97] Jeffrey PD, Russo AA, Polyak K, *et al.* Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. Nature 1995; 376: 313-20.
[98] Brown NR, Noble ME, Endicott JA, *et al.* The crystal structure of cyclin A. Structure 1995; 3: 1235-47.
[99] De Bondt HL, Rosenblatt J, Jancarik J, Jones HD, Morgan DO, Kim SH. Crystal structure of cyclin-dependent kinase 2. Nature 1993; 363: 595-602.
[100] Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med 2004; 10: 310-5.
[101] Gondeau C, Gerbal-Chaloin S, Bello P, Aldrian-Herrada G, Morris MC, Divita G. Design of a novel class of peptide inhibitors of cyclin-dependent kinase/cyclin activation. J Biol Chem 2005; 280: 13793-800.
[102] Canela N, Orzaez M, Fucho R, *et al.* Identification of an hexapeptide that binds to a surface pocket in cyclin A and inhibits the catalytic activity of the complex cyclin-dependent kinase 2-cyclin A. J Biol Chem 2006; 281: 35942-53.
[103] Adams PD, Sellers WR, Sharma SK, Wu AD, Nalin CM, Kaelin WG Jr. Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. Mol Cell Biol 1996; 16: 6623-33.
[104] Andrews MJ, McInnes C, Kontopidis G, *et al*. Design, synthesis, biological activity and structural analysis of cyclic peptide inhibitors targeting the substrate recruitment site of cyclin-dependent kinase complexes. Org Biomol Chem 2004; 2: 2735-41.
[105] Zheleva DI, McInnes C, Gavine AL, Zhelev NZ, Fischer PM, Lane DP. Highly potent p21(WAF1)-derived peptide inhibitors of CDK-mediated pRb phosphorylation: delineation and structural insight into their interactions with cyclin A. J Pept Res 2002; 60: 257-70.
[106] Atkinson GE, Cowan A, McInnes C, Zheleva DI, Fischer PM, Chan WC. Peptide inhibitors of CDK2-cyclin A that target the cyclin recruitment-site: structural variants of the C-terminal Phe. Bioorg Med Chem Lett 2002; 12: 2501-5.
[107] Kontopidis G, Andrews MJ, McInnes C, *et al.* Truncation and optimisation of peptide inhibitors of cyclin-dependent kinase 2-cyclin a through structure-guided design. ChemMedChem 2009; 4: 1120-8.
[108] Li W, Kotoshiba S, Kaldis P. Genetic mouse models to investigate cell cycle regulation. Transgenic Res 2009; 18: 491-8.

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PIN1

Phosphorylation is a key post-transcriptional event involved in cell cycle control, cellular growth, differentiation, stress response and many others processes. Serine or threonine followed by a proline are major phosphorylation motifs in the cells but their significance was obscured until the discovery of the PIN1 protein (protein interacting with NIMA (never in mitosis A)-1) [14]. PIN1 is a member of the evolutionarily conserved peptidyl-prolyl isomerase (PPIase) family of proteins. PIN1 has a two-domain structure that consists of an N-terminal WW domain (amino acids 1–39) and the C-terminal PPIase domain (amino acids 45–163). The WW domain binds only to specific pSer/Thr-Pro-motifs and the PPIase isomerase domain catalyzes the conformational switch from cis to trans of target proteins. This fact is especially important because Pro-directed kinases and phosphatases are conformation-specific and act only on the trans isoform [3, 4]. For this reason, PIN1 is important for many physiological activities of the cell (Fig. 1).

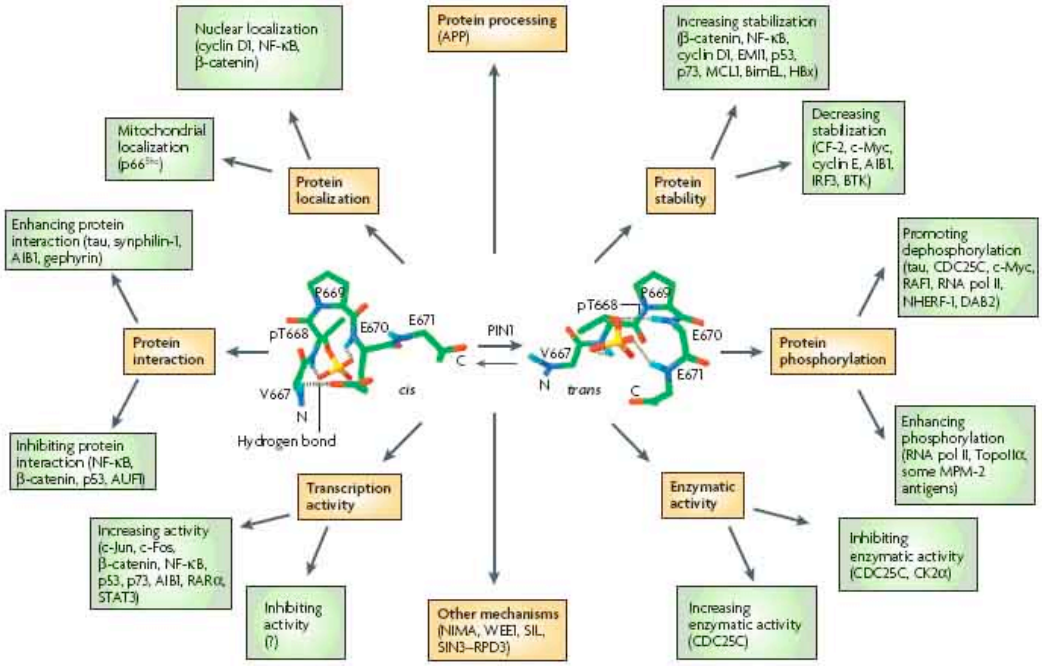


Fig. 1. The different roles of PIN1 in cellular physiology

In cell cycle control, PIN1 was originally identified and defined as a protein important in mitosis. Many of PIN1's substrates contain a single phosphorylation target in the form of CDC25, WEE1 and RPB1[15]. Others, like CK2 and Sil, have multi-phosphorylation sites, suggesting a different mechanism in PIN1 function [16, 17]. Depletion of PIN1 in yeast and human cells induces mitotic arrest and its over-expression blocks the cells in the G2 phase of the cell cycle [14]. Since the discovery of PIN1, a plethora of protein targets have been discovered, many of which are involved in the G0 and G1/S control [11]. Evidence emerged from in vitro and in vivo animal models. PIN1 controls Cyclin D1 mRNA levels and it is involved in regulation of CyclinD1, c-MYC and Cyclin E protein stability [5, 11]. PIN1 -/- MEF showed

proliferative defects in cell cycle entry after serum deprivation. In addition, PIN1 is a target of E2F transcription factors and its mRNA and protein levels fluctuate during cell cycle [18]. This data suggests a possible oncogene and conditional tumor suppressor function of PIN1, but more studies are necessary to distinguish PIN1's function in different cellular contexts. Moreover, these observations pointed toward a role of PIN1 in the cell cycle progression from G0 to G1 and in mitosis.

In cancer pathology, PIN1 over-expression was found in 38 different tumour types out of 60, including most common human cancers such as prostate, cervical, brain, ovary, lung, breast, liver cancer, and melanoma [8]. Moreover, PIN1 expression is an excellent prognostic marker in prostate cancer [9].

Overall this data confirms PIN1 as a fundamental player in cell cycle control and in the tumorigenesis process. Here, we demonstrated that PIN1 could control cell cycle proliferation through direct interaction with pRb and it regulates pRb phosphorylation.

RESULTS

T98G PIN1 knockdown cells show proliferative defects

Different papers reported that the loss of PIN1 in tumor cell lines causes a defect in cell proliferation. Mouse embryonic fibroblasts (MEFs) derived from PIN1 -/- mice showed a reduced proliferation rate [5], [6], [7]. The cause of this altered phenotype has been attributed to Cyclin D1. In fact, PIN1 loss in breast and MEF cells results in Cylin D1 downregulation and pRb hypo-phosphorylation [6]. On the contrary, analysis of several mouse tissues derived from PIN1 KO animals demonstrated that the Cyclin D1 expression level does not change in every tissue. For example in the heart and kidney, the level of cyclin D1 expression in normal and PIN1 KO mice is comparable [19]. These results suggest that PIN1 can control pRb activity through an indirect mechanism mediated by cyclin D1 or, since pRb contains many pS/T-P, a direct mechanism could be hypothesized.

In order to clarify the role of PIN1 in the RB/E2F pathway, we generated a PIN1 knock down (kd) T98G glioblastoma cell line. These cells are well described as being easily synchronized and cell cycle control depends on functional pRb [20]. In addition, cyclin D1 was expressed at very low levels, cyclin D2 levels were not detectable and cyclin D3 exhibited a half-life of less than one hour [21].

We decide to use an shRNA system for knockdown experiments. The technology of RNA interference emerged in its earliest form following a 1998 study in Caenorhabditis elegans and has since rapidly evolved to its current

form as a revolutionary tool for studying gene function, biological pathways, and the physiology of disease [22]. Pioneering studies in C. elegans provided breakthrough experiments that clearly established that double-stranded RNA (dsRNA) interfered with gene function. This discovery was called RNA interference or RNAi.

Further advances have shown that siRNAs can be expressed from DNA vectors within the host cell, providing methods for longer term silencing, inducible silencing, and a plasmid DNA format that can be replicated for unlimited supply. In addition, these vector-based RNAi platforms may be integrated with viral delivery systems allowing investigators to perform gene knockdown in a myriad of cell lines. Recent studies of endogenous microRNAs (miRNAs) suggested that synthetic miRNA mimics could be used to induce the RNAi pathway rather than directly using the standard 21 bp siRNA sequence. These synthetic forms of miRNA, termed short hairpin RNAs (shRNAs), are expressed from pol II or pol III promoters. The hairpin structure is recognized and cleaved by Dicer (RNase Enzyme) to form siRNA that is subsequently taken up by RISC (RNAi-Induced Silencing Complex) for silencing of the target gene. RISC unwinds the double strand siRNA and the activated complex with the associated anti-sense siRNA strand targets the homologous mRNA transcript for cleavage and subsequent degradation.

Five different plasmids expressing PIN1-shRNA were tested. Cells were infected with one MOI (Molteplicity of infection) PIN1-shRNA lentivirus and selected for 72 hours with puromycin. Stable polyclonal cells were analyzed

by western blot. Two shRNA (TRCN1033, TRCN10577) gave the strongest PIN1 downregulation. PIN1 kd cells (TRCN1033) showed a reduction of more than 90% protein level compared to normal and shRNA scrambled infected cells (Fig. 2a). We have used indifferently both shRNAs in the experiments.

We then evaluated the effect of PIN1 depletion with an in vitro cell proliferation assay. T98G cells were plated in 96 well plates and after 3 days were analyzed by XTT assay. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolically active cells [23]. Therefore, this conversion only occurs in viable cells. Cells, grown in a 96 well tissue culture plate, are incubated with the yellow XTT solution (final concentration 0.3 mg/ml) for 4–24 h. After this incubation period, orange formazan solution is formed, which is spectrophotometrically quantified using an ELISA plate reader. An increase in the number of living cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample. This increase directly correlates to the amount of orange formazan formed, as monitored by the absorbance.

As illustrated in fig. 2b, the PIN1 kd cells proliferate 25% less compared to normal cells ($p\_value < 0.001$). To discriminate which phase of the cell cycle was altered, cells were analyzed by FACS (fluorescent activated cell sorting) analysis. FACS is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a fluid stream and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical

characteristics of up to thousands of particles per second. Before analysis, the cells are permeabilized and treated with a fluorescent dye that stains DNA quantitatively, usually propidium iodide (PI). The fluorescence intensity of the stained cells at certain wavelengths will therefore correlate with the amount of DNA they contain. As the DNA content of cells duplicates during the S phase of the cell cycle, the relative amount of cells in the G0 phase and G1 phase (before S phase), in the S phase, and in the G2 phase and M phase (after S phase) can be determined as the fluorescence of cells in the G2/M phase will be twice as high as that of cells in the G0/G1 phase.

PIN1 kd cells have 7.1% more cells in G0/G1 cell cycle phase than normal cells (75.7% *vs* 68.6%, Fig. 2c).

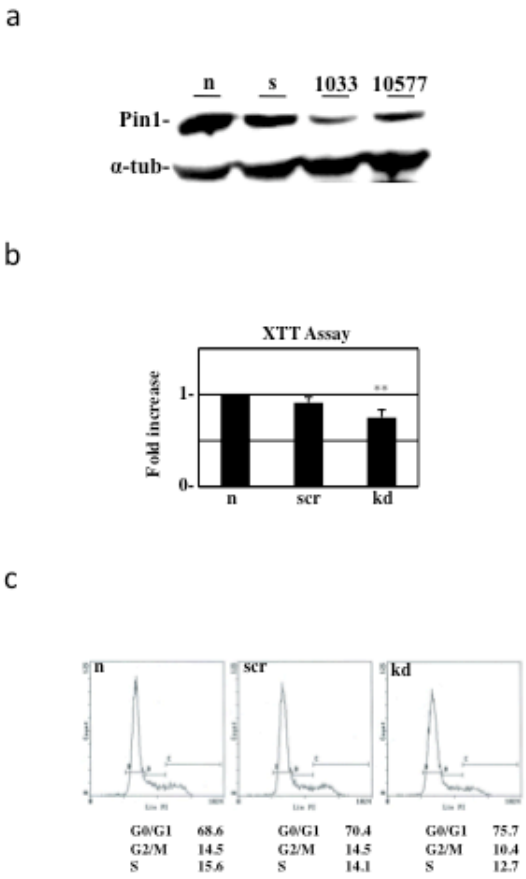


Fig. 2

Fig. 2. Cell proliferation defects in T98G PIN1 knockdown cells. a) T98G cells were untreated (n= normal) or treated with scrambled (scr), PIN1 (TRCN1033, TRCN10577) shRNAs. 50 μg of protein was loaded and analyzed by western blot with PIN1 specific antibodies. The samples were normalized with an α-tubulin antibody. b) XTT assay showing that PIN1 kd cells have reduced proliferation rate. The picture is the media of three experiments. ** p_value <.001, two tailed t-test . All the values were normalized to normal cells. c) A representative experiment that shows PIN1 kd cells (kd) have an increased number of cells in G1 compare to normal (n) and scrambled (scr) cells.

It has been previously demonstrated that siRNA targeting PIN1 in LNcaP and PC3 prostate cancer cells showed significantly reduced cell proliferation, anchorage-dependence, and -independent colony formation. In addition, increased cellular senescence and apoptosis was also observed after stress stimuli [24].

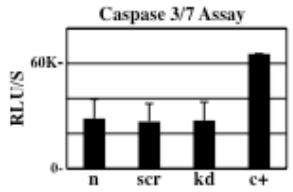
Since we observed reduced proliferation level in T98G cells, we tested as for prostate cancer cells [24], if apoptosis or senescence could be involved in our cellular model. Apoptosis was detected with a Caspase 3/7 assay. The assay includes a profluorescent caspase-3/7 consensus substrate, rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-aspartic acid amide) (Z-DEVD-R110). Upon cleavage on the C-terminal side of the aspartate residue in the DEVD peptide substrate sequence by caspase-3/7 enzymes, the rhodamine 110 becomes fluorescent when excited at a wavelength of 498nm. The emission maximum is 521nm. The amount of fluorescent product generated is representative of the amount of active caspase-3/7 present in the sample.

The caspase assay showed that the apoptotic pathway is not activated in PIN1 kd cells compared to normal cells (Fig. 3a). The results were confirmed by FACS analysis (Fig. 2c) in which no apoptotic cells were detected in normal and PIN1 kd cells. To corroborate these finding, microscopy analysis of normal and knockdown cells revealed no difference in cell morphology (Fig. 4a).

Senescence was detected by the β-galactosidase method. β-galactosidase is a hydrolase enzyme highly expressed and accumulated in lysosomes in

senescent cells and is able to hydrolyze β-D-galactosides. Senescence-associated β-galactosidase staining confirmed no statistical difference between PIN1 kd and normal cells (Fig. 3b).

a



b

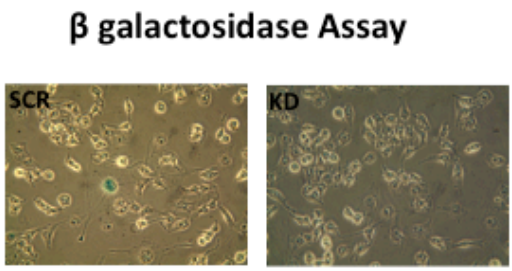


Fig. 3

Fig. 3 Cell proliferation defects in T98G PIN1 knockdown cells. a) Caspase assay. The figure shows the average of three experiments: (n) normal, (scr) scrambled, (kd) PIN1 knockdown cells. (c+) positive control cells were treated with 1 μM doxorubicin for 18 hours. The y axis shows the total value of RLU per second. b) β-galactosidase staining showed no difference between scrambled and PIN1kd cells.

G1/S cyclins and CDKs protein levels in T98G cells are unchanged

Starting from the observed phenotype in T98G cells, we analyzed the CDK/cyclin complex protein level that controls the G0 and G1/S transition cell cycle phase. Cells were synchronized in G0 and G1 through serum starvation and serum starvation combined with hydroxyurea treatment, respectively (see material and methods). The inhibitory mechanism of hydroxyurea is postulated to involve inhibition of the enzyme ribonucleotide reductase, and thus, the synthesis of deoxyribonucleotides from ribonucleotides. Synchronization was analyzed by FACS analysis. As an example, fig. 4c shows that normal cells are synchronized at more than 88%. Western blot analysis of cyclin D1, cyclin A, cyclin E and the relative partners CDK2 and CDK4 demonstrated that no differences were detectable between normal, scrambled and PIN1 knockdown cells (Fig. 4b).

To test the activity of the CDK4-, CDK6- and CDK2-cyclin complex, scrambled and PIN1 kd cells were immunoprecipitated with an antibody directed to CDK4, CDK6 and CDK2 proteins and tested for the ability to phosphorylate a specific substrate. The assay was performed in two steps; first, after the kinase reaction, an equal volume of ADP-Glo™ Reagent was added to terminate the kinase reaction and deplete the remaining ATP. Second, the Kinase Detection Reagent is added to simultaneously convert ADP to ATP and allow the newly synthesized ATP to be measured using a luciferase/luciferin reaction. The light generated is measured using a luminometer. Luminescence can be correlated to ADP concentrations by

using an ATP-to-ADP conversion curve. This assay is sensitive enough to detect very low amounts of ADP (20nM) and can detect generated ADP in a reaction containing up to 1mM ATP in a linear range. As shown in fig. 4d, PIN1 kd cells have similar CDK activity to scrambled cells.

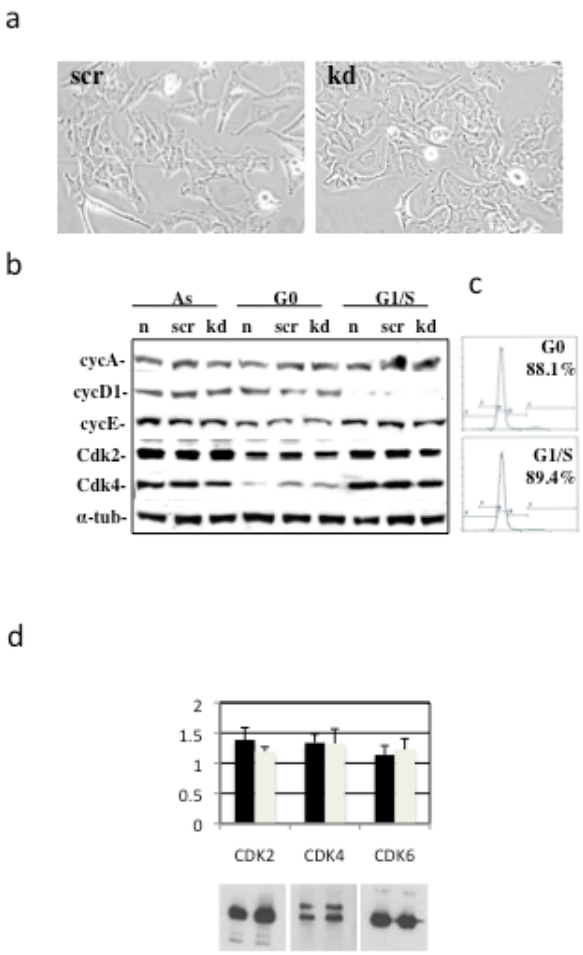


Fig. 4

Fig. 4. Cyclin/CDK complexes are unchanged. a) Light microscope images at 10X showing no difference between scrambled and PIN1 kd cells. b) Normal (n), scrambled (scr) and PIN1 knockdown (Kd) cells were synchronized as described in the material and methods. 50 μg of protein was loaded in each lane and analyzed by western blot with specific antibodies as specified on the left. (As) asynchronous, G0 and G1/S cell cycle phases. c) A representative example of T98G normal cells synchronized in G0 and G1/S and analyzed by FACS. d) Kinase assay. Upper panel. Total protein lysates were immunoprecipitated with indicated kinases and tested with ADP-glo kinase assay on scr (black) and PIN1kd (white) cells. An average of three experiments is shown. Lower panel. The IP (1/20) was loaded on a 10% polyacrylamide gel and probed with the same antibody as in the IP.

To verify the quantity of pRb protein and its phosphorylation level, scrambled and PIN1 knock down cells were synchronized in G0 and G1. Western blot analysis confirmed that no difference in total pRb was detectable in asynchronous, G0 and G1/S cells. Consistent with the previous observation, reduced phosphorylation was evident in asynchronous and G1 cells (Fig. 5a). To further confirm this result, a phospho-specific antibody against ser780 was utilized. A reduced phosphorylation level of pRb is clearly evident (Fig. 5a). At RNA level no difference was observed (Fig. 5b) suggesting a post-transcriptional mechanism by which PIN1 controls the pRb phosphorylation level.

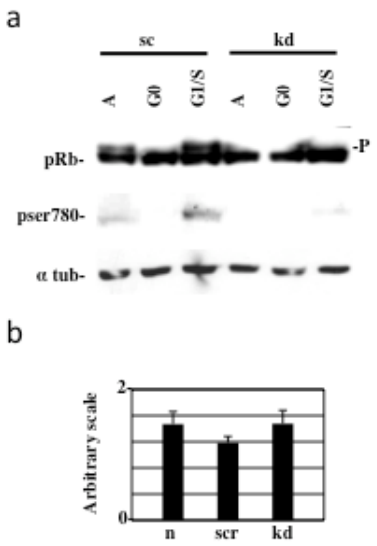


Fig. 5

Fig. 5 pRb is hypo-phosphorylated in T98G PIN1 knockdown cells. a) Cells were syncronized as described in the material and methods. 50 μg of protein was analyzed using a western blot analysis with pRb and pRbser780 phospho-specific antibodies. (p) phosphorylation. The membrane was normalized with α-tubulin. b) Real-time PCR of pRb in PIN1 knockdown cells.

PIN1 directly interacts with pRb

Since the CDK/cyclin complexes are not altered in PIN1 knock down cells and pRb is still hypo-phosphorylated, we hypothesized that the two proteins can directly interact. pRb has more than fifteen Ser/Thr-Pro motifs that can be phosphorylated and could be potential targets of PIN1 (Fig. 6a).

The interaction between PIN1 and pRb was demonstrated by GST (glutathione S-transferase)-pulldown. In a GST gene fusion system, the GST sequence is incorporated into an expression vector alongside the gene sequence encoding the protein of interest. Induction of protein expression from the vector's promoter results in expression of a fusion protein. This GST-fusion protein can then be purified from cells via its high affinity for glutathione. It is fused to the N-terminus of a protein. Agarose beads can be coated with glutathione, and such glutathione-Agarose beads bind GST-proteins. These beads are then washed, to remove contaminating bacterial proteins.

Total lysate proteins were pulled-down with GST or GST-PIN1. Fig. 6b demonstrated that pRb interacts with PIN1. The band has the same molecular weight of the hyper-phosphorylated form of pRb. It is widely accepted that PIN1 interacts with phosphorylated ser/thr motifs followed by proline. To test if this was the case for pRb, T98G cells were treated with shrimp alkaline phoshatase. In Fig. 6c the total input showed that the treatment completely dephosphorylated pRb. Again, the interaction was detected in GST-PIN1 but not in the GST pull-down of the T98G cell lysate and it is absent in the shrimp

alkaline phosphatase treated samples. Phospho- specific antibody directed to ser780 confirmed that the interaction is phosphorylation dependent (Fig. 6c).

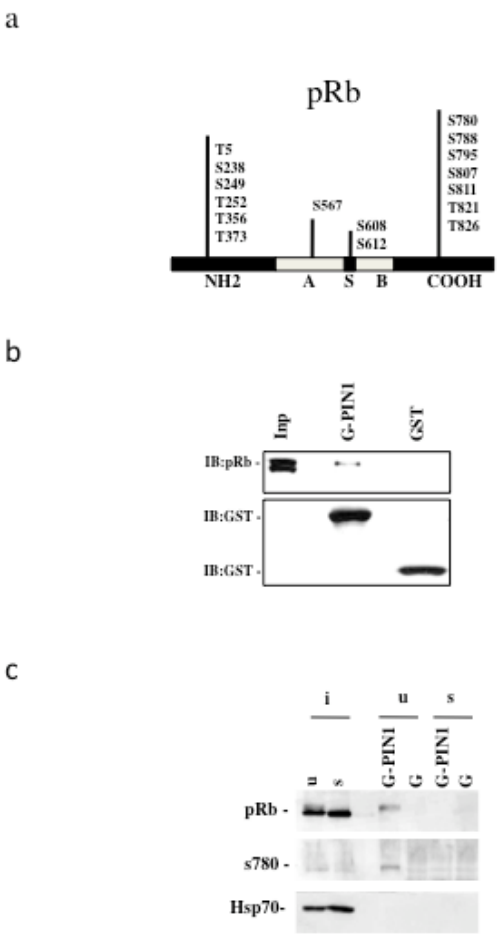


Fig. 6

Fig. 6. In vitro and in vivo interaction between PIN1 and pRb. a) Potential PIN1 binding site targets in pRb protein. b) GST-PIN1 interaction with pRb. A specific band was detected in the GST-PIN1 lane and no band is detected in GST control lane. Of note, the band corresponds to the high molecular weight of phospho-pRb. As a control, the membrane was probed with anti GST antibody. c) Total lysate were untreated (u) or phosphatase treated (s) and pull-down with GST or GST-PIN1 protein. The interaction between PIN1 and pRb is phospho-dependent as a band is clearly visible only in the untreated sample. Hsp 70 antibody was utilized to normalize. In the middle panel, the phospho-dependent interaction was confirmed with pRbser780 phospho-specific antibody. (I) input, (G-PIN1) GST-PIN1, (G) GST.

To assess the in vivo interaction between PIN1 and pRb, T98G cells were immunoprecipitated with anti-PIN1 antibody and analyzed by western blot with anti-pRb antibody.

Co-immunoprecipitation is a purification procedure to determine if two different proteins interact. An antibody specific to the protein of interest is added to a cell lysate. Then the antibody-protein complex is pelleted usually using protein-A/G agarose, which binds most antibodies. If there are any proteins that bind to the first protein, they will also be pelleted. Identification of proteins in the pellet can be determined by western blot.

Since the interaction is barely detectable (data not shown), we fractionated the nucleus from the cytoplasm. As shown in fig. 7a, the interaction is primarily localized in the nucleus. As a negative control, we immunoprecipitated the PIN1 kd cells as normal cells. α-tubulin is used to normalize and to test the quality of the nuclear-cytoplasm fraction. This data demonstrated that PIN1 and pRb form a macromolecular complex. To demonstrate direct interaction between PIN1 and pRb, FAR-western blot experiments were done [25].

In a classical Far-Western analysis, a labelled or antibody-detectable “bait” protein is used to probe and detect the target “prey” protein on the membrane. The sample (usually a lysate) containing the unknown prey protein is separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) or native PAGE and then transferred to the surface of the membrane, making the prey protein accessible to probing. After transfer, the

membrane was blocked and then probed with a known bait protein, which usually is applied in pure form. Following binding of the bait protein with the prey protein, a detection system specific for the bait protein is used to identify the corresponding band.

T98G cell lysate was immunoprecipitated with anti-pRb and transferred on a nitrocellulose membrane. The membrane was incubated with GST-PIN1 or GST and probed with anti-PIN1 antibody. The western blot showed a band that had the same molecular weight of pRb. HSP70 antibody was used to normalize samples (Fig. 7b).

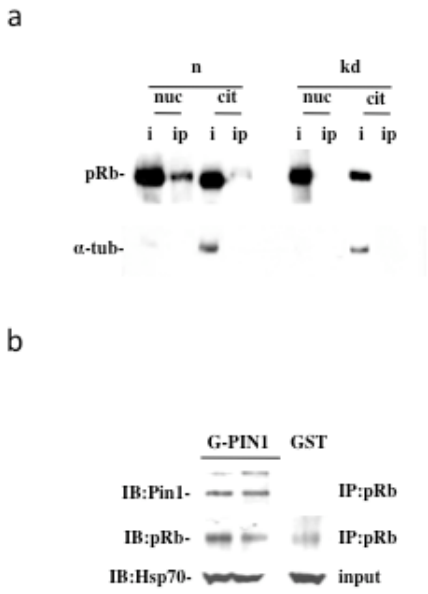


Fig. 7

Fig. 7 a) PIN1 interacts with pRb in the nucleus. Cells were immunoprecipitated with anti-PIN1 antibody, analyzed by western blot with anti-pRb antibody. As control, PIN1 kd cells are treated as normal cells. The interaction is evident in the nucleus. α-tubulin antibody was utilized to verify the nuclear/cytoplasmic fraction. (n) normal, (kd) PIN1 knockdown, (nuc) nucleus, (cit) cytoplasm, (i) input, (IP) immunoprecipitation, (IB) immunoblot. b) Far-western blot experiment showing direct interaction between PIN1 and pRb. Proteins were immunoprecipitated with anti-pRb and transferred onto a nitrocellulose membrane. Membrane was incubated with GST or GST-PIN1 (see material and methods). After washing, the membrane was probe with anti-PIN1 or anti-pRb (diluted four times) as control. 50 µg of input was probed with Hsp70 antibody to normalize samples. Two representative results are shown.

PIN1 controls proliferation through pRb

PIN1 is demonstrated to control the phosphorylation of many proteins that are important in the G1/S and G2/M cell cycle transition [11]. Many studies and herein suggested that the proliferation from G1 to S phases could be controlled through direct and indirect regulation of pRb. To demonstrate that slower proliferation of T98G PIN1 kd cells is due to pRb, we used a specific shRNA direct to pRb and generated T98G PIN1/pRb double knockdown cells (Fig. 8a). The cell proliferation was tested by XTT assay. As illustrated in fig. 8b, PIN1 kd cells proliferate less than wild-type cells whereas PIN1/pRb double knockdown cells proliferate as normal cells. pRb knockdown cells proliferate normally as previously observed in other cell lines [26, 27]. This data reveal that pRb is the major target of PIN1 in controlling the cell cycle without affecting cyclin/CDK protein complexes in T98G cells.

To further confirm the contribution of PIN1 and pRb in cell proliferation, we perform a colony forming assay. This method is based on the capacity of single tumor cells to grow and give visible colonies of progeny after specific staining. Thus, this method is a direct measure of proliferation activity.

As shown in fig. 8c, pRb kd cells rescue the proliferation defects of PIN1 kd cells.

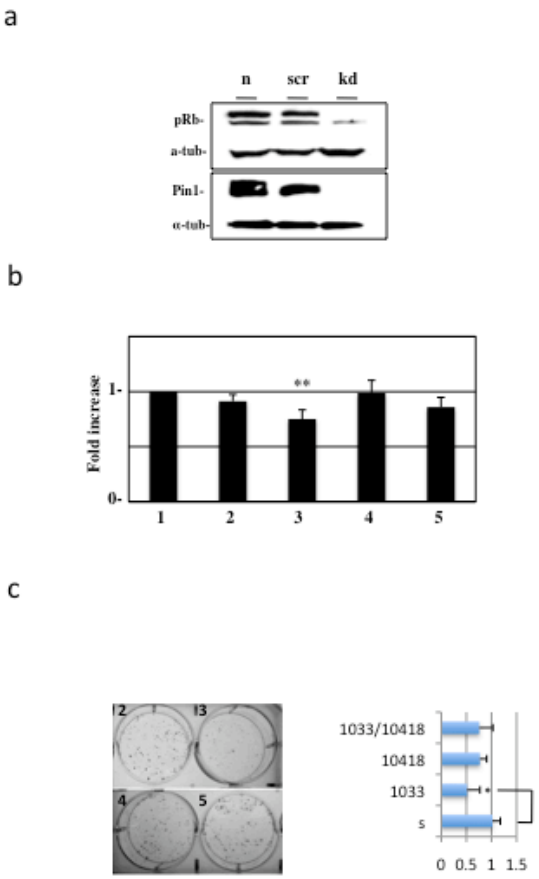


Fig. 8

Fig. 8. pRb is the effector of PIN1 in G1/S cell cycle control. a) T98G cells were untreated (n= normal) or treated with scrambled (scr), PIN1 and pRb shRNA. 50 μg of protein were loaded and analyzed by western blot with pRb and PIN1 specific antibodies. The samples were normalized with α-tubulin antibody. b) Cell proliferation was assessed by XTT assay. All the cells were normalized to control cells. c) Colony forming assay. A representative experiment showing pRb kd cells rescue the PIN1 null phenotype. On the right, the average of three independents experiments. The values were normalized to scrambled cells. 1) normal, (2) scrambled, (3) PIN1 kd, (4) pRb kd, (5) PIN1/pRb double kd cells. ** p value < .01.

Finally, we tested the expression level of some pRb/E2F target genes in PIN1 kd cells. Quantitative real time PCR was done on *E2F1/2/3, DHFR, POLA* and *c-MYC* genes. The results in fig. 9 demonstrated that *c-MYC* and *POLA* genes are down regulated. *E2F2* is an example of a gene whose expression was not altered. This data confirmed that the RB/E2F pathway in PIN1 T98G cells is changed and is responsible for reduced proliferation.

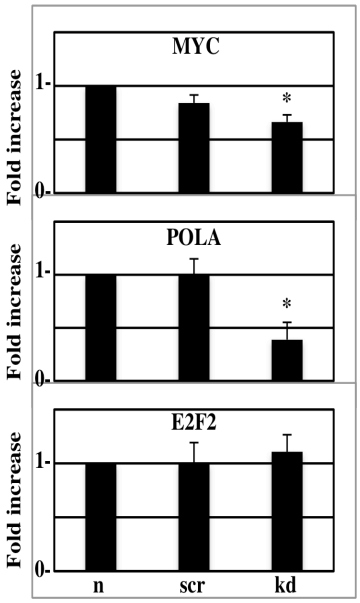


Fig. 9

Fig. 9. Real-time PCR of *c-MYC*, *POLA* and *E2F2* pRb/E2F target genes. The columns represent the average of at least two different experiments. All the values were normalized to control cells. * p value < .05.

These data are in favour of a model by which PIN1 can control the cell cycle through direct and indirect interactions with pRb. PIN1 can stabilize cyclin D1 [19] [28] with the final result of pRb phosphorylation or destabilized cyclin E [10]. As shown in this paper, PIN1 can also control pRb phosphorylation through direct interaction (Fig. 10). PIN1 is important for correct pRb phosphorylation and its over-expression in tumor samples could be responsible for pRb hyper-phosphorylation and inactivation.

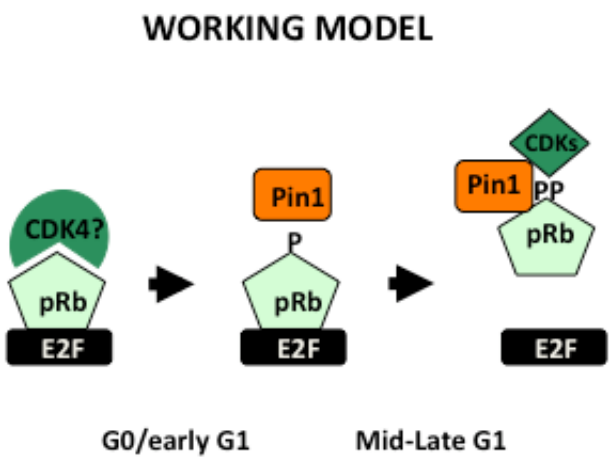


Fig. 10

Fig. 10 PIN1 and pRb interaction model. Direct interaction between PIN1 and pRb is sufficient to control pRb phosphorylation. An unidentified kinase allows the interaction between PIN1 and pRb. This step is necessary for complete pRb phosphorylation and inactivation.

DISCUSSION

Previous studies suggested that PIN1 could control cell proliferation through regulation of cyclin D1, cyclin E or more recently Kip1/p27 protein [29]. These data are also confirmed in *in vivo* models. From mouse models, mice homozygous for the targeted disruption of PIN1 gene in a mixed genetic background revealed several defects that resembled those previously reported to occur in cyclin D1 null mice including lack of breast epithelial expansion during pregnancy. These studies indicated that PIN1 controls the G1–S transition cell cycle phase in breast cancer cells through cyclin D1 [19] [28]. To reinforce these results, PIN1-null mouse embryo fibroblasts grow slower than wild-type cells and markedly delayed cell cycle re-entry in response to mitogenic signal [5]. Additional studies by an independent group confirmed the cell growth defects by showing that serum-arrested PIN1–/– MEFs were resistant to cell cycle re-entry in response to insulin-like growth factor 1 (IGF1). Western blot analysis demonstrated a reduced level of cyclin D1 and hypophosphorylation of pRb protein in knockdown cells. PIN1 -/- cells challenged with PIN1 restore the level of cyclin D1 but the pRb protein was still hypo-phosphorylated [6]. This data suggests that cyclin D1 and other cellular signals are necessary for correct pRb phosphorylation and different mechanisms should be considered.

In this paper, we provide evidence that PIN1 controls tumor cell proliferation through direct interaction with pRb protein. First, our hypothesis stemmed from the evidence that pRb has more than fifteen ser/thr motifs followed by

proline that when phosphorylated became a possible substrate of PIN1 [30]. As with many other regulators of cell cycle, the interaction could be through single or multiple sites. Currently, we are mapping the essential residues involved in the interaction. Importantly, since the decreased level of phosphorylation appears to involve different residues (loss of phosphorylation is evident with total pRb antibody), this analysis will identify the critical motif or motifs that regulate the total pRb phosphorylation. As a result of the isomerization process, pRb could be more prone to the activity of phosphatase enzymes or inhibit the activity of CDK/cyclins complexes. One of the well-characterized examples is the RNAPII CTD phosphorylation. PIN1 can stimulate CDK1 phosphorylation and inhibit the activity of FCP1 phosphatase [31]. Further experiments will clarify these points. Different from the effects seen in prostate cancer cells and in neuronal cells [14, 24, 32], PIN1 knockdown T98G glioblastoma cells have an increase in the number of cells in G1 without an increase in the number of apoptotic or senescent cells, suggesting a cell specific function of PIN1 in different pathways that control equilibrium between growth and death.

Second, cyclin/CDK complexes involved in pRb phsphorylation showed normal levels when comparing PIN1 knockdown and normal cells. Although the CDK complexes are unaltered, the pRb protein is still hypo-phosphorylated. Third, in vitro and in vivo experiments showed that PIN1 is in the same complex with pRb and the interaction is phosphorylation dependent. Since, pRb ser780 is a specific target of CDK4 kinase suggesting a

conformational structural inhibition of pRb mediated by PIN1 as a more likely mechanism. Strikingly, we demonstrate direct interaction between PIN1 and pRb suggesting a new mechanism for PIN1 to control cell cycle proliferation.

Fourth, the proliferation defect of PIN1 knockdown cells can be rescued by knockdown of the pRb protein, highlighting pRb as the major target of PIN1 to control the G1-S cell cycle transition. However, some RB/E2F target genes are down regulated in PIN1 deficient cells. Among them, *c-MYC* proto-oncogene is implicated in many tumors and tumoral transformation can be driven from its over-expression. In quiescence, *c-MYC* is barely detectable and its expression increases together with mitogenic signals during cell proliferation. After the exit from G0, pRb is phosphorylated and E2F binds the *c-MYC* promoter to increase its expression. How c-Myc functions as oncogene is a matter of debate. It is reported that c-Myc can control 10-15% of all cellular genes and recently, it can regulate chromatin structure in a global fashion [33]. In addition, c-Myc interacts with a pre-replicative complex and increases the replication of the replicative origin [34].

Another important point is to understand if the other two members of the pocket protein family, Rb2/p130 and p107, are substrates of PIN1. Due to the high homology, p130/Rb2 and p107 have different conserved ser/thr motifs followed by proline [30]. We hypothize that a similar mechanism of pRb could be involved. Further studies are necessary to clarify if Rb2/p130 and p107 are modulated by PIN1 during the cell cycle.

The interaction between pRb and E2F proteins is the “classical” model to control cell cycle. Moreover, there are at least one hundred different proteins that are reported to interact with pRb. Seventy percent of these proteins are transcription factors. Following this evidence, it could be interesting to analyze how PIN1 regulates these interactions with a major emphasis in cell cycle control.

Interestingly from a therapeutic point of view, PIN1 knockout mice develop normally. They showed a deficiency in cell proliferation only in specific organs. Most of the tissues that develop tumors with high incidence like prostate, lung and colon are unaffected. Loss of PIN1 in normal fibroblasts didn’t show any significant phenotype [24, 35]. These results suggest that PIN1 could be essential for tumors cells but not in normal cells. Different groups are currently developing small-molecule inhibitors that should be tested in vivo as effective anticancer therapy [35-40].

In summary, a number of studies suggest that PIN1 has a role in tumorigenesis. First, PIN1 is over-expressed in most common human cancers such as prostate, ovary, cervical, brain, lung, breast, liver cancer, and melanoma human cancers [8]. Second, mouse models confirm PIN1 as a gene involved in cancer pathology. PIN1 null mice prevent tumor formation induced by *Hras* or *Erbb2* [18, 41]. In p53 null mice, ablation of PIN1 accelerates thymic hyperplasia [42]. Ectopic expression of PIN1 in mammary cells induces tumor formation. In MMTV-PIN1 transgenic mice mammary epithelia hyperplasia is evident at 10 months and different types of mammary

tumors are evident at ages as early as 13 months. Molecular analysis showed that MMTV-PIN1 mice have an increased number of centrosomes. Analysis of primary cell culture showed that most of the cells undergo bipolar divisions, but in a short period a multipolar spindle appears resulting in chromosome missegregation and aneuploidy [43]. Third, PIN1 is a fundamental player in cell cycle control, one of the first steps in transforming a normal cell. Many phospho-substrate proteins are regulators of the G1/S and G2/M phases of the cell cycle. Our study adds a new layer on the complex mechanism of PIN1 action and finds pRb and most likely pocket proteins as the major actors in PIN1 cell cycle control.

MATERIALS AND METHODS

Materials and methods

Cells culture conditions

T98G glioblastoma cell lines were purchased from American Type Culture Collection (ATCC, Rochville, MD, USA). Cells were grown at 37 ^{o}C, in a 5% CO_2/95% atmosphere, in Dulbecco's modified Eagle's medium (Mediatech Inc., Herndon, VA, USA) supplemented with fetal bovine serum (FBS) (Mediatech Inc., Herndon, VA, USA).

For G0 synchronization experiments, cells were blocked by contact inhibition and serum starvation for 48 hours. For G1/S, cells were split and grown in DMEM 10% FBS in 2 µM hydroxyurea for 24 hours as described [44].

Lentiviral production

To generate knock down cells, lentiviral particles were produced as described (http://www.broadinstitute.org/genome_bio/trc/publicProtocols.html). Briefly, 1x10^6 293FT cells (Invitrogen Corp, Carlsbad, CA, USA) were transfected with 2.25 µg of PAX2 packaging plasmid, 0.75 µg of PMD2G envelop plasmid and 3µg of pLKO.1 hairpin vector utilizing 30 µl of Fugene HD (Roche Applied Science, Indianapolis, IN, USA) in 10 cm plate. The lentivirus were collected and filtered (45um) after 48 hours. 2.5x10^5 T98G cells were plated in a multi 6 wells plate. The following day, the cells were transduced with 1 MOI of lentiviral particle in 10% FBS DMEM medium. After 3 days post-infection, the

cells were selected with 2 μg/ml of puromycin (Sigma-Aldrich, St Louis, MO, USA) for 1 week.

Reagents

Antibodies were purchased from: PIN1 (600-401-A20), cyclin A (100-401-151) from Rockland Immunochemicals, Gilbertsville, PA, USA; pRb (sc-102), Hsp70 (sc-24), cyclin E (sc-481), CDK2 (sc-163), CDK4 (sc-260), CDK6 (sc-177), GST (sc-459) from Santa Cruz Biotechnology, Santa Cruz, CA, USA; α-tubulin (T-6074) from Sigma Inc., St Louis, MO, USA; cyclin D1 (556470) from BD Biosciences, San Jose, CA, USA; pRbser780 (9307) from Cell Signaling, Beverly, MA, USA.

shRNA plasmids for pRB (SHCLNG-NM_000321), PIN1 (SHCLNG-NM_006221) were from Sigma Inc., St Louis, MO, USA. Scrambled shRNA (17920), psPAX2 packaging plasmid (12260), pMDG.2 envelope plasmid (12259) were from Addgene Inc, Cambridge, MA, USA.

Fluorescent-activated cell sorting

Cultured cells were harvested after incubation with Trypsin solution (Sigma, St Louis, MO, USA). Cells were fixed by adding ice-cold 70% ethanol while vortexing. Fixed cells were stored at 4 ^{o}C for at least 30 min and then washed once with PBS. Cells were stained with 10 mg/ml propidium iodide (Sigma, St Louis, MO, USA), 250 mg/ml RNase (Sigma, St Louis, MO, USA) in PBS and

incubated at 37 ^{o}C for 30 min in the dark. The percentage of cells in the different phases of the cell cycle was measured with a FACS Calibur instrument (Becton-Dickinson, San Jose, CA, USA).

Cell proliferation assay

Cell proliferation assay was measured by using the Cell proliferating kit II (XTT, Roche Diagnostic, Basel, Switzerland). Briefly, 2x10^{3} T98G cells were plated in triplicate in 96- wells plate. After 3 hours, XTT solution was added to normalize all samples. Cell proliferation was tested after 60 hours. Statistical analysis was done with a two-tailed student's t-test.

Colony forming assay. 2x10^{3} cells were plated in a 6 multi-wells plate and grown for two weeks. Cells were stained with methylene blue/ethanol and counted by two independent investigators.

Caspase 3/7 assay

Caspase 3/7 activities were measured using a Caspase-Glo assay kit (G-7792, Promega, Madison, WI, USA). Briefly, the proluminescent substrate containing the DEVD sequence (sequence is a single letter amino acid code) is cleaved by caspase 3. After caspase cleavage, a substrate for luciferase (aminoluciferin) is released; this results in the luciferase reaction and the production of luminescent signal. Cellular extracts were obtained and lysed. The protein concentration of supernatant was adjusted to 1mg/ml. 10µg/10µl

of proteins were incubated with 10 µl of reconstituted caspase 3/7 glo reagent for 1h at room temperature. The luminescence of each sample was measured in a single tube luminometer (Berthold Technologies, GmbH & CO, Germany)

B-galactosidase assay.

Sub-confluent cells were treated as described [45]. Briefly, cells were washed in PBS, fixed for 3-5 min (room temperature) in 2% formaldehyde/0.2% glutaraldehyde, washed and incubated at 37ºC (no CO_2) with fresh senescence associated β-Gal (SA-β-Gal) stain solution:

- 1mg of 5-bromo-4-chloro-3-indolyl β-D-Galactoside (X-Gal) per ml (from 20mg/ml dimethylformamide stock)

- 40mM citric acid/ sodium phosphate, pH 6.0

- 5mM potassium ferrocyanide

- 5mM potassium ferricyanide

- 150 mM NaCl

- 2mM $MgCl_2$

Staining was evident after 12-16 hr. To detect lysosomal β-Galactosidase, the citric acid/sodium phosphate was pH 4.0.

Real-time PCR

Total RNA was prepared from tissues using the RNA extraction kit Rnaeasy (Qiagen Inc, Valencia, CA, USA). One µg of total RNA was reverse transcribed in a 20 µl reaction using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Primers to amplify *pRb, GAPDH, cMYC, POLA, E2F2* are the following: pRb/p105-f CACCAATACCTCACATTCCTC, pRb/p105-r 5-TTCTCAGAAGTCCCGAATG, GAPDH-f 5-GAAGGTGAAGGTCGGAGT, GAPDH-r 5-CATGGGTGGAATCATATTGGA, RTMYC-F 5-CCTTGCCGCATCCACGAAAC, RTMYC-R GGTGGGCAGCAGCTCGAATT, hPOLART-for 5-TAAATATGAAGTCGAGGACTTCAC, hPOLART-rev 5-GCGTGCTTTACCATCTTTTCCTT, RTE2f2-f 5-TAGCATCATGGAGCCCACAGC, RTE2f2-r TCAGTCTGCTGCAGGAGTGG. Quantitative Real Time PCR (qRT-PCR) was performed with SYBR Green PCR Master Mix (Roche Diagnostic, Basel, Switzerland) in a 7300 ABI instrument (Invitrogen, Carlsbad, CA, USA). Samples were run in triplicates and the efficiency of each primer was calculated utilizing an internal standard control [46]. All values were normalized for *GAPDH*.

Pull-down analyses

For GST pull-down experiment, the IMAGE: 3941595 clone was utilized to amplify the PIN1 human gene with the oligonucleotide primers PIN1-BamHIF GCGGATCCGCGGCAGGAGGGAAGATGG at the 5' end and PIN1-EcoRIR GCGAATTCCTGGGCTCCCCACCCTCAC at the 3' with BamHI and EcoRI

adaptor sequences, respectively. The PCR generated products were ligated in the pGEX-2T plasmid for the prokaryotic expression vector (Stratagene Inc., La Jolla CA, USA). All the plasmids were sequenced verified. GST and GST-PIN1 proteins were produced in BL21 bacteria cells. Cells were grown to mid log phase and then induced to express protein by adding 0.25mM of isopropyl-1-thio-b-D-galactopyranoside (IPTG, Roche Applied Science, Indianapolis, IN, USA). The cultures were shaken for 4 h; bacteria were then pelleted and resuspended in NENT buffer (20mM Tris (pH 8), 100mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 0.5% NP-40). Cell suspensions were sonicated and pelleted so that the supernatant could be collected. The remaining bacteria were then resuspended in NENT buffer plus 2% of N-lauryl-sarcosine, then pelleted and finally, the supernatants were collected again. The combined supernatants were incubated with glutathione agarose beads (Sigma Inc., St Louis, MO, USA) overnight at 4 ^{o}C. The agarose was then pelleted and washed three times in NENT buffer. The GST protein was analyzed by electrophoresis gel and blue coomassie staining. 1mg of protein was pulled-down with 10 ug of GST or GST-PIN1. To dephosphorylate proteins, 1mg of protein lysate was treated with 50 U of shrimp alkaline phosphatase for 1h at 37 ^{o}C.

Co-immunoprecipitation assay

Sub-confluent T98G cells were harvested and nuclear/cytoplasmatic proteins were prepared as follows: the cell pellet was resuspended in NP40 lysis

buffer (0.01M Tris-HCl, 0.01M NaCl, 0.003M MgCl2, 0.03M Sucrose, 0.5% NP40) to prepare the cytoplasmatic fraction. Afterwards, nuclei were pelleted and resuspended in lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% NP40, 2 mM EDTA). 1mg of proteins was immunoprecipitated, utilizing 4 μg of PIN1 antibody. 50 μg of total lysate and IP proteins were run on 8% polyacrylamide gel and probed with pRb antibody (1:250).

Far-western blot

Detection of protein-protein interaction was done essentially as described [47] by far western blotting. Briefly, 3 mg of proteins from total cell lysate were immunoprecipitated with 3 μg of pRb antibody. The samples were run on 7% acrylamide gel and transferred on a nitrocellulose membrane. After separation on SDS-PAGE, the resulting proteins were denatured by SDS. To preserve an intact 3D structure comprising of a functional interaction site, the membrane was subjected to a denaturing/renaturing process. AC buffer (100 mM NaCl, 20 mM Tris (pH 7.6), 0.5 mM EDTA, 10% glycerol, 0.1% Tween-20, 2% skim milk powder and 1 mM DTT. These solutions should be freshly prepared.) with varying concentrations of Guanidine HCl was used: an initial incubation was done in AC buffer containing 6M of Guanidine-HCL for 30 min a room temperature. The membrane was then washed with the AC buffer containing 3M Guanidine-HCL for 30 min a RT. This was followed by washing with the AC buffer containing 0.1 and no Guanidine-HCl at 4ºC, for 30 min and 1 h, respectively. The membrane was incubated with 5 μg of GST or

GST-PIN1 protein at 4ºC overnight. Finally, the membrane was probed with anti PIN1 antibody and anti-pRb antibody as control. 25 μg of total lysate were loaded on the gel and probed with anti-HSP70 to normalize.

Kinase assay

Detection of kinase activity was performed using Kinase Glo luminescence assay (Promega, Madison, WI, USA). 1 mg of protein from total cell lysate was immunoprecipitated with 1μg of CDK2, CDK4, CDK6 antibodies and rabbit polyclonal IgG (sc-66931, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as negative control. After overnight incubation at 4ºC, Protein A/G agarose beads (20421, Pierce, Rockford, IL 61105 USA) were added and after 3 hours washed three times with lysis buffer. Beads were resuspended in 10 μl kinase reaction buffer (40mM THCl, 20mM MgCl2, 0.1 mg/ml BSA, 0.2mM, ATP, 2mM DTT) and incubated 30 minutes at RT with specific substrate (CDk4/6: p107 and CDK2: p53). The reaction was terminated by adding 10 μlADP-GLO reagent 40 minutes at RT and 10 μl kinase detection reagent 5 minutes at RT. 1μl from each experiment was loaded and analyzed by western blot with CDK specific antibodies.

Future aims and prospective

The following future aims are designed to dissect the cooperation between PIN1 and RBf (pRb, Rb2/p130 and p107) in cancer development and to find which pathways cooperate with PIN1 in tumorigenesis.

AIM1. To dissect the molecular pathway of PIN1 and RBf interaction.

We will: - map which domains are involved in the interaction, defining in which phase of the cell cycle PIN1 and RBf interact, which protein kinases and specific growth factor signalling are involved. - correlate the expression of PIN1 and phospho-RBf in human tissue microarray (TMA) by immunonohistochemistry. The planned experiments will shed light on the molecular complexity that regulates RBf protein phsphorylation and activity by PIN1.

AIM2. To assess the in vivo cooperation between PIN1 and pRb in mouse models.

We will test: - if PIN1 is a driver of oncogenesis through the RB pathway in vitro and in vivo by ectopic expression of PIN1 by lentiviral and transgenic mouse (PIN1TG) approaches. - if PIN1 is important for tumor progression by a defined p53/pRb loss tumor model in epithelia ovarian cells. These experiments will provide information on PIN1 in the tumorigenesis process.

AIM3. To test new pathways that cooperates with PIN1 in oncogenesis utilizing a transposon-tagged insertional mutagenesis.

We will screen for genetic lesion that cooperate with PIN1 in the tumorigenesis process. PIN1TG will be crossed with transposon-tagged mice to discovery which pathways cooperate for tumor development. These experiments will provide new potential “druggable” targets to counteract the tumorigenesis process.

The proposed aims will have a broad impact on the mechanism that governs cell cycle control. Since the fundamental concept of target therapy is based on an understanding of the mechanisms that regulate the molecular pathways, the project will start from the molecular analysis of PIN1 and pRb interaction to move forward utilizing the innovative approach of transposon-tagged technology that will allow us to define new pathways that cooperate with PIN1 in tumorigenesis and discovery new players for target therapy.

REFERENCES

1. Giordano, A., et al., *A 60 kd cdc2-associated polypeptide complexes with the E1A proteins in adenovirus-infected cells.* Cell, 1989. **58**(5): p. 981-90.
2. Yeung, R.S., et al., *The retinoblastoma-related gene, RB2, maps to human chromosome 16q12 and rat chromosome 19.* Oncogene, 1993. **8**(12): p. 3465-8.
3. Lu, K.P., et al., *Prolyl cis-trans isomerization as a molecular timer.* Nat Chem Biol, 2007. **3**(10): p. 619-29.
4. Lu, K.P. and X.Z. Zhou, *The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease.* Nat Rev Mol Cell Biol, 2007. **8**(11): p. 904-16.
5. Fujimori, F., et al., *Mice lacking Pin1 develop normally, but are defective in entering cell cycle from G(0) arrest.* Biochem Biophys Res Commun, 1999. **265**(3): p. 658-63.
6. You, H., et al., *IGF-1 induces Pin1 expression in promoting cell cycle S-phase entry.* J Cell Biochem, 2002. **84**(2): p. 211-6.
7. Joseph, J.D., S.N. Daigle, and A.R. Means, *PINA is essential for growth and positively influences NIMA function in Aspergillus nidulans.* J Biol Chem, 2004. **279**(31): p. 32373-84.
8. Bao, L., et al., *Prevalent overexpression of prolyl isomerase Pin1 in human cancers.* Am J Pathol, 2004. **164**(5): p. 1727-37.
9. Ayala, G., et al., *The prolyl isomerase Pin1 is a novel prognostic marker in human prostate cancer.* Cancer Res, 2003. **63**(19): p. 6244-51.
10. Yeh, E.S., B.O. Lew, and A.R. Means, *The loss of PIN1 deregulates cyclin E and sensitizes mouse embryo fibroblasts to genomic instability.* J Biol Chem, 2006. **281**(1): p. 241-51.
11. Yeh, E.S. and A.R. Means, *PIN1, the cell cycle and cancer.* Nat Rev Cancer, 2007. **7**(5): p. 381-8.
12. Ding, L., et al., *Somatic mutations affect key pathways in lung adenocarcinoma.* Nature, 2008. **455**(7216): p. 1069-75.
13. *Comprehensive genomic characterization defines human glioblastoma genes and core pathways.* Nature, 2008. **455**(7216): p. 1061-8.
14. Lu, K.P., S.D. Hanes, and T. Hunter, *A human peptidyl-prolyl isomerase essential for regulation of mitosis.* Nature, 1996. **380**(6574): p. 544-7.
15. Shen, M., et al., *The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins.* Genes Dev, 1998. **12**(5): p. 706-20.
16. Messenger, M.M., et al., *Interactions between protein kinase CK2 and Pin1. Evidence for phosphorylation-dependent interactions.* J Biol Chem, 2002. **277**(25): p. 23054-64.
17. Campaner, S., et al., *Sil phosphorylation in a Pin1 binding domain affects the duration of the spindle checkpoint.* Mol Cell Biol, 2005. **25**(15): p. 6660-72.

18. Ryo, A., et al., *PIN1 is an E2F target gene essential for Neu/Ras-induced transformation of mammary epithelial cells.* Mol Cell Biol, 2002. **22**(15): p. 5281-95.
19. Liou, Y.C., et al., *Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes.* Proc Natl Acad Sci U S A, 2002. **99**(3): p. 1335-40.
20. Mayol, X., J. Garriga, and X. Grana, *Cell cycle-dependent phosphorylation of the retinoblastoma-related protein p130.* Oncogene, 1995. **11**(4): p. 801-8.
21. Garriga, J., et al., *A dynamic equilibrium between CDKs and PP2A modulates phosphorylation of pRB, p107 and p130.* Cell Cycle, 2004. **3**(10): p. 1320-30.
22. Fire, A., et al., *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.* Nature, 1998. **391**(6669): p. 806-11.
23. Gerlier, D. and N. Thomasset, *Use of MTT colorimetric assay to measure cell activation.* J Immunol Methods, 1986. **94**(1-2): p. 57-63.
24. Ryo, A., et al., *Stable suppression of tumorigenicity by Pin1-targeted RNA interference in prostate cancer.* Clin Cancer Res, 2005. **11**(20): p. 7523-31.
25. Gianni, M., et al., *Inhibition of the peptidyl-prolyl-isomerase Pin1 enhances the responses of acute myeloid leukemia cells to retinoic acid via stabilization of RARalpha and PML-RARalpha.* Cancer Res, 2009. **69**(3): p. 1016-26.
26. Dannenberg, J.H., et al., *Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions.* Genes Dev, 2000. **14**(23): p. 3051-64.
27. Sage, J., et al., *Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization.* Genes Dev, 2000. **14**(23): p. 3037-50.
28. Wulf, G.M., et al., *Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1.* EMBO J, 2001. **20**(13): p. 3459-72.
29. Zhou, W., et al., *Pin1 catalyzes conformational changes of Thr187 in p27Kip1 and mediates its stability through a poly-ubiquitination process.* J Biol Chem, 2009.
30. Gallo, G. and A. Giordano, *Are RB proteins a potential substrate of Pin1 in the regulation of the cell cycle?* J Cell Physiol, 2005. **205**(2): p. 176-81.
31. Xu, Y.X., et al., *Pin1 modulates the structure and function of human RNA polymerase II.* Genes Dev, 2003. **17**(22): p. 2765-76.
32. Lu, P.J., et al., *The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein.* Nature, 1999. **399**(6738): p. 784-8.
33. Knoepfler, P.S., *Myc goes global: new tricks for an old oncogene.* Cancer Res, 2007. **67**(11): p. 5061-3.
34. Dominguez-Sola, D., et al., *Non-transcriptional control of DNA replication by c-Myc.* Nature, 2007. **448**(7152): p. 445-51.
35. Hennig, L., et al., *Selective inactivation of parvulin-like peptidyl-prolyl cis/trans isomerases by juglone.* Biochemistry, 1998. **37**(17): p. 5953-60.

36. Guo, C., et al., *Structure-based design of novel human Pin1 inhibitors (I).* Bioorg Med Chem Lett, 2009. **19**(19): p. 5613-6.
37. Zhang, Y., et al., *Structural basis for high-affinity peptide inhibition of human Pin1.* ACS Chem Biol, 2007. **2**(5): p. 320-8.
38. Wu, B., et al., *Discovery and binding studies on a series of novel Pin1 ligands.* Chem Biol Drug Des, 2009. **73**(4): p. 369-79.
39. Dong, L., et al., *Structure-based design of novel human Pin1 inhibitors (II).* Bioorg Med Chem Lett. **20**(7): p. 2210-4.
40. Liu, T., et al., *Membrane permeable cyclic peptidyl inhibitors against human Peptidylprolyl Isomerase Pin1.* J Med Chem. **53**(6): p. 2494-501.
41. Wulf, G., et al., *Modeling breast cancer in vivo and ex vivo reveals an essential role of Pin1 in tumorigenesis.* EMBO J, 2004. **23**(16): p. 3397-407.
42. Takahashi, K., et al., *Ablation of a peptidyl prolyl isomerase Pin1 from p53-null mice accelerated thymic hyperplasia by increasing the level of the intracellular form of Notch1.* Oncogene, 2007. **26**(26): p. 3835-45.
43. Suizu, F., et al., *Pin1 regulates centrosome duplication, and its overexpression induces centrosome amplification, chromosome instability, and oncogenesis.* Mol Cell Biol, 2006. **26**(4): p. 1463-79.
44. Balciunaite, E., et al., *Pocket protein complexes are recruited to distinct targets in quiescent and proliferating cells.* Mol Cell Biol, 2005. **25**(18): p. 8166-78.
45. Dimri, G.P., et al., *A biomarker that identifies senescent human cells in culture and in aging skin in vivo.* Proc Natl Acad Sci U S A, 1995. **92**(20): p. 9363-7.
46. Rizzolio, F., et al., *Highly conserved non-coding sequences and the 18q critical region for short stature: a common mechanism of disease?* PLoS One, 2008. **3**(1): p. e1460.
47. Wu, Y., Q. Li, and X.Z. Chen, *Detecting protein-protein interactions by Far western blotting.* Nat Protoc, 2007. **2**(12): p. 3278-84.