UNIVERSITÀ
DEGLI STUDI
FIRENZE

DOTTORATO DI RICERCA IN SCIENZE BIOMEDICHE

CICLO XXVIII

COORDINATORE Prof. Persio Dello Sbarba

Serine 10 phosphorylation in $p27^{Kip1}$ metabolism: studies on wild type protein and Glycine 9 Arginine oncogenic mutant

Settore Scientifico Disciplinare MED/04

Dottoranda
Dott.ssa Negri Aide

Tutore
Prof. Calorini Lido

Co-Tutore
Prof. Della Ragione Fulvio

Coordinatore
Prof. Dello Sbarba Persio

Anni 2012/2015

INDEX

ABBREVIATION…….. 4

ABSTRACT…….. 5

1.INTRODUCTION……... 6

1.1 Cell cycle control mechanisms..... 6

1.2 p27^{Kip1} gene expression..... 11

1.3 p27^{Kip1}protein structure……. 13

1.4 p27^{Kip1} phosphoisoforms, metabolism and functions……... 16

1.4.1 Phosphorylation on Tyr74, Tyr88, Tyr89……... 18

1.4.2 Phosphorylation on Thr187 and p27^{Kip1} proteolysis……. 18

1.4.3 Phosphorylation on Thr157 and Thr198…… 20

1.4.4 Phosphorylation on Ser10…….. 21

1.5 p27^{Kip1} and cancer……. 25

1.5.1 p27^{Kip1} mutation in human tumors…… 30

2.AIMS OF THE STUDY……. 35

3.MATHERIALS AND METHODS……. 36

3.1Reagents and antibodies…… 36

3.2 Cell cultures and treatments…….. 37

3.3 Cell extracts preparation…… 38

3.4 Cell fraction preparation…… 38

3.5 Protein concentration determination……. 38

3.6 Western blot analysis…….. 38

3.7 Immunoprecipitation experiments……. 39

3.8 Two-dimensional electrophoresis……. 39

3.9 Phosphatase assay……. 39

3.10 Plasmids preparation and transfection…….. 40

3.11 Immunofluorescence analysis…….. 40

3.12 Wound healing…… 41

4.RESULTS…….. 42
4.1 p27^{Kip1} phosphoisoforms characterization…… 42
4.2 pSer10p27^{Kip1} metabolism…… 44
4.3 pSer10p27^{Kip1} biphosphorylated isoform identification…….. 47
4.4 p27^{Kip1} isoforms in cell cycle phases……. 48
4.5 pSer10p27^{Kip1} interaction with CDKs……. 51
4.6 pSer10p27^{Kip1} interaction with cyclins……. 53
4.7 p27^{Kip1}G9R mutation localization analysis……. 55
4.8 p27^{Kip1}G9R metabolism and phosphoisoforms pattern…….. 57
4.9 p27^{Kip1}G9R interaction with CDKs……. 62
4.10 p27^{Kip1}G9R functions in cell motility……. 65
5.DISCUSSION AND PERSPECTIVES…… 68
6.REFERENCES…….. 72

ABBREVIATIONS

CDK: cyclin-dependent kinase

IUP: intrinsically unfolded protein

CKI: cyclin-dependent kinase inhibitor

CIP/Kip: CDK interacting protein/kinase inhibitor protein

CD: circular dichroism

KID: kinase inhibitor domain

PCNA: Proliferative Cell Nuclear Antigen

NLS: nuclear localization signal

ITC: isothermal titration calorimetry

NES: nuclear export signal

NMR: nuclear magnetic resonance

SCF/Skp2: S-phase kinase associated protein 1/Cullin/F-box protein: S-phase kinase associated protein 2

KPC: Kip1 ubiquitylation-promoting complex

FOXO: Forkhead box O

MEN1: multiple endocrine neoplasia

miR: microRNA

ATRA: all-trans-retinoic acid

WB: western blot

2D: two-dimensional electrophoresis

IP: immunoprecipitation

NR: not related

IF: immunofluorescence

CHX: cycloheximide

PPase: phosphatase

Epox: epoxomicin

ABSTRACT

$p27^{Kip1}$ is a Cyclin-dependent Kinase Inhibitor (CKI) belonging to CIP/Kip protein family. It is essentially known for its inhibitory action on several cyclin/CDK complexes (specifically cyclin E(A)/CDK2 and cyclin A(B)/CDK1), suggesting a role as tumor suppressor. However, when localized in cytosol, $p27^{Kip1}$ has a number of CDK-independent functions, including the regulation of apoptosis, cell motility and differentiation. Some of these activities can enhance malignant transformation and/or metastasization under specific conditions. $p27^{Kip1}$ is characterized by the lack of a stable tertiary structure that favors its "adaptability" to bind different targets and contributes to the heterogeneity of its functions. Because of this peculiar structure, the presence of several post translational modifications (especially phosphorylation) has a key relevance for the CKI cellular localization, metabolism and functions. In this study, we have investigated the turn-over and cyclins/CDK interactions of phosphoserine10-$p27^{Kip1}$ ($pSer10p27^{Kip1}$), the main CKI phosphoisoform. $Ser10p27^{Kip1}$ has been suggested to: i) increase $p27^{Kip1}$ stability; ii) allow CKI cytosol translocation, and iii) induce cyclin D/CDKs complexes assembly and nuclear import. We have observed by several different approaches (immunoprecipitations, western blot (WB) and bidimensional analysis associated to WB) that serine 10 phosphorylation confers more stability to $p27^{Kip1}$ allowing the CKI to escape the proteasome-dependent degradation mechanisms. We also established that $pSer10p27^{Kip1}$ does not bind to CDK1, but interacts mostly with cyclin E/CDK2 complex. Conversely, cyclin A/CDK2, associates mainly with unmodified $p27^{Kip1}$. These data demonstrated, for the first time, that the CKI phosphorylations might modulate its binding to cyclin/CDK complexes. Moreover, we demonstrated the absence of Thr187 phosphorylation on $pSer10p27^{Kip1}$ suggesting that this isoform acts as inhibitor of cyclin E/CDK2 complex, and not as substrate. Thus, it is possible to conclude that the phosphorylation of serine 10 acts as a modification that increases the antiproliferative (oncosoppressive) feature of the CKI. We also examined the localization, metabolism, phosphoisoforms pattern and interaction of a cancer-associated mutant form of the CKI (namely $G9Rp27^{Kip1}$) in which glycine 9 is substituted by an arginine. Our attention focused on this $p27^{Kip1}$ mutant since the residue change (i.e. glycine 9) occurs in the amino acid preceding serine 10 thus allowing the hypothesis that the mutation affects Ser10 post translational modification. Unexpectedly, our data demonstrated that the protein is hyperphosphorylated (when compared to the wild-type CKI) and is mostly localized in the nucleus. Part of the increased phosphorylation occurs Ser10. In turn, this reduces the interaction and inhibition of $p27^{Kip1}$ mutant with CDK1. The lower inhibition could lead to an enhanced CDK1 activity that might represent a possible cause of $p27^{Kip1}$G9R tumorigenic property.

1.INTRODUCTION

1.1 Cell cycle control mechanisms

Cell division cycle.

Cell division cycle is a series of biochemical events that leads to cell division and consists of five distinct stages. Senescent cells are found in the G0 phase of the cell cycle and exist in a state where mRNA and protein syntheses are minimal. These cells (as well differentiated cells) may remain quiescent for long periods of time, but under specific condition, such as external signals stimulating cell growth, they can exit the senescent state and re-enter in the cycle at the first gap phase (G1). During G1, cells synthesize proteins required for DNA replication (S phase) following which cells enter into a second gap phase (G2). During G2, the synthesis of proteins required to assemble the cell division machinery occurs and, finally, cells proceed to mitosis (M phase), where the parental cells are divided into two daughter cells. In the cell cycle exists a number of checkpoints playing important roles in: i) the identification of defects that could take place during processes as DNA duplication or chromosome segregation, and ii) the induction of a cell cycle arrest until the defects are repaired. The first cell cycle checkpoint, called restriction point in mammalian cells, takes place at the G1-S transition. If cells pass this point they are committed to entering into S phase. Another cell cycle checkpoint, also known as the DNA damage checkpoint, occurs at the G2-M transition, it ensures that only cells with correct DNA replication enter into mitosis. The last checkpoint, known as mitotic spindle checkpoint, takes place in the M phase (specifically in metaphase) and it prevents the separation of the duplicated chromosomes until each chromosome is correctly attached to the spindle apparatus. This checkpoint is necessary to maintain the right number of chromosomes after each cell division.[1].

Cell division cycle progression is regulated by the formation, activation, and then inactivation of a series of binary complexes made by a catalytic subunit, a Ser/Thr kinase constitutively expressed by the cells and called cyclin-dependent kinase (CDK), and a regulatory subunit, a cyclin, whose concentration, instead, changes periodically during the cell cycle. As a consequence of its crucial roles within the cell, CDK enzymatic activity is regulated at different levels: cyclin binding, subunit phosphorylation (made by enzymes as CAK kinase) and de-phosphorylation (due to enzymes as cdc25 phosphatase), association with and inhibition by a group of small regulatory proteins[2]. In particular two classes of CDK inhibitors (CKI) have been

[1] Li J. M. and Brooks G. "Cell cycle regulatory molecules (cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors) and the cardiovascular system; potential targets for therapy?" European Heart Journal (1999) 20, 406–420.

[2] Guan KL, Jenkins CW, Li Y, Nichols MA, Wu X, O'Keefe CL, Matera AG, Xiong Y. "Growth suppression by p18, a $p16^{INK4/MTS1-}$ and $p\ 14^{INK4B/MTS2-}$ related CDK6 inhibitor, correlates with wild-type pRb function". Genes & Development (1994) 8:2939-2952.

identified: the INK4 and the CIP/Kip family[3;4] differing in structure and function. The first (i.e. INK4) includes $p15^{INK4b}$ [5], $p16^{INK4a}$ [6], $p18^{INK4c}$ [2], and $p19^{INK4d}$ [7], which are folded proteins containing tandem repeats of an ankyrin-like sequence binding specifically CDK4 and CDK6 and inhibiting their activation by D-type cyclins[8]. The second includes $p21^{CIP1/WAF1}$ [9], $p27^{Kip1}$ [10] and $p57^{Kip2}$ [11], which are intrinsically unfolded proteins (IUPs) interacting with and inhibiting a wide range of cyclin/CDK complexes (especially cyclin E(A)/CDK2 and cyclin A(B)/CDK1) with a conserved N-terminal domain that embraces both cyclin and CDK binding sites.

The interaction between cyclin/CDK complexes and CIP/Kip proteins is critical for an appropriate regulation of cell cycle progression[12]. In this thesis, we have focalized our attention on the study of one of the CIP/Kip family members, namely $p27^{Kip1}$.

The CIP/Kip family members. The members of CIP/Kip (CDK interacting protein/kinase inhibitor protein) family were at first identified because of their ability to bind and inhibit or, in general, regulate cyclins/CDKs complexes and consequently to block cell cycle progression. Thus, they were mainly considered as tumor suppressors. More recently, however they were also recognized to be able to have several CDK-independent functions as regulation of transcription, apoptosis, cell migration and differentiation, and cytoskeleton remodeling. Importantly, some of these functions may be oncogenic under certain circumstances. In particular, it seems that the three CKIs play opposing roles in relation to their localization: they act as oncogenes when localized in the cytoplasm, and as tumor suppressors when occurring in the nucleus.

---

[3] Sherr CJ, Roberts JM "Inhibitors of mammalian G 1 cyclin-dependent kinases" Genes & Development (1995) 9; 1149-1163.

[4] Elledge SJ "Cell cycle checkpoints: preventing an identity crisis". Science (1996) 274; 1664-72.

[5] Hannon GJ, Beach D "$p15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest." Nature (1994) 371; 257-61.

[6] Serrano M, Hannon GJ, Beach D "A new regulatory motif of cell-cycle control causing specific inhibition of cyclin D/CDK4". Nature (1993) 366; 704-7.

[7] Chan FK, Zhang J, Cheng L, Shapiro DN, Winoto A "Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to $p16^{INK4}$". Molecular and Cellular Biology (1995) 2682–2688.

[8] Della Ragione F, Takabayashi K, Mastropietro S, Mercurio C, Oliva A, Russo GL, Della Pietra V, Borriello A, Nobori T, Carson DA, Zappia V "Purification and characterization of recombinant human 5'-methylthioadenosine phosphorylase: definite identification of coding cDNA." Biochemical and Biophysical Research Communications (1996) 223; 514-519.

[9] Gu Y, Turck CW, Morgan DO "Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit". Nature (1993) 366; 707-10.

[10] Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A "p27 Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-13 and contact inhibition to cell cycle arrest". Genes & Development (1994) 8; 9-22

[11] Lee MH, Reynisdóttir I, Massagué J "Cloning of $p57^{Kip2}$, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution". Genes & Development (1995) 9; 639-649.

[12] Ou L, Ferreira AM, Otieno S, Xiao L, Bashford D, Kriwacki RW "Incomplete Folding upon Binding Mediates Cdk4/Cyclin D Complex Activation by Tyrosine Phosphorylation of Inhibitor p27 Protein." The Journal of Biological Chemistry (2011) 286; 30142–30151.

Regarding $p21^{CIP1/WAF1}$, $p27^{Kip1}$ and $p57^{Kip2}$ CDK-dependent function, it is known that they are inhibitors of cyclin E(A)/CDK2 and cyclin A(B)/CDK1 complexes, but it is also reported a contribution of these CKIs in the assembly and transport from cytoplasm to the nucleus of cyclin D/CDK4(6) complexes[13;14]. In turn, CIP/Kip proteins sequestration into cyclin D/CDK4(6) complexes could allow the downstream activation of cyclin E/CDK2[15].

The three CIP/Kip CKIs have, also, different roles in cell cycle regulation and the activity of one of them is only partially surrogated by the other family members. $p21^{Cip1/WAF1}$ is mainly regulated by two different pathways: a p53-dependent signaling which is induced in response to DNA damage and mediates cell cycle arrest in G1 and G2 phases with possible DNA repair or induction of apoptosis; a p53-independent signaling, mediated by cell growth factors as PDGF, FGF and EGF[16;17]. $p27^{Kip1}$ regulates the transition from G0, through G1, into S phase and its expression is usually increased in response to cell density, differentiation signals, loss of adhesion to the extracellular matrix, TGF-β signaling and it is rapidly down-regulated as cells enter the cell cycle[10;18]. Finally, studies on $p57^{Kip2}$ have demonstrated an increased association between cyclin D3 and CDK6 when the expression of this CKI is induced, but evidences of an increased stability of this ternary complex, are still lacking[19]. $p57^{Kip2}$ has also an important role in the regulation of cell cycle during embryonic development and its transcriptional regulation is mediated by factors that play critical roles during embryogenesis such as Notch/Hes1, MyoD, BMP-2 and -6, and p73[13].

CIP/Kip proteins are intrinsically unfolded, or rather, they lack secondary and tertiary structure under physiological condition. They can assume specific tertiary conformations and execute their biological functions only after the binding to specific biomolecules in a process called folding-upon-binding[20;21]. However, circular dichroism (CD) studies of $p21^{Cip1/WAF1}$ [22], $p27^{Kip1}$[20;23] and

[13] LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E. "New functional activities for the p21 family of CDK inhibitors". Genes & Development (1997) 11;847-62.

[14] Cheng M, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM, Sherr CJ. "The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts". The EMBO Journal (1999) 18; 1571–1583.

[15] Cheng M, Sexl V, Sherr CJ, Roussel MF. "Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1)". Proc Natl Acad Sci U S A. (1998) 95;1091-6.

[16] Starostina NG, Kipreos ET. "Multiple degradation pathways regulate versatile CIP/Kip CDK inhibitors". Trends Cell Biology (2012) 1; 33-41

[17] Pérez-Sayáns M, Suárez-Peñaranda JM, Gayoso-Diz P, Barros-Angueira F, Gándara-Rey JM, García-García A. "The role of p21Waf1/CIP1 as a Cip/Kip type cell-cycle regulator in oral squamous cell carcinoma". Med Oral Patol Oral Cir Bucal. (2013) 18; 219-25.

[18] Coats S, Flanagan WM, Nourse J, Roberts JM. "Requirement of $p27^{Kip1}$ for restriction point control of the fibroblast cell cycle. Science (!996) 272; 877-80.

[19] Li G, Domenico J, Lucas JJ, Gelfand EW. "Identification of multiple cell cycle regulatory functions of p57Kip2 in human T lymphocytes". The Journal of Immunology (2004) 173;2383-91.

[20] Besson A, Dowdy SF, Roberts JM. "CDK inhibitors: cell cycle regulators and beyond". Developmental Cell (2008) 2; 159-69.

$p57^{Kip2}$ [24] indicate the presence of a nascent α-helical secondary structure in their CDK inhibitor domain.

The conformational flexibility of CIP/Kip CKIs confers them some functional advantages compared to more structured proteins. First, they bind specifically to more than one target regulating various cellular functions. Second, most of CKIs residues are accessible for post-translational modifications, these sites are involved in the control of protein function, localization and turnover. For example, phosphorylation events and protein-protein interactions may alter CKIs folding and modify their capacity to inhibit cyclin/CDK complexes[18;31].

All the three CKIs have a homologous N-terminal domain that contains a conserved region aimed to bind and inhibit the CDK kinase activity (the KID: kinase inhibitor domain)[25]. Instead, CIP/Kip proteins C-terminal domain, binds different effectors: $p21^{CIP1/WAF1}$ and $p57^{Kip2}$ present a PCNA (Proliferative Cell Nuclear Antigen) binding domain that, when bound, prevents the stimulation of DNA synthesis by PCNA[26;27]; $p27^{Kip1}$ and $p57^{Kip2}$ present within their C-terminus a QT domain that contains a threonine residue (Thr187 in $p27^{Kip1}$ and Thr310 in $p57^{Kip2}$) that, when phosphorylated by CDK2, determines a SCF/Skp2-dependent ubiquitination of $p27^{Kip1}$ [28;29] and $p57^{Kip2}$ [30;31].

---

[21] Lacy ER, Filippov I, Lewis WS, Otieno S, Xiao L, Weiss S, Hengst L, Kriwacki RW. "p27 binds cyclin-CDK complexes through a sequential mechanism involving binding-induced protein folding". Nature Structural & Molecular Biology (2004) 4;358-64.

[22] Kriwacki RW, Hengst L, Tennant L, Reed SI, Wright PE. "Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk2-bound state: conformational disorder mediates binding diversity". Proc Natl Acad Sci U S A (1996) 93; 11504-9.

[23] Bienkiewicz EA, Adkins JN, Lumb KJ. "Functional consequences of preorganized helical structure in the intrinsically disordered cell-cycle inhibitor p27(Kip1)." Biochemistry (2002) 41; 752-9.

[24] Adkins JN, Lumb KJ. "Intrinsic structural disorder and sequence features of the cell cycle inhibitor p57Kip2". Proteins (2002) 46;1-7.

[25] Goubin F, Ducommun B. "Identification of binding domains on the p21Cip1 cyclin-dependent kinase inhibitor". Oncogene (1995) 10;2281-7.

[26] Waga S, Hannon GJ, Beach D, Stillman B. "The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA". Nature (1994) 369;574–578.

[27] Watanabe H, Pan ZQ, Schreiber-Agus N, DePinho RA, Hurwitz J, Xiong Y. "Suppression of cell transformation by the cyclin-dependent kinase inhibitor p57KIP2 requires binding to proliferating cell nuclear antigen". Proc Natl Acad Sci U S A (1998) 95;1392–1397.

[28] Montagnoli A, Fiore F, Eytan E, Carrano AC, Draetta GF, Hershko A, Pagano M. "Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation". Genes &Development (1999) 13;1181–1189.

[29] Nguyen H, Gitig DM, Koff A. "Cell-free degradation of p27(kip1), a G1 cyclin-dependent kinase inhibitor, is dependent on CDK2 activity and the proteasome". Molecular Cell Biology (1999) 19;1190–1201.

[30] Kamura T, Hara T, Kotoshiba S, Yada M, Ishida N, Imaki H, Hatakeyama S, Nakayama K, Nakayama KI. "Degradation of p57Kip2 mediated by SCFSkp2-dependent ubiquitylation". Proc Natl Acad Sci USA (2003) 100;10231–10236. [

[31] Galea CA, Wang Y, Sivakolundu SG, Kriwacki RW. "Regulation of cell division by intrinsically unstructured proteins: intrinsic flexibility, modularity, and signaling conduits". Biochemistry (2008) 47;7598–7609.

Another role assigned to the C-terminal region of all three CKIs is the regulation of actin filaments organization and cell migration[32;33;34]. It has been reported that CIP/Kip proteins have a common function in the mobilization of tumor cells and, in turn, in the development of metastases.

The aim of the thesis is to understand the role of the principal nuclear phosphoisoforms occurring in wild-type and mutated $p27^{Kip1}$, as well as to get information on their interactions with the cyclin/CDK complexes. To clarify our objective, the following sections will report a brief description of $p27^{Kip1}$ gene expression, structure, metabolism and its role in human cancer.

[32] Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevsky SA, Dowdy SF. "Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration". Nature Medicine (1998) 4; 1449-52.

[33] McAllister SS, Becker-Hapak M, Pintucci G, Pagano M, Dowdy SF. "Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions". Molecular and Cellular Biology (2003) 1;216-228.

[34] Yokoo T, Toyoshima H, Miura M, Wang Y, Iida KT, Suzuki H, Sone H, Shimano H, Gotoda T, Nishimori S, Tanaka K, Yamada N. "p57Kip2 regulates actin dynamics by binding and translocating LIM-kinase 1 to the nucleus". Journal of Biological Chemistry (2003) 278;52919-23.

1.2 p27^{Kip1} gene expression

p27^{Kip1} gene, named *CDKN1B*, is located on chromosome 12p13 and consists of two exons (a total of 541 bp) originating a protein of 198 amino-acids. p27^{Kip1} cell content is generally controlled by protein degradation mechanisms, however several transcription factors have been reported as involved in *CDKN1B* transcriptional control, including members of the Forkhead box O (FOXO) transcription factor family, menin, E2F1, and Sp1[35].

Members of FOXO transcription factors family play important roles in cell cycle progression[36], apoptosis[37], oxidative stress[36] and DNA repair[38]. Their activity is negatively regulated by the Ser/Thr kinase Akt. Specific extracellular signals activate Akt that can phosphorylate three members of the FOXO family, FOXO1, FOXO3a and FOXO4[39]. This modification induces their detachment from DNA, subsequent translocation and sequestration into the cytoplasm. Thus, FOXO factors are inactivated and consequently, the expression of some genes involved in cell cycle regulation like *CDKN1B* is suppressed.

Menin is encoded by the tumor suppressor gene MEN1 (multiple endocrine neoplasia) and represents another transcription factor able to regulate *CDKN1B* expression. Specifically it acts in differentiated pancreatic islet cells[40;41]. Recent works have shown that mature mice with mutated MEN1 gene, present reduced p27^{Kip1} level and develop insulinomas; young animals, instead, express p27^{Kip1} at normal levels in hyperplastic and dysplastic islets, indicating that loss of p27^{Kip1} in these insulinomas requires additional molecular events[40;42]. In rats it has also been found a *CDKN1B* germline fremshift mutation that reduces p27^{Kip1} levels and causes multiple

[35] Borriello A, Bencivenga D, Criscuolo M, Caldarelli I, Cucciolla V, Tramontano A, Borgia A, Spina A, Oliva A, Naviglio S, Della Ragione F. "Targeting p27Kip1 protein: its relevance in the therapy of human cancer". Expert Opin Ther Targets (2011) 15;677-93.

[36] Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. "Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress". Nature (2002) 419;316-21.

[37] Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. "Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor". Cell (1999) 96;857-68.

[38] Tran PT, Erdeniz N, Dudley S, Liskay RM. "Characterization of nuclease-dependent functions of Exo1p in Saccharomyces cerevisiae". DNA Repair (2002) 1;895-912.

[39] Burgering BM, Kops GJ. "Cell cycle and death control: long live Forkheads". Trends in Biochemical Science (2002) 27;352-60.

[40] Chu IM, Hengst L, Slingerland JM. "The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy". Nature Reviews Cancer (2008) 8;253-267.

[41] Karnik SK, Hughes CM, Gu X, Rozenblatt-Rosen O, McLean GW, Xiong Y, Meyerson M, Kim SK. "Menin regulates pancreatic islet growth by promoting histone methylation and expression of genes encoding p27Kip1 and p18INK4c". Proc Natl Acad Sci USA. (2005) 102;14659-64.

[42] Fontanière S, Casse H, Bertolino P, Zhang CX. "Analysis of p27(Kip1) expression in insulinomas developed in pancreatic beta-cell specific Men1 mutant mice". Familial Cancer (2006) 5;49-54.

endocrine neoplasia (MENX). Furthermore, *CDKN1B* germline mutations have also been identified in some human patients which present MEN1 phenotype[40;43].

E2F1 is activated by cyclin/CDK2 complexes and can subsequently activate *CDKN1B* promoter and determine a feedback inhibition of its own activity[40;44]. Finally, other transcription factors as SP1 and NFY, HES1 and vitamin D3 receptor (VDR) regulate $p27^{Kip1}$ expression and might control CKI levels during development[40;45;46].

$p27^{Kip1}$ expression can also be modulated with a translational control, effectuated by miRNAs. $p27^{Kip1}$ mRNA was, in fact, identified from several groups as a target for two polycistronic miRNAs: miR-221 and miR-222[35;47;48]. These two miRs can bind $p27^{Kip1}$ in the same seed sequence localized in the CKI 3'-UTR and affect its translation. Regulation of $p27^{Kip1}$ repression, so, is essential for cell growth and may also have a role in carcinogenesis. miR-221 and miR-222 levels, in fact, are elevated in several types of cancer, as hepatocellular carcinoma[49], chronic lymphocytic leukemia[50] and ovarian cancer[51], and correlate inversely with $p27^{Kip1}$ levels[35].

[43] Pellegata NS, Quintanilla-Martinez L, Siggelkow H, Samson E, Bink K, Höfler H, Fend F, Graw J, Atkinson MJ. "Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans". PNAS (2006) 103;15558-63.

[44] Wang C, Hou X, Mohapatra S, Ma Y, Cress WD, Pledger WJ, Chen J. "Activation of p27Kip1 Expression by E2F1. A negative feedback mechanism". Journal of Biological Chemistry (2005) 280;12339-43.

[45] Murata K, Hattori M, Hirai N, Shinozuka Y, Hirata H, Kageyama R, Sakai T, Minato N. "Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1". Molecular and Cellular Biology (2005) 25; 4262-71.

[46] Huang YC, Chen JY, Hung WC. "Vitamin D3 receptor/Sp1 complex is required for the induction of p27Kip1 expression by vitamin D3". Oncogene (2005) 23; 4856-61.

[47] Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafrè SA, Farace MG. "miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1". Rhe Journal of Biological Chemistry (2007) 282; 23716-24.

[48] Visone R, Russo L, Pallante P, De Martino I, Ferraro A, Leone V, Borbone E, Petrocca F, Alder H, Croce CM, Fusco A. "MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle". Endocrine Related Cancer (2007) 14;791-8.

[49] Fu X, Wang Q, Chen J, Huang X, Chen X, Cao L, Tan H, Li W, Zhang L, Bi J, Su Q, Chen L. "Clinical significance of miR-221 and its inverse correlation with $p27Kip^1$ in hepatocellular carcinoma". Mol Biol Rep. (2011) 38;3029-35.

[50] Frenquelli M, Muzio M, Scielzo C, Fazi C, Scarfò L, Rossi C, Ferrari G, Ghia P, Caligaris-Cappio F. "MicroRNA and proliferation control in chronic lymphocytic leukemia: functional relationship between miR-221/222 cluster and p27". Blood (2010) 115; 3949-59.

[51] Wurz K, Garcia RL, Goff BA, Mitchell PS, Lee JH, Tewari M, Swisher EM. "MiR-221 and MiR-222 alterations in sporadic ovarian carcinoma: Relationship to CDKN1B, CDKNIC and overall survival". Genes Chromosomes Cancer (2010) 49;577-84.

1.3 p27^{Kip1} protein structure

p27^{Kip1} protein sequence can be divided into two principal regions: the N-terminus and the C-terminus. The first contains the KID domain (residues 25-90) and the nuclear export signal (NES, residues 32-46). It is principally involved in the inhibition of cyclin/CDK complexes and therefore it is responsible of p27^{Kip1} major oncosoppressive function. The second includes the QT domain which embraces several phosphorylatable residues, some of which are critical for p27^{Kip1} metabolism, and a bipartite nuclear localization signal (NLS, residues 152-153 and 166-168). C-terminus functions were initially unknown, but recent studies reported the interaction of this region with proteins apparently not correlated with cell cycle control. Some of these interactions, however, probably favor p27^{Kip1}oncogenic funtions[35] (Fig. 1).

p27^{Kip1} N-terminal domain. The KID contains: a cyclin-binding subdomain (D1, residues 25-36), a CDK-binding subdomain (D2, residues 62-90), and a linker helix subdomain (LH, residues 38-60) that connects D1 and D2. Although p27^{Kip1}shows a disorder conformation before the interaction with its target, it also presents a nascent secondary structure (α-helix) in the LH domain that may have a function in molecular recognition[23] (Fig.2B).

As the other CIP/Kip family members, p27^{Kip1} assumes an order conformation when bound to its partners. This folding-upon-binding has been well characterized for the ternary complex p27^{Kip1}-cyclin A/CDK2 through kinetic and thermodynamic analyses realized with isothermal titration calorimetry (ITC) and surface plasmon resonance. The accumulated data showed that p27^{Kip1} KID domain binds cyclin A/CDK2 with a sequential mechanism[21].

At first, p27^{Kip1} subdomain D1 rapidly binds to cyclin A causing subtle structural changes in the cyclin. Then, the LH subdomain folds into an α-helix and induces the complete reorganization of subdomain D2 that slowly binds to CDK2. Finally, subdomain D2 forms a β-hairpin and an intermolecular β-sheet with CDK2 and the subdomain 3_{10} forms a 3_{10}-helix that inserts into CDK2 ATP binding pocket[12;31;52] (Fig.2A). These structural features reveal that p27^{Kip1} inhibits cyclin A/CDK2 in three different ways: by blocking the substrate binding site on cyclin A; by remodeling CDK2 catalytic cleft; and by occupying CDK2 ATP-binding pocket[53].

Studies using CD, ITC, computational docking, and biochemical assays have analyzed and compared interaction of cyclin D/CDK4 and cyclin A/CDK2 with p27^{Kip1}-KID and various p27^{Kip1}-KID deletion constructs. It has been proposed that p27^{Kip1} utilizes different combinations of residues within its D1 and D2 subdomains to bind and inhibit different cyclin/CDK

[52] Russo AA, Jeffrey PD, Patten AK, Massagué J, Pavletich NP. "Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex". Nature (1996) 382;325-31.

complexes. Although exist an elevated sequence conservation between cyclin D/CDK4 and cyclin A/CDK2, $p27^{Kip1}$ subdomains have different affinity in the bind to these complexes[12].
The NES domain regulates $p27^{Kip1}$ export from the nucleus into the cytoplasm through CKI interaction with the exportin CRM1, the signalosome component Jab1 and nucleoporines as mNPAP60.

$p27^{Kip1}$ C-terminal domain. When localized in the cytoplasm, $p27^{Kip1}$ interacts through its C-terminal domain with different proteins as RhoA, Rac, Stathmin, Grb2 and 14-3-3. $p27^{Kip1}$ C-terminus interaction with RhoA has not been exactly mapped, however coimmunoprecipitation experiments have proved that the CKI can bind RhoA directly and that it can inhibit its pathway blocking RhoA activation mediated by GEF exchanger and thus determining increased cell motility[54;55]. Rac GTPase binds $p27^{Kip1}$ in the region defined as "scatter domain" (residues 118–158), the binding is required for $p27^{Kip1}$-dependent movement[54;56]. Stathmin is a microtubule-destabilizing protein, it interacts with 170-198 residues of $p27^{Kip1}$. In HT-1080 fibrosarcoma cells, stathmin function is inhibited by $p27^{Kip1}$, this causes the accumulation of stabilized microtubules and the subsequent inhibition of mesenchymal cells motility[54;57]. Grb2 binds to $p27^{Kip1}$ in a region that contain the proline rich domain (residues 90–96). Due to this possible interaction, $p27^{Kip1}$ competes with Sos, a Ras GTP exchanger, for binding to Grb2, and prevent Ras activation[54;58]. The 14-3-3 proteins are a family of acidic polypeptides. They bind to pSer/Thr motifs in a sequence-specific manner and regulate the activity of protein involved in signal transduction and cell cycle. It has been demonstrated that $p27^{Kip1}$ interacts with various members of 14-3-3 family and that this interaction can be induced by AKT-dependent phosphorylation on Thr157 or Thr198. The formation of this complex should inhibit the CKI entry into the nucleus, inducing its cytosolic accumulation[54;59].

---

[53] Yoon MK, Mitrea DM, Ou L, Kriwacki RW. "Cell cycle regulation by the intrinsically disordered proteins p21 and p27". Biochemical Society Transactions (2012) 40;981-88.
[54] Borriello A, Cucciolla V, Oliva A, Zappia V, Della Ragione F. "p27Kip1 metabolism: a fascinating labyrinth". Cell Cycle (2007) 6;1053-61.
[55] Besson A, Gurian-West M, Schmidt A, Hall A, Roberts JM. "p27Kip1 modulates cell migration through the regulation of RhoA activation". Genes & Development (2004) 18;862-76.
[56] McAllister SS, Becker-Hapak M, Pintucci G, Pagano M, Dowdy SF. "Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions". Molecular Cell Biology (2003) 23;216-28.
[57] Baldassarre G, Belletti B, Nicoloso MS, Schiappacassi M, Vecchione A, Spessotto P, Morrione A, Canzonieri V, Colombatti A. "p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion". Cancer Cell (2005) 7;51-63.
[58] Moeller SJ, Head ED, Sheaff RJ. "p27Kip1 inhibition of GRB2-SOS formation can regulate Ras activation". Molecular Cell Biology (2003) 23;3735-52.
[59] Fujita N, Sato S, Katayama K, Tsuruo T. "Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization". Journal of biological chemistry (2002) 277;28706-13.

Finally, the NLS domain is recognized by the α/β importins and regulates p27^{Kip1} transport into the nucleus[60]. (Fig. 1).

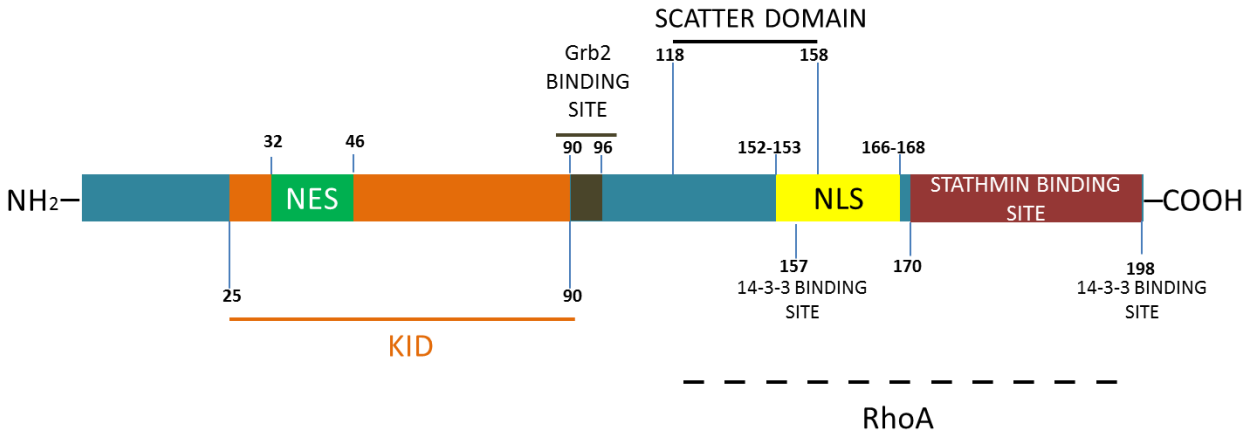

Figure 1 p27^{Kip1} protein domains.

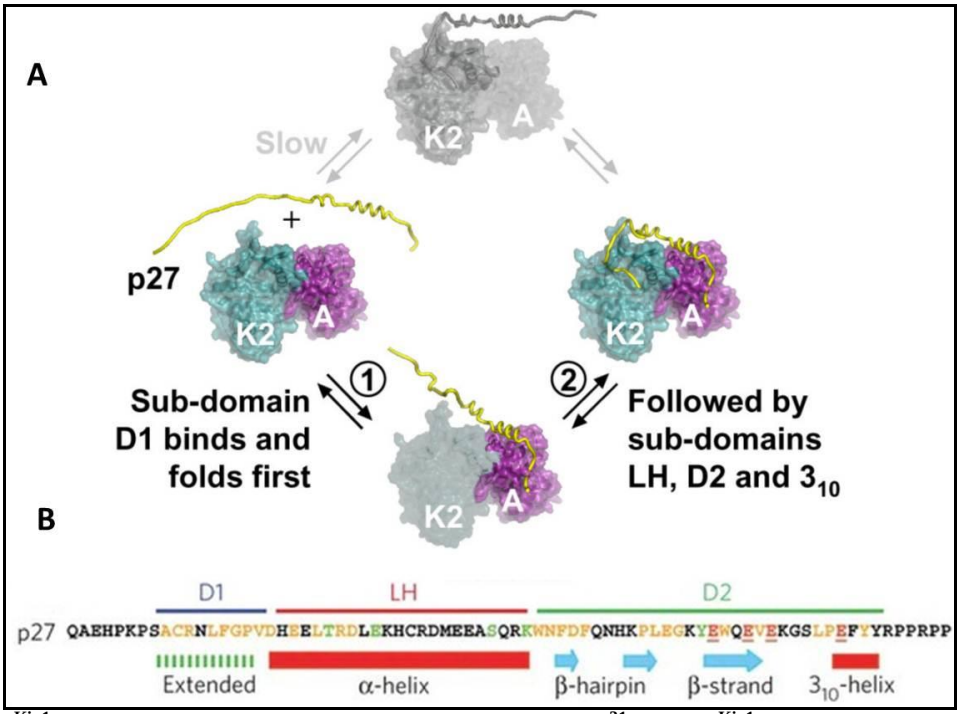

Figure 2 A) p27^{Kip1} folding-upon-binding to cyclin A/CDK2 complex[31]; B) p27^{Kip1} N-terminal subdomains[61].

[60] Zeng Y, Hirano K, Hirano M, Nishimura J, Kanaide H. "Minimal requirements for the nuclear localization of p27(Kip1), a cyclin-dependent kinase inhibitor". Biochem Biophys Res Commun. (2000) 274;37-42.
[61] Wang Y, Fisher JC, Mathew R, Ou L, Otieno S, Sublet J, Xiao L, Chen J, Roussel MF, Kriwacki RW. "Intrinsic disorder mediates the diverse regulatory functions of the Cdk inhibitor p21". Nature Chemical Biology (2011) 7; 214-21.

1.4 p27^{Kip1} phosphoisoforms, metabolism and functions

p27^{Kip1} is an atypical tumor suppressor that controls G0 to S phase transitions by binding and regulating CDKs activity. In G0 and early G1, p27^{Kip1} levels are very elevated and the CKI binds and inhibits cyclin D/CDK4(6) and cyclin E(A)/CDK2 complexes causing the subsequent inhibition of pRb phosphorylation and the block of cell cycle progression[54]. The progressive reduction in p27^{Kip1} levels during G1 and S phases and the CKI export in the cytoplasm, after the phosphorylation of some residues (including Ser10), permits to cyclin E(A)/CDK2 to trigger the transcription of genes required for G1-S transition and to participate in the initiation of DNA replication. p27^{Kip1} levels are mainly controlled by translational regulation and nuclear and cytoplasmic ubiquitination-dependent proteolysis. In early G1, p27^{Kip1} cytoplasmic ubiquitination is regulated by the KPC complex, at the G1-S transition, instead, nuclear ubiquitination is regulated by a two-step mechanism that involves p27^{Kip1} multiple phosphorylations, first on Tyr88 by Src kinase and second on Thr187 by CDK2 (all these mechanisms will be widely discuss below)[40]. In G2/M phases p27^{Kip1} levels increase again, however CKI role in late phases of cell cycle is less clear. Several investigations speculate that p27^{Kip1} could have a function in mitosis and in the maintenance of genomic integrity and DNA damage response[62;63;64].

D-type cyclin/CDKs are activated by mitogens and causes G0 exit and the re-enter in G1 phase. However, p27^{Kip1} has an opposite role in cyclin D/CDK4(6) regulation: in some cases, as contact inhibition, it can inhibit cyclin D/CDK4(6); instead, in early G1 to mid G1, p27^{Kip1} phosphorylated on Thr157 and/or Thr198 favors cyclin D1/CDK4(6) assembly and nuclear translocation[40]. This and many others dual functions of p27^{Kip1} allow us to presume how intricate are the CKI roles in cell physiology.

Moreover, several *in vivo* studies on p27^{Kip1} knock out effects have been done. It has been observed that p27^{Kip1} homozygous null mice are viable but are bigger than wild-type models[38;65;66]. In particular, they present multiple organ hyperplasia, pituitary tumors and, in

[62] Besson A, Gurian-West M, Chen X, Kelly-Spratt KS, Kemp CJ, Roberts JM. "A pathway in quiescent cells that controls p27Kip1 stability, subcellular localization, and tumor suppression". Genes & Development (2006) 20;47-64.

[63] Nakayama K, Nagahama H, Minamishima YA, Miyake S, Ishida N, Hatakeyama S, Kitagawa M, Iemura S, Natsume T, Nakayama KI. "Skp2-mediated degradation of p27 regulates progression into mitosis". Developmental Cell (2004) 6;661-72.

[64] See WL, Miller JP, Squatrito M, Holland E, Resh MD, Koff A. "Defective DNA double-strand break repair underlies enhanced tumorigenesis and chromosomal instability in p27-deficient mice with growth factor-induced oligodendrogliomas". Oncogene (2010) 29;1720-31.

[65] Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai LH, Broudy V, Perlmutter RM, Kaushansky K, Roberts JM. "A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice". Cell (1996) 85;733-44.

some cases, retinal dysplasia, thymic hyperplasia, female sterility, and hyperplasia of the adrenal gland[38;47;48]. Thus $p27^{Kip1}$ does not seem a vital gene, but a protein that controls, through its local inhibitory action on the cell cycle, body and organ size[38].

$p27^{Kip1}$ CDK-dependent and also independent activity is controlled at different levels, by its concentration, subcellular localization and phosphorylation status. In particular, $p27^{Kip1}$ post-translational modifications have a critical role and a highly impact on protein functions, because of the absence of a CKI well-defined folding. Thus, some of the most frequent and important phosphorylations that regulate $p27^{Kip1}$ levels and metabolism, subcellular localization, and its main CDK-dependent and independent functions are described below (Fig.3).

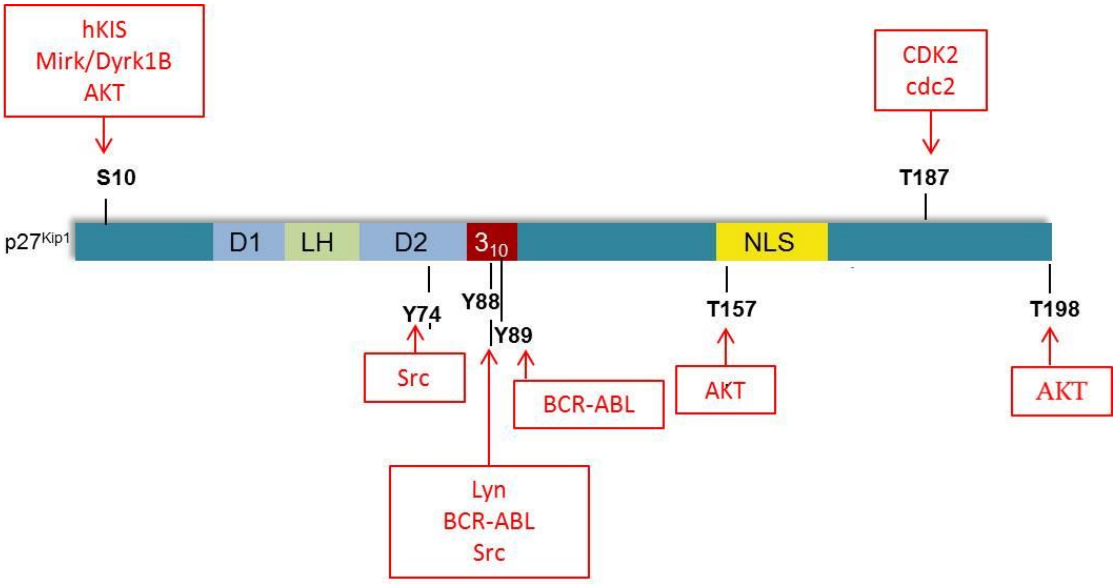

Fig. 3 Major $p27^{Kip1}$ phosphorylations and the most accreditate kinases responsible of these post-translational modifications.

[66] Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY, Nakayama K. "Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors". Cell (1996) 85;707-20.

1.4.1 Phosphorylation on Tyr74, Tyr88, Tyr89

There are three tyrosines in $p27^{Kip1}$ at residues 74, 88 and 89, all within the KID. Tyrosine 88 (Tyr88) is highly conserved among all CIP/Kip family members, whereas tyrosine 74 (Tyr74) is specific for $p27^{Kip1}$. Three kinases responsible of tyrosine phosphorylation are ABL, LYN and SRC[40]. In particular, the nonreceptor tyrosine kinase LYN phosphorylates $p27^{Kip1}$ on Tyr88; ABL phosphorylates $p27^{Kip1}$ at the residues 88 and 89; SRC phosphorylates *in vitro* $p27^{Kip1}$ on Tyr74 and Tyr88 (*in vivo* it seems that the phosphorylation on these residues is decreased because of SRC functional inactivation)[67;68;69].

Tyrosine modification causes a reduced ability of $p27^{Kip1}$ to inhibit CDK2, by affecting the interaction with the cyclin/CDK complex. Crystal structure analysis, in fact, show that Tyr88 is a part of a 3_{10}-helix that inserts into the CDK2 catalityc cleft and displaces ATP inactivating the kinase; Tyr74 forms hydrophobic interactions with CDK2. $p27^{Kip1}$ affinity for CDK2 is weakly decreased by Tyr88 phosphorylation, however NMR analysis demonstrate that when this amino acid is phosphorylated, the 3_{10}-helix is expelled from cyclin/CDK2 catalytic cleft, opening up the ATP binding pocket and determining kinase activation. Thus, phosphorylation of $p27^{Kip1}$ at Tyr88 causes the subsequent CKI phosphorylation at Thr187 residue by the active cyclin E(A)/CDK2 complex. This kind of post-translational modification, determines, so, the transition of $p27^{Kip1}$ from inhibitor of cyclin E(A)/CDK2 to a substrate of the same complex. Because the phosphorylation at Thr187 activates SCF/Skp2 mediated $p27^{Kip1}$ proteolysis, the modification on tyrosine residues probably results in a $p27^{Kip1}$ increased instability[40;50;51] (Fig. 4).

1.4.2 Phosphorylation on Thr187 and $p27^{Kip1}$ proteolysis

Phosphorylation on Thr187 represents the only post translational modification consistently demonstrated and accepted, it is catalyzed by the cyclin E(A)/CDK2 complex and also by cyclin B/CDK1[70;71]. During the G1-S transition the nuclear Thr187 phosphorylation is required for $p27^{Kip1}$ polyubiquitylation by SCF/Skp2/E3 ubiquitin ligase (S-phase kinase associated protein 1/Cullin/F-box protein: S-phase kinase associated protein 2) and for the subsequent degradation

[67] Kardinal C, Dangers M, Kardinal A, Koch A, Brandt DT, Tamura T, Welte K. "Tyrosine phosphorylation modulates binding preference to cyclin-dependent kinases and subcellular localization of p27Kip1 in the acute promyelocytic leukemia cell line NB4". Blood (2006) 107;1133-40.

[68] Grimmler M, Wang Y, Mund T, Cilensek Z, Keidel EM, Waddell MB, Jäkel H, Kullmann M, Kriwacki RW, Hengst L. "Cdk-inhibitory activity and stability of p27Kip1 are directly regulated by oncogenic tyrosine kinases". Cell (2007) 128; 269-80.

[69] Chu I, Sun J, Arnaout A, Kahn H, Hanna W, Narod S, Sun P, Tan CK, Hengst L, Slingerland J. "p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2". Cell (2007) 128;281-94.

[70] Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. "Cyclin E-CDK2 is a regulator of p27Kip1". Genes & Development (1997) 11;1464-78.

[71] Vlach J, Hennecke S, Amati B. "Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27". (1997) 16;5334-44.

by 26S-proteasome. The SCF/Skp2 RING-type ubiquitin ligase consists of SKP1, CUL1, the ring finger protein RBX1 (also known as ROC1), the F-box protein Skp2 and the accessory protein CSK1B[72]. The cyclin/CDK-bound pThr187p27^{Kip1}, binds to the phosphate binding site of CSK1B, to Skp2 and to the CSK1B-Skp2 interface. p27^{Kip1} binding to cyclin E(A)/CDK2 causes, not only phosphorylation on Thr187, but it also stimulates p27^{Kip1} recruitment to SCF/Skp2, because cyclin A binds Skp2 and CDK2 binds CSK1B. It was initially thought that the SCF/Skp2 complex was able to regulate only the G1-S transition, but recent studies show that it plays an important role also in S and G2 phases[40;73] (Fig. 5).

pThr187-independent p27^{Kip1} proteolysis. p27^{Kip1} nuclear proteolysis mediated by Skp2 can also occur in G1 independently of Thr187 phosphorylation[74]. Exists, in fact, an additional Skp2-dependent ubiquitin ligase complex that does not require CKS1B for p27^{Kip1} recruitment. This complex is constituted by DDB1, a subunit of the damaged-DNA binding protein DDB, that functions also as an adaptor for Cul4A, a member of the cullin family of E3 ubiquitin ligase; the Cul4A-DDB1 complex is also associated with the COP9 signalosome. Recently the complex made by Skp2-Cul4A-DDB1-COP9, has been shown to target p27^{Kip1} for proteolysis[75]. Further studies illustrate a p27^{Kip1} degradation mediated by Cul4A in S-phase, independently of Skp2 presence. Thus, it has been proposed that Cul4 acts to reprogram S-phase progression and to replace some functions of SCF/Skp2[35;76].

In early G1, in addition to the nuclear proteolysis, p27^{Kip1} can also have a cytoplasmic degradation mediated by a Skp2-and Thr187-independent pathway[54]. The CKI export in early G1, can be promoted by p27^{Kip1} phosphorylation on serine 10 (Ser10). Some kinases responsible of this post-translational modification are KIS, MIRK and DIRK1, which increase p27^{Kip1} bind, through the NES domain, to CRM1 promoting, in turn, the CKI nuclear export[77;78]. p27^{Kip1} export requires loss of cyclin/CDK2 binding, because CRM1 binds p27^{Kip1} in its CDK2

[72] Nakayama KI, Nakayama K. "Ubiquitin ligases: cell-cycle control and cancer". Nature Review Cancer (2006) 6;369-81.
[73] Bloom J, Pagano M "Deregulated degradation of the cdk inhibitor p27 and malignant transformation". Seminars in Cancer Biology (2003) 13;41-7.
[74] Malek NP, Sundberg H, McGrew S, Nakayama K, Kyriakides TR, Roberts JM. "A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase". Nature (2001) 413;323-7.
[75] Bondar T, Kalinina A, Khair L, Kopanja D, Nag A, Bagchi S, Raychaudhuri P. "Cul4A and DDB1 associate with Skp2 to target p27Kip1 for proteolysis involving the COP9 signalosome". Molecular and Cellular Biology (2006) 26;2531-9.
[76] Li B, Jia N, Kapur R, Chun KT. "Cul4A targets p27 for degradation and regulates proliferation, cell cycle exit, and differentiation during erythropoiesis". Blood (2006) 107;4291-9.
[77] Ishida N, Hara T, Kamura T, Yoshida M, Nakayama K, Nakayama KI. "Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export". The Journal of Biological Chemistry (2002) 277;14355-8.

interaction site. Cytoplasmic $p27^{Kip1}$ can be ubiquitylated by the ubiquitin ligase Kip1 ubiquitylation-promoting complex (KPC). KPC complex is constituted of two subunits that act cooperatively: KPC1 and KPC2. KPC1 contains the catalytic domain of the complex, responsible for the poly-ubiquitination of the protein; KPC2 instead transfers the labeled protein to the proteasome[54].

In addition other two removal processes need to be mentioned: the first is dependent on the activity of calpain, a Ca^{2+}-dependent protease, and has been demonstrated in MAPK-activated OCM-1 (a choroidal melanoma tumor-derived cell line)[35;79;80]. The second requires an initial interaction between $p27^{Kip1}$ and annexin 6 that commits the CKI to endolysosomal degradation. The process has been demonstrated in several cell lines and might contribute to $p27^{Kip1}$ downregulation after serum stimulation[35;81].

1.4.3 Phosphorylation on Thr157 and Thr198

$p27^{Kip1}$ phosphorylation of on Thr157 and Thr198 by Akt kinase inhibits nuclear import of the CKI, causing $p27^{Kip1}$ accumulation in cytosol and the inhibition of G1 arrest[82;83]. Further studies have demonstrated that also the oncogenic Ser/Thr kinase, Pim, phosphorylates $p27^{Kip1}$ on the same residues, promoting cell cycle progression. When Thr157 or Thr198 are phosphorylated, it is generated a recognition domain for the 14-3-3 proteins that competes with the α-importin to bind $p27^{Kip1}$ (Thr157 is, in fact, located in the NLS) and leads to sequestration of the CKI in the cytoplasm[59;84] (Fig. 6).

Phosphorylation of Thr157 and Thr198 promotes assembly but not activation of $p27^{Kip1}$–cyclin D/CDK complexes. PI3K-mediated activation of Akt, p90RSK1, mTOR and SGK may

---

[78] Connor MK, Kotchetkov R, Cariou S, Resch A, Lupetti R, Beniston RG, Melchior F, Hengst L, Slingerland JM. "CRM1/Ran-mediated nuclear export of p27(Kip1) involves a nuclear export signal and links p27 export and proteolysis". Molecular Biology of the Cell (2003) 14;201-13.

[79] Delmas C, Aragou N, Poussard S, Cottin P, Darbon JM, Manenti S. "MAP kinase-dependent degradation of p27Kip1 by calpains in choroidal melanoma cells. Requirement of p27Kip1 nuclear export". Journal of Biological Chemistry (2003) 278;12443-51.

[80] Akashiba H, Matsuki N, Nishiyama N. "Calpain activation is required for glutamate-induced p27 down-regulation in cultured cortical neurons". Journal of Neurochemistry (2006) 99;733-44.

[81] Fuster JJ, González JM, Edo MD, Viana R, Boya P, Cervera J, Verges M, Rivera J, Andrés V. "Tumor suppressor p27(Kip1) undergoes endolysosomal degradation through its interaction with sorting nexin 6". FASEB J. (2010) 24;2998-3009.

[82] Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. "PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest". Nature Medicine (2002) 8;1153-60.

[83] Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, Fusco A, Santoro M. "Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer". Nature Medicine (2002) 8;1136-44.

[84] Fujita N, Sato S, Tsuruo T. "Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization". Journal of Biological Chemistry (2003) 278;49254-60.

contribute to $p27^{Kip1}$ phosphorylation at Thr157 and Thr198 and favor $p27^{Kip1}$ –cyclin D/CDK complexes association in several cell types[40].

These modifications can also interfere with the cytoskeleton normal structure: pThr198, in fact, can favor $p27^{Kip1}$ bind to RhoA, preventing its GEFs activation and increasing cell motility and metastatic capability. Furthermore, several works have shown that phosphorylation on Thr198 may have a role on $p27^{Kip1}$ stabilization: in HeLa and MCF7 cell lines treated with Phorbol-12-myristate-13-acetate (PMA), an activator of PKC, the protein kinase C is able to phosphorylate Thr198 residue, this modification results in the increase of $p27^{Kip1}$ protein levels[85]. In early G1 this modification seems to increase the CKI stability, but it is not connected to the protein sub-cellular localization; in this case, the stabilization could be probably due to the combination of this phosphorylation with Thr157 and/or Ser10 phosphorylations[86].

1.4.4 Phosphorylation on Ser10

Phosphorylation on Ser10 might be considered the mayor $p27^{Kip1}$ post-translational modification, it represents the 70–75% of CKI phosphoisoforms, and was identified for the first time by Nakayama's group in 2000[87]. Different kinases have been supposed to catalyze the phosphorylation at Ser10 residue, some approved are: MAPK (Erk1/2)[88], human kinase interacting stathmin (hKis)[89], Akt[84], Minibrain related kinase (Mirk)/dual specificity tyrosine-phosphorylation-regulated-kinase 1B (DirkB1)[90], CDK5[91] and calcium calmodulin-dependent protein kinase II (CaMKII)[92]. The involvement of these kinases in Ser10 phosphorylation has been principally demonstrated with *in vitro* assays or with overexpression experiments. Therefore, the "physiological" role of these enzymes in $p27^{Kip1}$ phosphorylation and metabolism needs to be confirmed[35].

[85] De Vita F, Giuliani F, Silvestris N, Rossetti S, Pizzolorusso A, Santabarbara G, Galizia G, Colucci G, Ciardiello F, Orditura M. "Current status of targeted therapies in advanced gastric cancer". Expert Opinion on Therapeutic Targets (2012) 16;S29-34.

[86] Motti ML, De Marco C, Califano D, Fusco A, Viglietto G. "Akt-dependent T198 phosphorylation of cyclin-dependent kinase inhibitor p27kip1 in breast cancer". Cell Cycle (2004) 3;1074-80.

[87] Ishida N, Kitagawa M, Hatakeyama S, Nakayama K. "Phosphorylation at serine 10, a major phosphorylation site of p27(Kip1), increases its protein stability". The Journal of Biological Chemistry (2000) 275;25146-54.

[88] Alessandrini A, Chiaur DS, Pagano M. "Regulation of the cyclin-dependent kinase inhibitor p27 by degradation and phosphorylation". Leukemia (1997) 11;342-5.

[89] Boehm M, Yoshimoto T, Crook MF, Nallamshetty S, True A, Nabel GJ, Nabel EG. "A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression". EMBO (2002) 21; 3390-401.

[90] Deng X, Mercer SE, Shah S, Ewton DZ, Friedman E. "The cyclin-dependent kinase inhibitor p27Kip1 is stabilized in G(0) by Mirk/dyrk1B kinase". The Journal of Biological Chemistry (2004) 279; 22498-504.

[91] Kawauchi T, Chihama K, Nabeshima Y, Hoshino M. "Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration". Nature Cell Biology (2006) 8;17-26.

[92] Kajihara R, Fukushige S, Shioda N, Tanabe K, Fukunaga K, Inui S. "CaMKII phosphorylates serine 10 of p27 and confers apoptosis resistance to HeLa cells". Biochem Biophys Res Commun. (2010) 401;350-5.

Main roles correlated with Ser10 phosphorylation are $p27^{Kip1}$ nuclear export[77] and protein stabilization[87]. In early G1 this modification facilitates $p27^{Kip1}$ binding to CRM1 and subsequent cytoplasm translocation and degradation by KPC complexes (Fig. 7). Studies on mouse fibroblasts have shown that the mutation of Ser10 to Ala10 reduces $p27^{Kip1}$ nuclear export, and causes its accumulation in the nucleus[56;77;78;93]. Furthermore, several studies speculate that Ser10 phosphorylation stabilizes the CKI in quiescent cells. Mutation of Ser10 to Ala10 or Glu10 destabilizes or stabilizes $p27^{Kip1}$ respectively[87;94] and similar results have been also reported by studies on Ser10Ala $p27^{Kip1}$-knock-in mouse[62]. It seems that, in quiescent cells, Ser10 phosphorylation could increase protein stability by modulating CKI interactions with cyclin/CDK complexes, although these mechanisms are still unknown. Borriello et al. have shown that in LAN-5 (neuroblastoma cell line), ATRA (all-trans-retinoic acid) treatment increases $p27^{Kip1}$ levels[95]. This increase is not due to impairment of CKI degradation in that ATRA-treatment does not down-regulate Skp2 and CSK1 and does not impair $p27^{Kip1}$ nuclear export. Using two-dimensional PAGE/immunoblotting, it has been demonstrated that, after retinoic treatment, the nuclear monophosphorylated CKI isoform is up-regulated and it has also been proved, using immunological analysis, that this isoform corresponds to $pSer10p27^{Kip1}$. Furthermore, ATRA-treated nuclear LAN-5 extracts show an enhanced capability of phosphorylating $p27^{Kip1}$ on Ser10, thus explaining the nuclear up-regulation of the isoform. Thus, it seems that another mechanism by which ATRA can explicate its antiproliferative activity is the up-regulation of Ser10 phosphorylation. This event causes $p27^{Kip1}$ stabilization and its accumulation in the nuclear compartment, suggesting a possible role of $pSer10p27^{Kip1}$ in the regulation of cell cycle progression[96].

Other $pSer10p27^{Kip1}$ roles are linked to cortical neuron differentiation and migration[91] and apoptosis regulation[92]. Neural migration control is associated to CDK5 activation by p35 in post mitotic neurons[97;98;99;100]. It seems that after p35-mediated activation, CDK5 could act as $p27^{Kip1}$

[93] Shin I, Rotty J, Wu FY, Arteaga CL. "Phosphorylation of p27Kip1 at Thr-157 interferes with its association with importin alpha during G1 and prevents nuclear re-entry". The Journal of Biological Chemistry (2005) 280;6055-63.
[94] Rodier G, Montagnoli A, Di Marcotullio L, Coulombe P, Draetta GF, Pagano M, Meloche S. "p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis". EMBO (2001) 20;6672-82.
[95] Borriello A, Cucciolla V, Criscuolo M, Indaco S, Oliva A, Giovane A, Bencivenga D, Iolascon A, Zappia V, Della Ragione F. "Retinoic acid induces p27Kip1 nuclear accumulation by modulating its phosphorylation". Cancer Research (2006) 66;4240-8.
[96] Borriello A, Cucciolla V, Criscuolo M, Indaco S, Oliva A, Giovane A, Bencivenga D, Iolascon A, Zappia V, Della Ragione F. "Retinoic acid induces p27Kip1 nuclear accumulation by modulating its phosphorylation". Cancer Research (2006) 66;4240-8.
[97] Tsai LH, Takahashi T, Caviness VS Jr, Harlow E. "Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system". Development (1993) 119;1029-40.
[98] Lee MH, Nikolic M, Baptista CA, Lai E, Tsai LH, Massagué J. "The brain-specific activator p35 allows Cdk5 to escape inhibition by p27Kip1 in neurons". PNAS (1996) 93;3259-63.

positive regulator, phosphorylating the CKI on Ser10 residue in G0-arrested neurons. Subsequently, this determines microtubule and actin reorganization through the suppression of RhoA activity and through cofilin activation[91]. *In vivo* $p27^{Kip1}$ suppression leads to neuronal abnormal migration and cytoskeleton disorders.

Furthermore, Kajihara and colleagues have demonstrated that, in HeLa cells, CaMKII increases $p27^{Kip1}$ expression and stability through Ser10 phosphorylation and that the wild-type protein overexpression, but not the Ser10Ala mutant increases Bcl-xL expression and confers resistance to apoptosis[92].

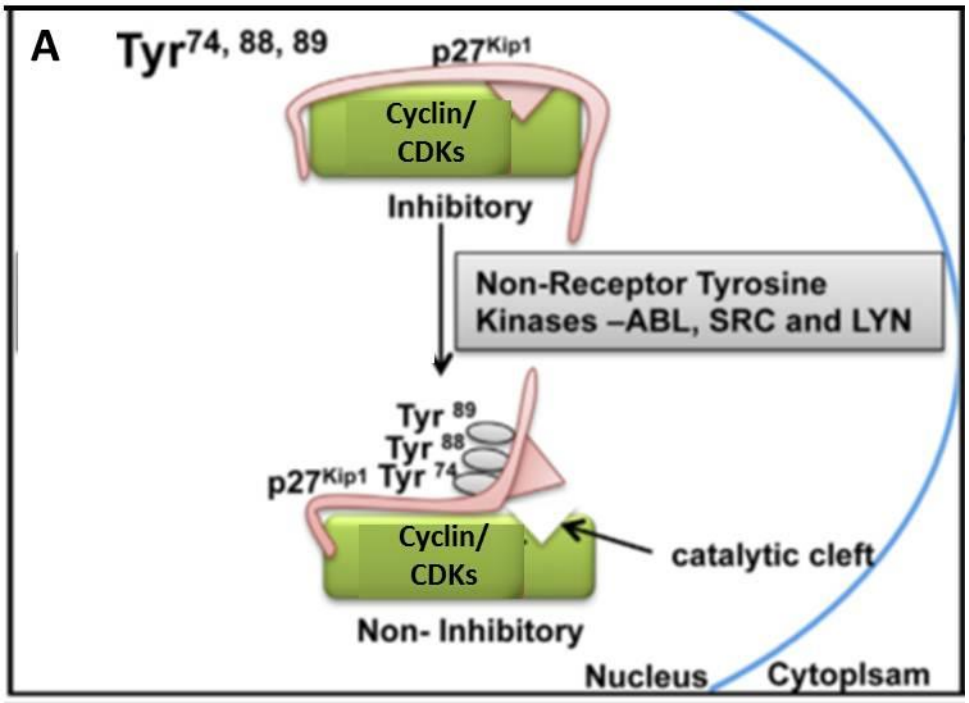

Fig. 4 Effect of tyrosine phosphorylations on $p27^{Kip1}$ inhibitory functions.

[99] Brinkkoetter PT, Olivier P, Wu JS, Henderson S, Krofft RD, Pippin JW, Hockenbery D, Roberts JM, Shankland SJ. "Cyclin I activates Cdk5 and regulates expression of Bcl-2 and Bcl-XL in postmitotic mouse cells". The Journal of Clinical Investigation (2009) 119;3089-101.

[100] Su SC, Tsai LH. "Cyclin-dependent kinases in brain development and disease". Annu Rev Cell Dev Biol. (2011) 27;465-91.

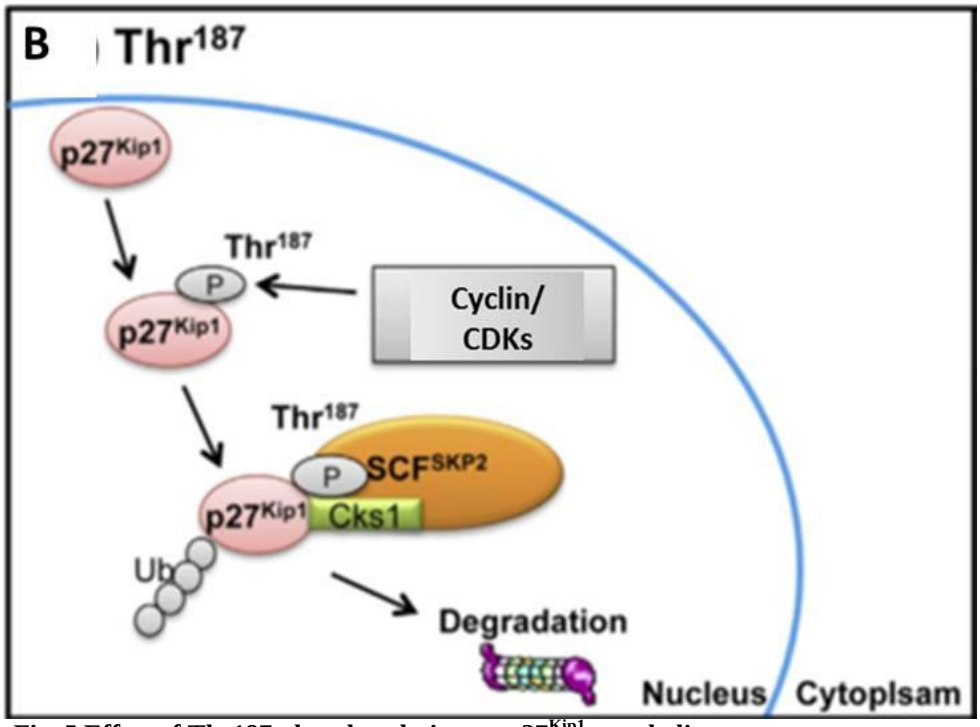

Fig. 5 Effect of Thr187 phosphorylation on $p27^{Kip1}$ metabolism.

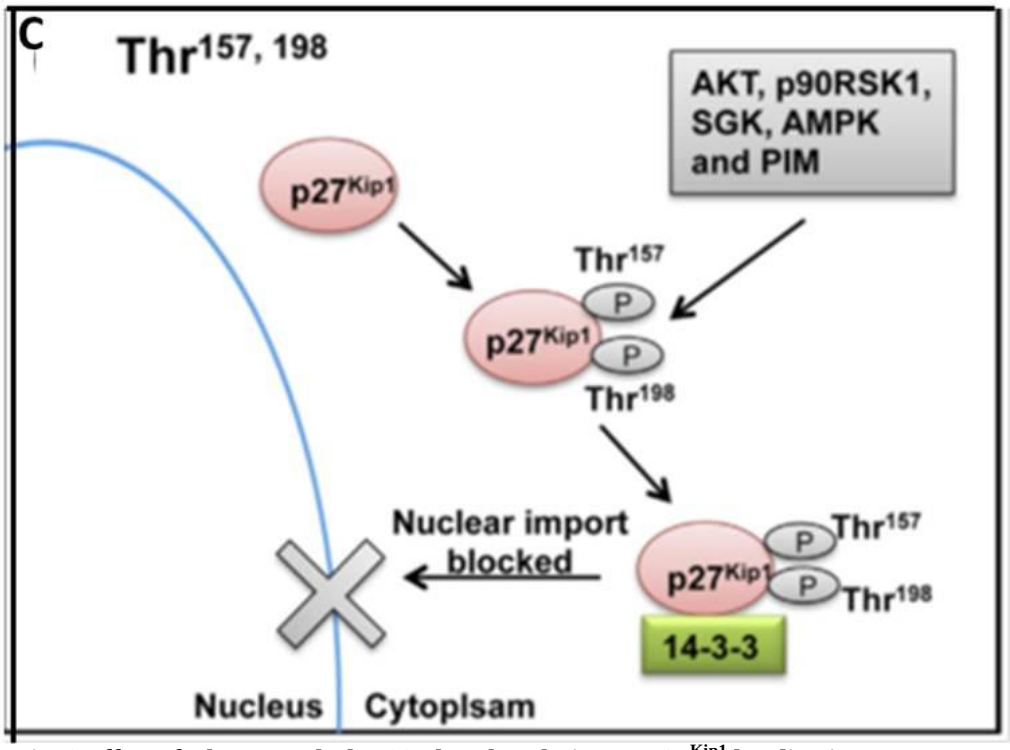

Fig. 6 Effect of Thr157 and Thr198 phosphorylation on $p27^{Kip1}$ localization.

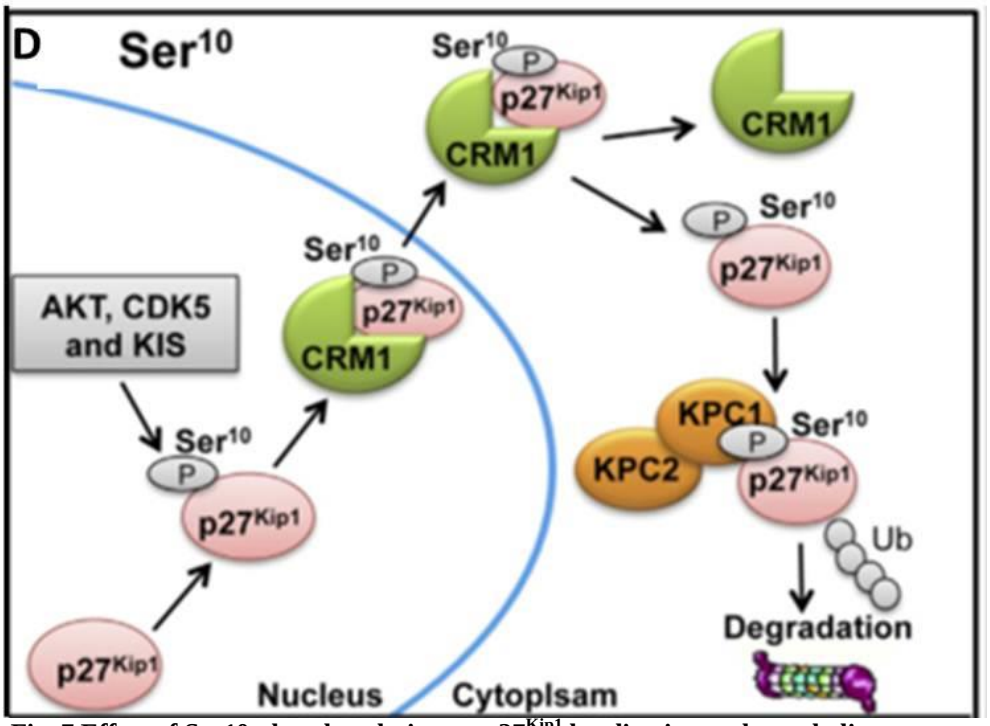

Fig. 7 Effect of Ser10 phosphorylation on p27^{Kip1} localization and metabolism.

1.5 p27^{Kip1} and cancer

p27^{Kip1} was previously considered only a key regulator of cell proliferation, which explicates its activity mainly interfering with cyclin/CDK complexes, suggesting a role as tumor suppressor. Consistent with this view, many tumors show CKI decreased levels due to its impaired synthesis or accelerated degradation, indicating that p27^{Kip1} expression levels could have both prognostic and therapeutic implications (Fig. 8). However, in the last decade, p27^{Kip1} has emerged both as an oncosoppressor and as a potential oncogene. As described previously, while p27^{Kip1} opposes cell cycle progression by binding to and inhibiting cyclin/CDK complexes, phosphorylation on Thr157 or Thr198 promotes the assembly and the nuclear translocation of D-type cyclin/CDKs; and phosphorylation on Ser10 promotes p27^{Kip1} mislocalization in the cytoplasmatic compartment, where it can play important role in cell motility and migration. In addition, in human cancers, also the oncogenic activation of mitogenic signaling (as PI3K signaling pathway) results in p27^{Kip1} cytoplasmic translocation. Thus, because of its anti-oncogenic and, at the same time, pro-oncogenic properties, p27^{Kip1} was defined by Slingerland and co-workers as "Dr. Jekyll and Mr. Hyde"[101]. Now we are going to describe some aspects of p27^{Kip1} involvement in cancer.

[101] Larrea MD, Wander SA, Slingerland JM. "p27 as Jekyll and Hyde: regulation of cell cycle and cell motility". Cell Cycle (2009) 8;3455-61.

p27^{Kip1} deregulation in human cancers. Normal human epithelial cells express p27^{Kip1} elevated levels in the nucleus. In several human cancers as breast, colon, lung, prostate carcinomas, lymphomas and gliomas, instead, it has been shown decreased p27^{Kip1} protein levels, even if mRNA levels remain normal. p27^{Kip1} low levels in primary tumors have been associated with reduced time to disease relapse and/or reduced patient survival[100;102]. Initial works proposed that an increased proteolysis was the principle cause of p27^{Kip1} loss in human tumors[103], however recent findings suggest that a reduction in p27^{Kip1} translation might contribute to the CKI down-regulation in cancers[104]. le Sage et al. have demonstrated that two miRNAs (miR221 and miR222) are involved in p27^{Kip1} translational inhibition in different human tumors, specifically glioblastoma, papillary thyroid carcinoma, pancreatic adenocarcinoma, colon and stomach cancers and chronic lymphocytic leukemia[100;105]. Thus, p27^{Kip1} protein reduction in malignancies, may be due to: accelerated proteolysis; miR-mediated inhibition of translation, or both these processes. Mechanisms of CKI translational regulation are still not well defined, however have been proposed some hypotheses to explain the accelerated p27^{Kip1} degradation. For example, several cancers show an increased Skp2 expression or, alternatively, some kinases (as Src) could be up-regulated increasing p27^{Kip1} tyrosine phosphorylation and its proteasomal degradation[35;106].

Some cancers, however, present high p27^{Kip1} levels, this can be due to p27^{Kip1} sequestering in cyclin D/CDK4 complexes. As described before, this interaction is required for the cytosolic formation of the active complex and the subsequent translocation into the nucleus. However, an excessive upregulation of cyclin D/CDK4 can sequester p27^{Kip1} in a ternary complex, hindering its ability to bind and inhibit cyclin E/CDK2. Moreover, cyclin D2/CDK4 complex can induce p27^{Kip1} export from the nucleus to the cytosol, thus allowing p27^{Kip1} degradation[35;107].

In several human tumors, it has been also described an increased p27^{Kip1} cytoplasmic translocation. Specifically, in breast cancer p27^{Kip1} (mainly pThr198p27^{Kip1}) is mislocalized in the cytoplasm in association with Akt activation; in breast carcinomas, acute myelogenous

[102] Tsihlias J, Kapusta L, Slingerland J. "The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer". Annu Rev Med (1999) 50;401-23.
[103] Slingerland J, Pagano M. "Regulation of the cdk inhibitor p27 and its deregulation in cancer". Journal of Cellular Physiology (2000) 183;10-7.
[104] le Sage C, Nagel R, Agami R. "Diverse ways to control p27Kip1 function: miRNAs come into play". Cell Cycle (2007) 6;2742-9.
[105] le Sage C, Nagel R, Egan DA, Schrier M, Mesman E, Mangiola A, Anile C, Maira G, Mercatelli N, Ciafrè SA, Farace MG, Agami R. "Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation". EMBO (2007) 26; 3699-708.
[106] Hershko DD. "Oncogenic properties and prognostic implications of the ubiquitin ligase Skp2 in cancer". Cancer (2008) 112; 1415-24.
[107] Susaki E, Nakayama K, Nakayama KI. "Cyclin D2 translocates p27 out of the nucleus and promotes its degradation at the G0-G1 transition". Molecular and Cellular Biology (2007) 27; 4626-40.

leukemia, pancreatic cancer and ovarian carcinomas an increase of $p27^{Kip1}$ cytoplasmic localization is correlated with a poor prognosis[82;100].

$p27^{Kip1}$ and cell motility. Directed cell migration is a process essential for embryonic development, tissue repair and regeneration, immune responses, angiogenesis and tumor metastasis[100]. In several cell types, $p27^{Kip1}$ accumulation in the cytoplasm increases cell motility, whereas, in other cell models, it exerts a negative effect on cell migration. This opposite CKI role could be due to $p27^{Kip1}$ interaction with distinct targets and might have important consequences in cancer development and therapy[35].

Rho GTPases is a protein family principally involved in cell motility regulation, cytokinesis and morphology. It includes 23 members of which RhoA, Rac and Cdc42 are the best characterized. RhoA exists in two states: in a GDP-bound inactive state and a GTP-bound active state. RhoA-GTP activates Rho-kinases, ROCK1 and ROCK2, which subsequent activate LIM domain-containing protein kinase (LIMK). LIMK phosphorylates and inhibits the actin depolymerization factor, cofilin (Fig. 9). Thus, the activation of this signaling determines an increase in actin polymerization and in the assembly and stability of focal adhesions. Conversely, the inhibition of RhoA pathway leads to a decrease in stress fibers and focal adhesions stabilization and to a subsequent increase in cell motility[100;108]. Cytosolic $p27^{Kip1}$ is able to bind to RhoA and prevent its activation by GEFs, therefore an increase of $p27^{Kip1}$ cytoplasmic levels can result in enhanced cell motility. Nagahara et al. were the first to show that cytoplasmic $p27^{Kip1}$ promoted cell migration[109] and then McAllister et al. demonstrated that this function was dependent on the CKI C-terminal domain[33;100]. Then, Besson and co-workers, showed that $p27^{Kip1}$ null mouse embryonic fibroblasts (MEFs) have reduced motility compared to wild type MEFs and re-expression of $p27^{Kip1}$ rescued the motility defect of $p27^{Kip1}$ null MEFs. They also showed that re-expression of $p27^{Kip1}$ mutant unable to bind cyclins and CDKs ($p27^{Kip1}$CK-) restored cell motility, suggesting that $p27^{Kip1}$ effect on cell migration was not dependent on its cell cycle role. In the same study, Besson and colleagues demonstrated by immunoprecipitation experiments, that $p27^{Kip1}$ interacts directly with RhoA preventing the binding with GEF interactors and leading to the protein inhibition[55;100]. Further investigations reported that $p27^{Kip1}$ with mutations in the NLS domain (and thus with a cytosolic localization) decreases RhoA activation and

[108] Bar-Sagi D, Hall A. "Ras and Rho GTPases: a family reunion". Cell (2000) 103;227-38.

[109] Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevsky SA, Dowdy SF. "Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration". Nature Medicine (1998) 4;1449-52.

increases cell motility in MCF-7 breast cancer cells[100;110]. Moreover, cytoplasmic expression of $p27^{Kip1}$ in murine melanoms models, causes an enhanced cell migration and also an increased capability of the tumor to metastasize[100;111]. Recently, Larrea and colleagues, demonstrate that phosphorylation on Thr198 by p90 ribosomal S6 kinase (RSK1) induces cytoplasmic $p27^{Kip1}$ accumulation and enhances motility of melanoma cell lines through RhoA/ROCK inhibition[112]. In conclusion, all these evidences support the hypothesis that increased $p27^{Kip1}$ cytoplasmic levels improve cell migration and favor the metastasization process.

However, in contrast to the reported findings, different studies have demonstrated that cytoplasmic $p27^{Kip1}$ can also inhibit cell migration. This effect has been observed in vascular smooth muscle cells, umbilical vein endothelial cells, neurons and oral cancer cells. In glioblastoma cell lines, Schiappacassi et al. demonstrated that $p27^{Kip1}$ overexpression induces cell cycle arrest and inhibition of cell motility, invasion and tumor-induced neoangiogenesis[35;113]. Cytosolic $p27^{Kip1}$ antimigratory activity depends on its ability to interact with and inhibit the microtubule-destabilizing protein stathmin. In particular, cytosolic $p27^{Kip1}$ upregulation or downregulation of stathmin levels result in the inhibition of mesenchymal cell motility. Conversely, high stathmin and low cytoplasmic $p27^{Kip1}$ expression correlate with the metastatic phenotype of human sarcomas *in vivo*[35;57]. Belletti et al. reported that the interaction between $p27^{Kip1}$ and stathmin also influences morphology and motility of fibroblasts in three-dimensional matrices. When cells lacking $p27^{Kip1}$ are immersed in 3D environments, they show reduced microtubule stability, rounded shape and a mesenchymal-amoeboid conversion. These modification in $p27^{Kip1}$ null cells were determined by the concomitant genetic stathmin ablation, suggesting that a balanced expression of $p27^{Kip1}$ and stathmin is critical for the cytoskeletal organization[35;114].

Thus, it is clear that cytosolic $p27^{Kip1}$ significantly modulates cell motility, but its effect is not definitely clarified, suggesting that $p27^{Kip1}$ activity is strongly dependent on cell phenotype[35].

---

[110] Wu FY, Wang SE, Sanders ME, Shin I, Rojo F, Baselga J, Arteaga CL. "Reduction of cytosolic p27(Kip1) inhibits cancer cell motility, survival, and tumorigenicity". Cancer Research (2006) 7;51-63.

[111] Denicourt C, Saenz CC, Datnow B, Cui XS, Dowdy SF. "Relocalized p27Kip1 tumor suppressor functions as a cytoplasmic metastatic oncogene in melanoma". Cancer Research (2007) 67;9238-43.

[112] Larrea MD, Hong F, Wander SA, da Silva TG, Helfman D, Lannigan D, Smith JA, Slingerland JM. "RSK1 drives p27Kip1 phosphorylation at T198 to promote RhoA inhibition and increase cell motility". PNAS (2009) 106;9268-73.

[113] Schiappacassi M, Lovat F, Canzonieri V, Belletti B, Berton S, Di Stefano D, Vecchione A, Colombatti A, Baldassarre G. "p27Kip1 expression inhibits glioblastoma growth, invasion, and tumor-induced neoangiogenesis". Molecular Cancer Therapeutics (2008) 7;1164-75.

[114] Belletti B, Pellizzari I, Berton S, Fabris L, Wolf K, Lovat F, Schiappacassi M, D'Andrea S, Nicoloso MS, Lovisa S, Sonego M, Defilippi P, Vecchione A, Colombatti A, Friedl P, Baldassarre G. "p27kip1 controls cell morphology and motility by regulating microtubule-dependent lipid raft recycling". Molecular and Cellular Biology (2010). 30;2229-40.

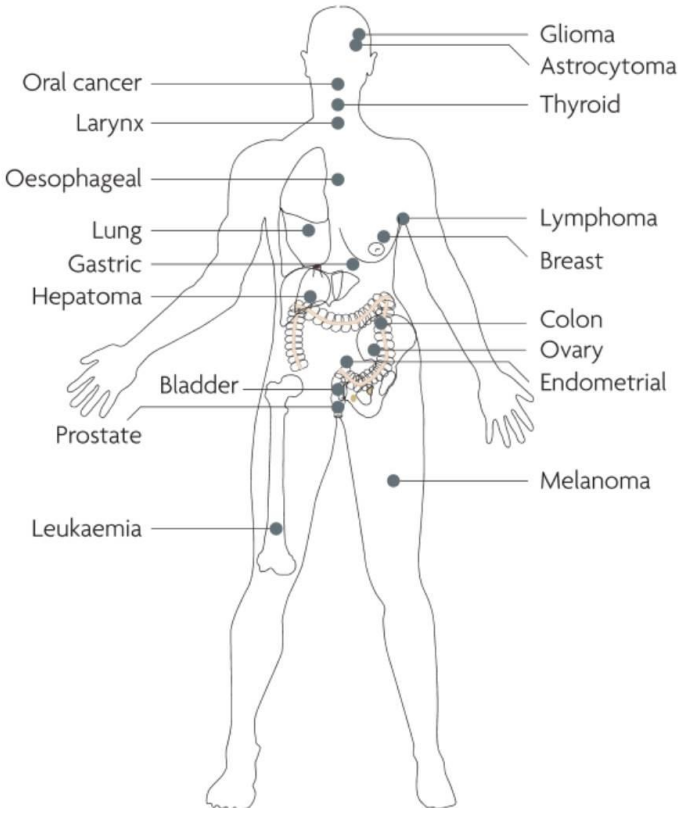

Fig. 8 p27^{Kip1} expression levels and cancer[40]

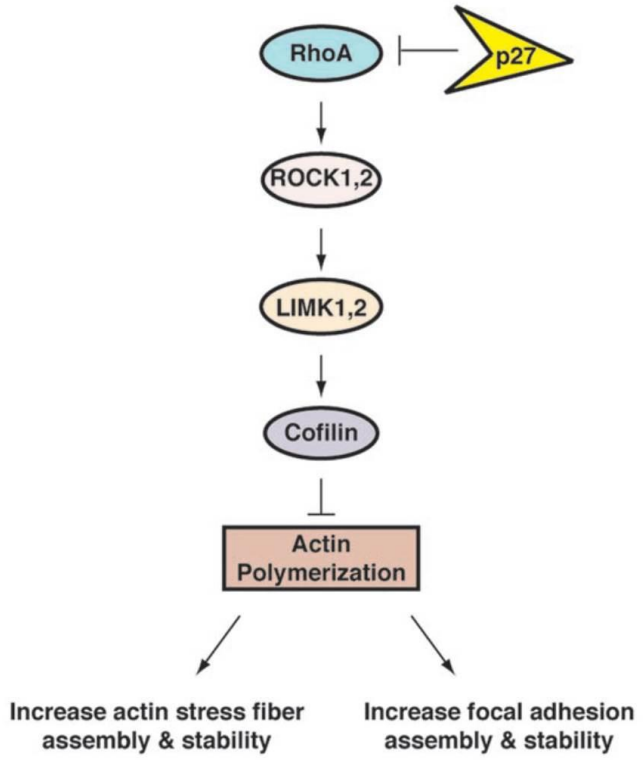

Fig. 9 p27^{Kip1} inhibition of RhoA signaling[100]

1.5.1 p27^{Kip1} mutation in human tumors

Differently from other tumor suppressors, *CDKN1B* was initially reported as rarely inactivated in human cancers. However, more recent investigations are subverting this initial hypothesis. Monoallelic inactivation of *CDKN1B*, in fact, has been demonstrated to increase significantly cancer development. *CDKN1B* haploinsufficiency seems caused mainly by deletion or frameshift; missense changes, instead, have been identified, but their effects on the protein dosage/function have been poorly characterized. Furthermore it seems that mutated p27^{Kip1} cannot function as a dominant negative[115].

Data supporting the importance of *CDKN1B* haplosinsufficiency were initially shown in 1998 by Fero and co-workers in a study on *CDKN1B* knockout mice and were then confirmed by others[116]. Recently, *CDKN1B* was demonstrated inactivated in several human tumors as: parathyroid adenomas[117], MEN4[118], small intestine neuroendocrine tumors (SI-NET)[119], pancreatic neuroendocrine tumors (PNET)[120], luminal breast cancer[121] and hairy cell leukemia[122]. Moreover, although the importance of *CDKN1B* mutations in human cancers is now evident, the mechanisms by which these alterations drive carcinogenesis are still obscure[114].

CDKN1B mutations in MEN syndroms. MEN are autosomal dominant syndromes characterized by the manifestation of tumors involving two or more neuroendocrine glands. The

[115] Borriello A, Bencivenga D, Della Ragione F. "The unpredictable consequences of CDKN1B/p27Kip1 mutations in cancer". Cell Cycle (2015) 14;1-2.

[116] Fero ML, Randel E, Gurley KE, Roberts JM, Kemp CJ. "The murine gene p27Kip1 is haplo-insufficient for tumour suppression". Nature (1998) 396;177-80.

[117] Costa-Guda J, Marinoni I, Molatore S, Pellegata NS, Arnold A. "Somatic mutation and germline sequence abnormalities in CDKN1B, encoding p27Kip1, in sporadic parathyroid adenomas". Journal of Clinical Endocrinology and Metabolism (2011) 96;E701-E706.

[118] Marinoni I, Pellegata NS. "p27kip1: a new multiple endocrine neoplasia gene?" Neuroendocrinology (2011) 93;19-28.

[119] Francis JM, Kiezun A, Ramos AH, Serra S, Pedamallu CS, Qian ZR, Banck MS, Kanwar R, Kulkarni AA, Karpathakis A, Manzo V, Contractor T, Philips J, Nickerson E, Pho N, Hooshmand SM, Brais LK, Lawrence MS, Pugh T, McKenna A, Sivachenko A, Cibulskis K, Carter SL, Ojesina AI, Freeman S, Jones RT, Voet D, Saksena G, Auclair D, Onofrio R, Shefler E, Sougnez C, Grimsby J, Green L, Lennon N, Meyer T, Caplin M, Chung DC, Beutler AS, Ogino S, Thirlwell C, Shivdasani R, Asa SL, Harris CR, Getz G, Kulke M, Meyerson M. "Somatic mutation of CDKN1B in small intestine neuroendocrine tumors". Nature Genetics (2012) 45;1483-6.

[120] Maxwell JE, Sherman SK, Li G, Choi AB, Bellizzi AM, O'Dorisio TM, Howe JR. "Somatic alterations of CDKN1B are associated with small bowel neuroendocrine tumors". Cancer Genetics (2015) 208;564-570.

[121] Stephens PJ, Tarpey PS, Davies H, Van Loo P, Greenman C, Wedge DC, Nik-Zainal S, Martin S, Varela I, Bignell GR, Yates LR, Papaemmanuil E, Beare D, Butler A, Cheverton A, Gamble J, Hinton J, Jia M, Jayakumar A, Jones D, Latimer C, Lau KW, McLaren S, McBride DJ, Menzies A, Mudie L, Raine K, Rad R, Chapman MS, Teague J, Easton D, Langerød A; Oslo Breast Cancer Consortium (OSBREAC), Lee MT, Shen CY, Tee BT, Huimin BW, Broeks A, Vargas AC, Turashvili G, Martens J, Fatima A, Miron P, Chin SF, Thomas G, Boyault S, Mariani O, Lakhani SR, van de Vijver M, van 't Veer L, Foekens J, Desmedt C, Sotiriou C, Tutt A, Caldas C, Reis-Filho JS, Aparicio SA, Salomon AV, Børresen-Dale AL, Richardson AL, Campbell PJ, Futreal PA, Stratton MR. "The landscape of cancer genes and mutational processes in breast cancer". Nature (2012) 486;400-4.

[122] Dietrich S, Hüllein J, Lee SC, Hutter B, Gonzalez D, Jayne S, Dyer MJ, Oleś M, Else M, Liu X, Słabicki M, Wu B, Troussard X, Dürig J, Andrulis M, Dearden C, von Kalle C, Granzow M, Jauch A, Fröhling S, Huber W,

two syndromes principally characterized are the MEN type 1 (MEN1) and the MEN type 2 (MEN2). MEN1 is caused by loss-of-function of the tumor suppressor gene *MEN1*, and affected patients develop multiple parathyroid adenomas, pancreatic islet cell neoplasia, and anterior pituitary adenomas. MEN2 is caused by activating germline mutations in the *RET* proto-oncogene and the two subtypes are MEN2A and MEN2B. The first is characterized by the onset of medullary thyroid carcinoma (MTC), pheochromocytoma and parathyroid adenomas; the second (also known as MEN3), causes MTC, pheochromocytoma, marphanoid habitus and mucosal and digestive ganglioneuromatosis[117].

Recently, a MEN syndromes variant has been discovered in rats and named MENX, affected animals develop endocrine tumors that overlap the spectrum of human MEN1 and MEN2, but they do not present germline mutations in the canonical genes. A germline loss-of-function mutation in *CDKN1B* has been identified, with linkage analysis followed by positional cloning, as the gene responsible of this new syndrome. Affected rats carry a tandem duplication of eight nucleotides in exon 2 of *CDKN1B*, which causes a frameshift. At protein level, the mutated allele encodes a protein with a novel C-terminal sequence starting at codon 177 ($p27^{Kip1}$fs177). The wild-type $p27^{Kip1}$ ($p27^{Kip1}$wt) protein is 198 amino acids long, whereas the mutated protein, as a result of the frameshift, is predicted to be 221 amino acids long. This *CDKN1B* mutation was identified in homozygosity in all MENX-affected rats tested, and it was never observed in unaffected littermates[123;124]. To understand the possible roles of mutated *CDKN1B* in MENX development, functional *in vitro* studies have been done. These analyses have demonstrated that $p27^{Kip1}$fs177 mutant protein retains some properties of the $p27^{Kip1}$wt: it can localize into the nucleus and interact with CDK2, CDK4 and with cyclins. However, differently from $p27^{Kip1}$wt, the mutant is more unstable and it is rapidly degraded, at least in part, with a Skp2-dependent proteasomal proteolysis. The $p27^{Kip1}$-unrelated C-terminal domain of $p27^{Kip1}$fs177 is responsible for this rapid degradation, probably through protein misfolding[125].

Heterozygous germline mutations in the human homologue *CDKN1B* gene have been identified in few patients presenting multiple endocrine tumors and no germline mutations in *MEN1* and *RET* genes. Thus, these findings lead to the identification of a novel MEN syndrome, named MEN4 (OMIM No. 610755) characterized by mutations in *CDKN1B* and by the development of

---

Meggendorfer M, Haferlach T, Ho AD, Richter D, Brors B, Glimm H, Matutes E, Abdel Wahab O, Zenz T. "Recurrent CDKN1B (p27) mutations in hairy cell leukemia". Blood (2015) 126;1005-8.

[123] Pellegata NS. "MENX and MEN4". Clinics (2012) 67;13-18.

[124] Pellegata NS, Quintanilla-Martinez L, Siggelkow H, Samson E, Bink K, Höfler H, Fend F, Graw J, Atkinson MJ. "Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans". PNAS (2006) 103;15558-63.

[125] Molatore S, Kiermaier E, Jung CB, Lee M, Pulz E, Höfler H, Atkinson MJ, Pellegata NS. Characterization of a naturally-occurring p27 mutation predisposing to multiple endocrine tumors". Molecular Cancer (2010) 9;116.

pituitary tumors in affected patients. In 2006, Pellegata and colleagues screened for the presence of *CDKN1B* germline mutations several patients. They identified a germline heterozygous TGG<TAG nonsense mutation at codon 76 ($p27^{Kip1}$W76X) in a female proband presenting symptoms characteristic of the MEN1 spectrum[123]. The mutated protein has lost its NLS and so it is mislocalized in the cytoplasm (as demonstrated with both *in vitro* and *in vivo* experiments). $p27^{Kip1}$W76X cannot bind to and inhibit cyclin/CDK complexes and as a consequence, cell growth is not decreased.

Other germline mutations have been identified in patients with features suggestive of MEN1. Georgitsi et al. identified a heterozygous 19-bp duplication in exon 1 of the *CDKN1B* gene. The duplication causes a frameshift, and the variant mRNA is predicted to encode a $p27^{Kip1}$ protein 69 amino acids shorter than $p27^{Kip1}$wt and with a different amino acid sequence after codon 25 ($p27^{Kip1}$K25fs)[122;126].

Subsequently, three new germline *CDKN1B* changes were identified by Agarwal and co-workers: ATG-7(g<c); c.283C<T ($p27^{Kip1}$Pro95Ser); and c.592G<G (stop.Gln, stop.Q). ATG-7(g<c) change affects the Kozac consensus sequence, which plays a major role in the initiation of mRNA translation. The mutation reduces the translation efficiency of the variant allele, which, subsequently, determines a reduction in $p27^{Kip1}$ amount. $p27^{Kip1}$P95S variant, instead, causes a change in Grb2 binding site, inheriting $p27^{Kip1}$ binding to the adaptor protein. This may ultimately impair the activation of Ras signal transduction[127].

Molatore et al identified a heterozygous germline change (c.206C<T) which, at the protein level, substitutes a Pro with a Leu at codon 69 ($p27^{Kip1}$P69L)[128]. The mutated residue is located in KID domain, so it seems that $p27^{Kip1}$P69L binds to CDK2 with lower affinity and that it is less efficient then $p27^{Kip1}$wt to inhibit cell growth. Furthermore, *in vitro* the mutant protein is more unstable and it is expressed at reduced levels both in transfected cells and in mutation-positive patients.

CDKN1B mutations in NETs and endocrine disease. NETs are rare neoplasms that develop from endocrine precursor cells and occur mostly in lung, pancreas, and small intestine. *MEN1*

[126] Georgitsi M, Raitila A, Karhu A, van der Luijt RB, Aalfs CM, Sane T, Vierimaa O, Mäkinen MJ, Tuppurainen K, Paschke R, Gimm O, Koch CA, Gündogdu S, Lucassen A, Tischkowitz M, Izatt L, Aylwin S, Bano G, Hodgson S, De Menis E, Launonen V, Vahteristo P, Aaltonen LA. "Germline CDKN1B/p27Kip1 mutation in multiple endocrine neoplasia". The Journal of Clinical Endocrinology and Metabolism (2007) 92;3321-5.

[127] Agarwal SK, Mateo CM, Marx SJ. "Rare germline mutations in cyclin-dependent kinase inhibitor genes in multiple endocrine neoplasia type 1 and related states". The journal of Clinical Endocrinology and Metabolism (2009) 94;1826-34.

[128] Molatore S, Marinoni I, Lee M, Pulz E, Ambrosio MR, degli Uberti EC, Zatelli MC, Pellegata NS. "A novel germline CDKN1B mutation causing multiple endocrine tumors: clinical, genetic and functional characterization". Human Mutation (2010) 31;E1825-35.

germline and somatic mutations are frequent in some kinds of NETs, especially lung and pancreatic[118]. SI-NET is one of the most known small intestinal neoplasia and it is characterized by the secretion of monoamines as serotonin or peptide hormones. These hormones secretion can led to the development of carcinoid syndrome, that represents the principal cause of morbidity and mortality in affected patients. Moreover, SI-NETs present relatively low cellular proliferation but they are also characterized by a strong invasive capability[129]. Recently Francis et al identified through exome/genome sequence analysis of SI-NETs, some heterozygous somatic mutations and genomic deletions of *CDKN1B* gene. It seems that *CDKN1B* haploinsufficiency lead to loss of its tumor suppressor function, suggesting a possible role of 27^{Kip1} in SI-NET cell cycle regulation and pathogenesis[118]. As in other types of NETs, $p27^{Kip1}$ is reportedly important in the progression of pancreatic islet cells into the S phase of the cell cycle. Disruption of this pathway could lead to PNET tumorigenesis. Recently Maxwell et al. screened a population of patients affected by small bowel neuroendocrine tumors (SB-NET) and PNET . They found both in SI-NET primary tumors and in their metastasis, frameshift and missense mutations and hemizygous deletions and duplications of *CDKN1B* gene; instead in PNET primary tumors and metastasis they do not found *CDKN1B* frameshift mutations. Immunohistochemistry analysis of patients tissues revealed that mutated $p27^{Kip1}$ localizes mainly in the cytoplasm compared to the nucleus. However in one missense mutation found in PNET, $p27^{Kip1}$ presents a prevalent nuclear localization in both primary tumor and lymph node metastasis. Thus, also these investigations let suppose that *CDKN1B* mutations and copy number variants could be involved in NETs alterations of cell cycle control and that these aberrations could have implications for new treatment modalities[119].

In addition to patients with a MEN1-like phenotype, also patients which present only a predisposition to sporadic parathyroid adenomas or to pituitary adenomas may have alterations in *CDKN1B* gene. Recently, Costa-Guida et al, found two novel germline *CDKN1B* mutations analyzing 90 common sporadic parathyroid adenomas with non-familial presentation. Specifically, one patient presented the heterozygous single nucleotide substitution c.397C<A, directing a Pro133Thr ($p27^{Kip1}$P133T) in the translated protein. Another patient carried a heterozygous germline single nucleotide change c.25G<A in *CDKN1B* exon 1, which results in a Gly9Arg ($p27^{Kip1}$G9R) substitution in the translated protein. Gly in position 9 of $p27^{Kip1}$ is highly conserved across species, and its substitution to Arg might affect phosphorylation on the adjacent Ser10 residue, influencing $p27^{Kip1}$ subcellular localization and stability. Costa-Guida *in*

[129] Crona J, Gustavsson T, Norlén O, Edfeldt K, Åkerström T, Westin G, Hellman P, Björklund P, Stålberg P. "Somatic Mutations and Genetic Heterogeneity at the CDKN1B Locus in Small Intestinal Neuroendocrine Tumors". Annals of Surgical Oncology (2015).

vitro experiments show a p27^{Kip1}G9R lower stability then wild type protein, which in turn determines p27^{Kip1} low levels in tumor tissue of mutation carrier individuals[116].

Finally, sequence analysis of 124 affected individuals which belonged to a familial pituitary adenoma (FIPA) families, that did not present any mutations in the AIP gene (the canonical gene responsible of this neoplasia), revealed the presence of a point mutation in *CDKN1B* gene: c.356T<C, which results in the substitution of an Ile to a Thr (p27^{Kip1}I119T). p27^{Kip1}I119T analysis through polyacrylamide electrophoresis reveled a slower migration pattern in the mutated protein compared to p27^{Kip1}WT. This different pattern does not seem to be caused by an increased phosphorylation at Thr119, but it could be the consequence of other post translational modification as glycosylation, which in turn could confer more stability to the protein[130;131].

The interplay between cancer and *CDKN1B* haploinsufficiency should, however, be clarified in more details to understand how *CDKN1B* mutations could affect p27^{Kip1} level, post translational modifications, localization and function, and subsequently, how these aberrations could influence not only cell cycle regulation, but also tumorigenesis, in order to open novel opportunities for therapeutic strategy.

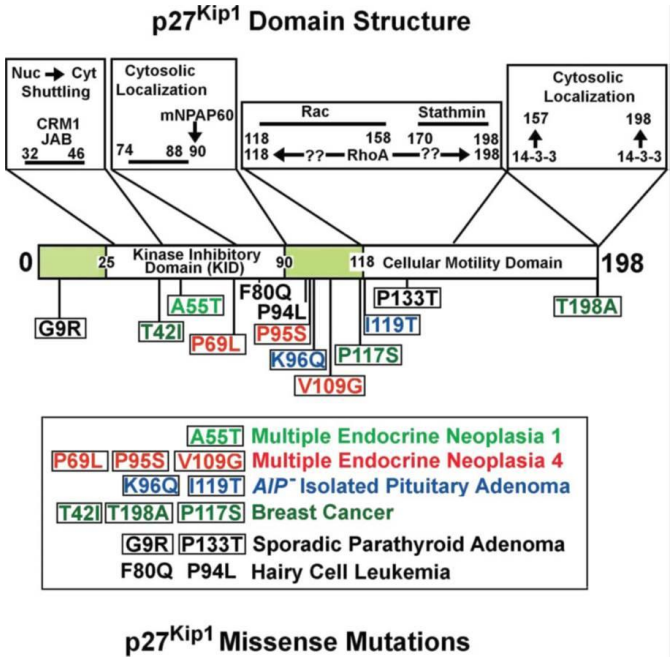

Fig. 10 Principal CDKN1B mutations identified in human tumors[114].

[130] Lee M, Pellegata NS. "Multiple endocrine neoplasia syndromes associated with mutation of p27". The Journal of Endocrinological Investigation (2013) 36;781-7.

[131] Tichomirowa MA, Lee M, Barlier A, Daly AF, Marinoni I, Jaffrain-Rea ML, Naves LA, Rodien P, Rohmer V, Faucz FR, Caron P, Estour B, Lecomte P, Borson-Chazot F, Penfornis A, Yaneva M, Guitelman M, Castermans E, Verhaege C, Wémeau JL, Tabarin A, Fajardo Montañana C, Delemer B, Kerlan V, Sadoul JL, Cortet Rudelli C, Archambeaud F, Zacharieva S, Theodoropoulou M, Brue T, Enjalbert A, Bours V, Pellegata NS, Beckers A. "Cyclin-dependent kinase inhibitor 1B (CDKN1B) gene variants in AIP mutation-negative familial isolated pituitary adenoma kindreds". Endocrine Related Cencer (2012) 19;233-41.

2. AIMS OF THE STUDY

$p27^{Kip1}$ is a protein characterized by an elevated percentage of post translational modifications, principally phosphorylation, that regulate its levels, cellular localization and functions. The relevance of $p27^{Kip1}$ post translational changes is also strengthened by the absence of a well-defined tertiary structure. Moreover, several evidence suggest that phosphorylations could shift $p27^{Kip1}$ tumor suppressor properties to oncogenic activities and *vice versa*. Finally, a number of investigations have revealed that also mutations in $p27^{Kip1}$ encoding gene, namely *CDKN1B*, can be involved in different human tumors oncogenesis, especially in endocrine neoplasias.

In a previous study, the research group in which I developed my PhD thesis, characterized $p27^{Kip1}$ phosphoisoforms pattern in neuroblastoma cell line, confirmed that $pSer10p27^{Kip1}$ is the most quantitatively abundant $p27^{Kip1}$ phosphoisoform and found the occurrence of CKI mono- and bi-phosphorylated derivatives. Since $pSer10p27^{Kip1}$ represents, in some circumstances, about 30-40 % of the total nuclear $p27^{Kip1}$ content, we decided to evaluate its metabolism and interactions in the nucleus.

The first aim of this research has been to investigate the possible causes of $pSer10p27^{Kip1}$ abundance. Thus, we took into consideration the main mechanism that control nuclear $p27^{Kip1}$ levels, i.e. ubiquitin-proteasomal dependent degradation pathway. To examine this pathway, we evaluated whether phosphorylation on serine 10 could also allow a phosphorylation on pThr187. Phosphorylation on Thr187 is, in fact, a prerequisite for $p27^{Kip1}$ nuclear degradation. Furthermore, considering $pSer10p27^{Kip1}$ high levels, its consistent nuclear localization, and the increased stability that it confers to $p27^{Kip1}$, we have investigated the possibility that this phosphorylation could be important in modulating the interaction of the CKI with specific cyclin/CDK complexes.

The second aim of this research has been to analyze the possibility that Ser10 phosphorylation play a role in the metabolism and function of $p27^{Kip1}$ mutant forms recently identified in human cancers. Specifically we focalized our attention on $p27^{Kip1}$G9R, because residue change (glycine 9 into arginine) is adjacent to Ser10 residue. Thus, through site-directed mutagenesis and 2D/WB approaches, we characterized $p27^{Kip1}$G9R post translational modifications and investigate their possible role in mutant metabolism and activity.

3. MATERIALS AND METHODS

3.1 Reagents and antibodies

All-trans-retinoic acid (ATRA), phenylmethylsulphoniyl fluoride (PMSF), reduced dithiothreitol (DTT), leupeptin, trypsin inhibitor, sodium ortho-vanadate (Na3VO4), sodium fluoride (NaF), sodium pyrophosphate, Igepal CA-630, Mg132, isopropyl-ß-galattosyltiopiranoside (IPTG), 3-[(3-Chloramidopropyl)dimethylammonium]-1-propanolsulphonate (CHAPS), Protein A sepharose and anti-actin rabbit polyclonal antibody were supplied by Sigma Chemical Company (St Louis, MO, USA). Substrates for immune complexes detection by chemiluminescence (ECL: Enhanced ChemoLuminescence) was from Amersham Biosciences, (Buchs, UK). Epoxomicin, cycloheximide were from BIOMOL International, LP (Plymouth Meeting, PA). siRNA for Skp2 gene silencing was supplied by Applied Biosystems (Ambion, 2130 Woodward St. Austin, USA). Transfectant agent Turbo-fect and cyclin E mAb were obtained from Thermo Fisher Scientific, USA. Human wild type $p27^{Kip1}$ coding sequence, cloned into the pcDNA3 plasmid, were gently given by Dr. Michele Pagano (Department of Pathology, New York University School of Medicine and New York University Cancer Institute, New York, NY, USA). QuikChange II Site-Directed Mutagenesis Kit was from Agilent Technologies (Santa Clara, CA, USA). Gel strips "IPG strip pH 3-10" and oil "DryStrip cover fluid" were supplied by Amersham Pharmacia Biotech (Little Chalfont , UK). Mouse mAb anti-$p27^{Kip1}$ was provided from BD Transduction Laboratories (Franklin Lakes, NJ, USA).

Lambda protein phosphatase, rabbit pAbs directed against $p27^{Kip1}$(C19), HDAC1, CDK1, CDK2, CDK4, CDK6, CDK7, Skp2, phospho(T187) $p27^{Kip1}$, cyclin E, cyclin A, phospho(S10)$p27^{Kip1}$, RhoA, cofilin, mouse mAb PKM2, CDK2, CDK1, CDK5, cyclin D1, cyclin E, cyclin A, cyclin B1, pyruvate kinase M2 (PKM2), p-cofilin, not related pAb were provided by Santa Cruz Biotechnologies (Santa Cruz, CA, USA). pAb against pT198-$p27^{Kip1}$ were from Aviva System Biology (San Diego, CA, USA) and RD System (Minneapolis, MN, USA).

All other reagents, of the highest purity available, were obtained from commercial sources.

3.2 Cell cultures and treatments

Human neuroblastoma cell line Lan-5 was grown in 100 cm^2 plates in OptiMEM and RPMI 1640 culture medium (Invitrogen Corporation CA, USA) supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Mouse embryonic fibroblast cell line MEF (gently given by Dr. Marcos Malumbres, Cell Division and Cancer Group, Spanish National Cancer Research Centre, Madrid, Spain) was grown in 100 cm^2 plates in DMEM

culture medium (Invitrogen Corporation) supplemented with 10% bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Prostate cell line EPN was grown in 100 cm^2 plates in DMEM-F12 culture medium (Invitrogen Corporation) supplemented with 5% bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Mouse fibroblast cell line NIH3T3 was grown in 100 cm^2 plates in DMEM culture medium (Invitrogen Corporation) supplemented with 10% bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Chronic myeloid leukemia cell line K562 was grown in 100 cm^2 plates in RPMI culture medium (Invitrogen Corporation) supplemented with 10% bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). All cell lines were cultured in a humidified incubator at a temperature of 37° C and 5% CO_2. Cells were plated at low density (2.5 - 3.0 x 103 cells/cm^2) to avoid contact inhibition. Generally, cells were left in culture for 24 hours before the addition of ATRA (diluted from a stock solution in ethanol $5x10^{-3}$M), epoxomicine ($5x10^{-6}$M), cycloheximide ($3.6x\ 10^{-6}$M) and before transfection.

p27^{Kip1} half-life evaluation. To evaluate $p27^{Kip1}$ half-life and metabolism, Lan-5 and K562 were grown with ATRA (5μM) for 8 hours, with cycloheximide (36 μM), with Mg132 (1μM) and epoxomicin (5μM). To evaluate $p27^{Kip1}$WT and $p27^{Kip1}$G9R half-life and metabolism, NIH3T3 were treated, after 24 h of transfection (see below) with cycloheximide (36 μM) for 6 hours and then (in some experiments) with Mg132 (1μM) for other 2 hours. After treatments cells were harvested and processed as described below (i.e, analyzed by WB or 2D/WB).

Cell lines synchronization. 1) G0 phase: Lan-5 at 30-40% confluency were washed twice with 1X PBS (Phosphate Buffered Saline, 0.8% NaCl, 10 mM sodium phosphate pH 7.4, and 2.7 mM KCl) and fresh medium serum free was added. After 72h cells were collected. 2) G1 phase: after 72 hours of starvation, serum free medium was removed and Lan-5 were maintained for 3 hours with 10% serum medium to release cells and then collected. 3) S phase: Lan-5 at 30-40% confluency were wash twice with 1X PBS, and 5 mM thymidine was added to the medium. After 48h cells were collected. 4) G2/M phases: after thymidine treatment, thymidine-containing medium was removed, Lan-5 were washed twice with PBS1X and then medium without thymidine were added to release cells. After 15 hours cells were collected. Cell synchronization was evaluated by FACS analysis and cyclins blot .

3.3 Cell extracts preparation

Cell pellets were suspended in RIPA 1X lysis buffer (100 mM Tris-HCl pH 7.5, 300 mM NaCl, 2% deoxycholate, 2% Triton X-100, 0.2% SDS, 0.57 mM PMSF, 0.27 mM TPCK, 21 μM leupeptin, 0.83 μg/ml chymostatin, 10 μg/ml trypsin inhibitor, 0.5 mM DTT, 16 mM PNPP,

1mM Na_3VO_4, 40 mM NaF and 1 mM sodium pyrophosphate) and then left for 1 hour at 4°C. Samples were centrifuged at 16,000*g* for 10 minutes in order to remove cell debris, and the supernatants recovered and stored at -80°C.

3.4 Cell fraction preparation

Cytoplasmic and nuclear extracts were prepared suspending cell pellets in hypotonic lysis buffer 1X (10 mM Hepes pH 7,9, 1.5 mM $MgCl_2$, 10 mM KCl) for 15 min at 4°C. Then Igepal 0,06% were added and samples were centrifuged at 16,000 g for 30 sec. Samples supernatants which contain cytoplasmic fraction, were recovered and stored at -80°C. Samples pellets were suspended with lysis buffer 1X (50 mM Tris, 150 mM NaCl, 0,1% Igepal) for 45 min at 4°C. Samples were centrifuged at 16,000 *g* for 10 minutes in order to remove cell debris, and the supernatants which contain nuclear fraction were recovered and stored at -80°C.

The occurrence of cross-contamination were assessed by western blotting with anti-HDAC1 (nuclear marker) and PKM2 (cytoplasmic marker).

3.5 Protein concentration determination

Protein concentration was determined according to the method of Bradford (1976) using the reagent "Protein Assay Kit" of Bio-Rad Laboratories (Richmond, CA, USA). Calibration curve was constructed using serial dilutions of a solution of known concentration (1.5 mg/ml) of standard proteins (Bio-Rad).

3.6 Western blot analysis

Protein extracts were separated by electrophoresis on vertical polyacrylamide gel (SDS/PAGE) using a 12% resolving gel. After separation and electroblotting on a nitrocellulose filter, protein of interest were identified by specific antibodies and immunoreactive bands were detected by secondary HRP (horseradish peroxidase) conjugated antibodies and ECL detection method. After 1 minute of ECL reagent (Amersham Biosciences) incubation, membranes were exposed to an autoradiographic hyperfilm (Hyperfilm ECL, Amersham Biosciences) and immunoreactive bands were identified. Different exposure times were used to verify the reproducibility and proportionality of signals.

Western blot images obtained by scanning were acquired and processed by using Adobe Photoshop 3.0 installed on a Power Macintosh 5400/180. Bands intensity was determined using the program NIH Image 1.61.

3.7 Immunoprecipitation experiments

Immunoprecipitation experiments (IP) were realized incubating 0.05-4 mg of cell extract proteins with antibodies directed against the protein of interest overnight at 4 °C under mild stirring. Immune complexes were then bound to Protein A-Sepharose (Sigma) incubating protein-antibody mixture with blocked resin for 2 hours at room temperature (RT). Then, the mixture was centrifuged for 2 min. IP supernatants were removed and immunoprecipitates were suspended in the sample loading buffer for monodimensional SDS PAGE analysis (WB). When immunoprecipitated materials were analyzed by two-dimensional electrophoresis, immune complexes were eluted from the resin with buffer 100 mM Glycine-HCl pH 2.5. Proteins recovered in eluate were precipitated with 10% trichloroacetic acid (TCA). After 40 min at 4°C, samples were centrifuged at 16,000 g for 20 minutes and then TCA was removed. Precipitated proteins were washed twice with cold acetone and pellets were suspended in specific two-dimensional analysis buffer (see below) and stored at -20°C.

3.8 Two-dimensional electrophoresis

Samples were suspended in buffer isoelectrofocusing (urea 8M, CHAPS 4% w / v, DTE 65mm, 40mM Tris, bromophenol blue and 0.2% anfoline) and loaded on gel strips (IPG strip pH 3 -10 linear, Amersham Biosciences) and coated with oil (DryStrip cover fluid, Amersham Biosciences). The hydration and the subsequent focusing were performed using a system Protean IEF Cell (Biorad). After this first separation according to isoelectric point, strips were placed on polyacrylamide gels (12%) and covered with 2 ml of a Tris-Glycine buffer pH 8.8, containing 4% SDS, 50 mM Dithiothreitol, 0.5% agarose. Electrophoresis allowed to proteins second separation according to molecular weight. Gels were transferred onto nitrocellulose membranes that were subsequently incubated with specific antibodies. On the basis of $p27^{Kip1}$ isoforms theoretical isoelectric points, calculated using ExPASy Proteomics Tools program (www.expasy.org/tools), the pIs in which they appeared as different immunoreactive signals (spots) were determined through a pI calibration curve (made by means of proteins with known pIs).

3.9 Phosphatase assay

For this assay was used λ phosphatase (BioLabs): 100 units of λ phosphatase remove 250 picomoles of phosphate in 30 minutes at 30°C.

500 μg of nuclear Lan-5 cells extract (in lysis buffer including protease and phosphatase inhibitors) were incubated with rabbit pAbs anti-$pS10p27^{Kip1}$ and immuneprecipitated with

protein A Sepharose. Immunoprecipitate was incubated at 30°C for 1 hour in 50 ml of reaction buffer with 400 units of enzyme. At the end of reaction, immunoprecipitate was centrifuged at 4000 x g and recovered. Proteins, linked to resin, were extracted with 100 mM Glycine-HCl pH 2.5 and subsequently precipitated with TCA 10%, as reported above. Precipitate was suspended in buffer for two-dimensional analysis.

3.10 Plasmids preparation and transfection

Plasmids were prepared starting from the coding sequence of wild type $p27^{Kip1}$ cloned in pcDNA3. Particularly, plasmids containing the following mutations were prepared: $p27^{Kip1}$G9R, $p27^{Kip1}$G9RS10A, $p27^{Kip1}$G9RT187A, $p27^{Kip1}$G9RS10AT187A, and $p27^{Kip1}$G9RT198V. To mutagenize was used QuikChange II Site-Directed Mutagenesis Kit, from Agilent Technologies (Santa Clara, CA, USA). Each plasmid sequence was then confirmed by direct sequencing.
Oligonucleotides employed for mutagenesis are:

$p27^{Kip1}$G9R mutants	**Primers sequence**
Fw $p27^{Kip1}$G9R	5’ GCG AGT GTC TAA CAG GAG CCC TAC GCT GG 3’
Rev $p27^{Kip1}$G9R	5’ CCA GGC TAG GGC TCC TGT TAG ACA CTC GC 3’
Fw $p27^{Kip1}$G9RS10A	5’GCG AGT GTC TAA CAG GGC CCC TAG CCT GG 3’
Rev $p27^{Kip1}$G9RS10A	5’ CCA GGC TAG GGG CCC TGT TAG ACA CTC GC 3’
Fw $p27^{Kip1}$T187A	5’ GTT CTG TGG AGC AGG CGC CCA AGA AGC CTG 3’
Rev $p27^{Kip1}$T187A	5’ CAG GCT TCT TGG GCG CCT GCT CCA CAG AAC 3’
Fw $p27^{Kip1}$T198V	5’ CCT CAG AAG ACG TCA AGT GTA AAA TTC TGC 3’
Rev $p27^{Kip1}$T198V	5’ GCA GAA TTT TAC ACT TGA CGT CTT TCT GAG G 3’

3.11 Immunofluorescence analysis

MEF transfected with $p27^{Kip1}$WT and $p27^{Kip1}$G9R were plated on cover slips for confocal microscopy. Cells were fixed with 4% of paraformaldehyde (PFA) for 30 min at room temperature and permeabilizated with 1X PBS + Triton 0,5% for 10 min. MEF were treated with a blocking solution (5% horse serum) for 30 min at room temperature and then incubated overnight at 4°C with anti-$p27^{Kip1}$ mouse monoclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ, USA). Goat anti-mouse FITC-conjugate (ImmunoReagents, Inc Raleigh

NC) was used as secondary antibody, nuclei were stained with Hoechst. Slides were examined with ZEISS Confocal Microscope and image analyzed with ZEISS software.

3.12 Wound healing

NIH3T3 were plated at high confluence, starved and transfected with $p27^{Kip1}$WT and $p27^{Kip1}$G9R vectors. After 24 hours of transfection, wounds were made by sterile pipet tip and the cell photographed (time 0). After different time intervals (up to 42 hours) the healing process was monitored and images were taken by DM IRB Microscope and Optica Vision 6.0 software.

4. RESULTS

4.1 p27^{Kip1} phosphoisoforms characterization

In a previous study, Borriello and colleagues demonstrated that p27^{Kip1} is characterized by multiple phosphoisoforms in nuclear and cytosolic compartments of neuroblastoma cell line Lan-5. In particular, after 8 hours of ATRA treatment, a high pSer10p27^{Kip1} up-regulation was found in Lan-5 cytoplasm and also in the nucleus[95]. To clarify the mechanisms of pSer10p27^{Kip1} increase, p27^{Kip1} phosphoisoforms characterization was performed through IP followed by two-dimensional electrophoresis (2D) and western blot (WB). IP was performed, when available, with anti-phospho-p27^{Kip1} specific antibodies[95]. Borriello et al demonstrated that classical WB analysis was not sufficient to characterize p27^{Kip1} isoforms because of the contemporaneously presence of multiple p27^{Kip1} post translational modification on multiple residues, the recurrently low specificity of anti-phospho-p27^{Kip1} antibodies and the faint level of some p27^{Kip1} isoforms. Instead, they found that the use of 2D/WB, associated at the occurrence with IP, lead to a better and direct phosphoisoforms identification.

Borriello and co-workers demonstrated that pattern spots 0 and 2 correspond respectively to unmodified and monophosphorylated p27^{Kip1} forms, and that signal 4 corresponds to biphosphorylated isoforms. Signal 1, defined as "intermediate form", was associated with a covalent modification (perhaps an acetylation) still unidentified. Spot 3 corresponds to the monophosphorylated derivative of "intermediated form" (Fig. 11)[95]. In Borriello's report, to obtain a precise characterization of Ser10 phosphorylation, Lan-5 nuclear extract was also immunoprecipitated using anti-pSer10p27^{Kip1} antibody [95]. 2D/WB analysis of pSer10p27^{Kip1} IP, indicated the presence of at least three p27^{Kip1} forms phosphorylated on Ser10 residue: spots 2, 3 and 4 (Fig. 12A)[95]. In pSer10p27^{Kip1} IP supernatant, a small amount of CKI phosphorylated forms was still visible (Fig. 12A)[95], proving the existence of nuclear p27^{Kip1} phosphoisoforms not phosphorylated on Ser10 residue[95]. Furthermore, to confirm data on Ser10 phosphorylation, pSer10p27^{Kip1} IP was treated with λ phosphatase (λPPase) and analyzed by 2D/WB. As shown in Fig. 12B**,** only spots 0 and 1 were visible after λPPase treatment, demonstrating that forms 2 and 3 corresponded respectively to the monophosphorylated derivatives of unmodified and "intermediated". Spot 4 represented, probably, unmodified biphosphorylated isoforms containing both pSer10 and an additional modified residue[95].

Successively, the authors examined other nuclear p27^{Kip1} phosphoisoforms. In particular, Borriello et al investigated pThr187p27^{Kip1}-containing isoforms, since Thr187 modification represents the most frequently demonstrated and accepted p27^{Kip1} phosphorylation that is

principally required for $p27^{Kip1}$ removal in S and G2/M phases[70-73]. Fig. 13 showed $pThr187p27^{Kip1}$ pattern characterization performed through 2D/WB analysis of $pThr187p27^{Kip1}$ IP on Lan-5 nuclear extract: signals 2 and 4 corresponded respectively to $pThr187p27^{Kip1}$ mono- and bi-phosphorylated forms. Conditions employed for $pThr187p27^{Kip1}$ detection were different from those employed to analyze $pSer10p27^{Kip1}$ because of $pThr187p27^{Kip1}$ scarce amount (Fig. 13)[95].

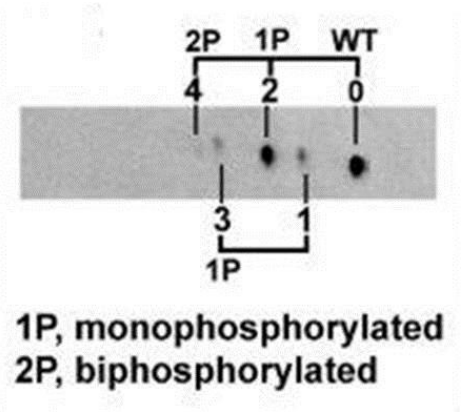

Fig. 11 $p27^{Kip1}$ phosphoisoforms pattern characterization. 2D/WB analysis of Lan-5 nuclear extract analyzed with anti-$p27^{Kip1}$ mouse monoclonal antibody[95].

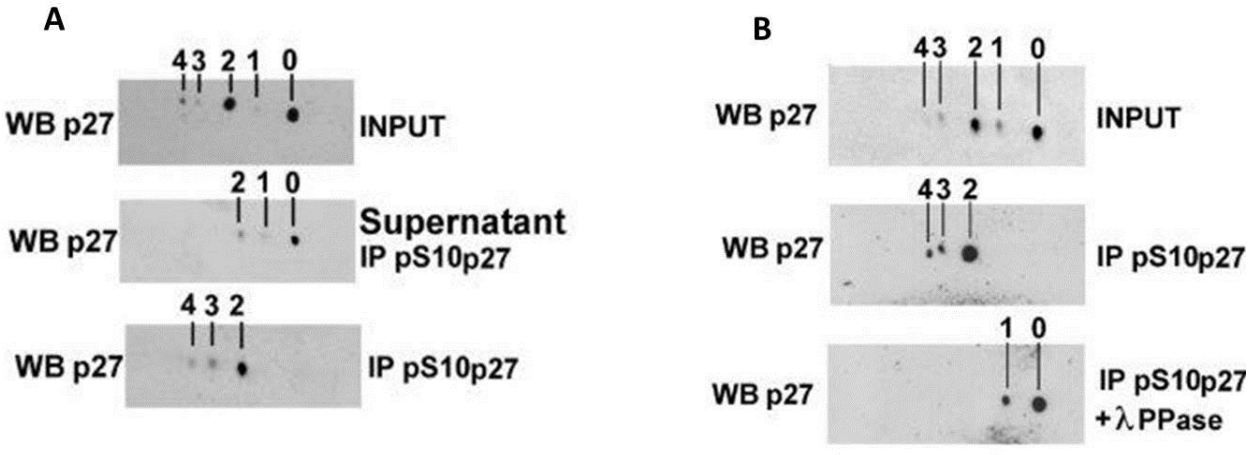

Fig. 12A-B $pSer10p27^{Kip1}$ isoforms characterization. A) 2D/WB of Lan-5 nuclear extract (INPUT), Lan-5 nuclear extract immunoprecipitated (1mg) with anti-$pSer10p27^{Kip1}$ rabbit polyclonal antibody and $pSer10p27^{Kip1}$ IP supernatant. All the samples were analyzed with anti-$p27^{Kip1}$ mouse monoclonal antibody. B) 2D/WB of Lan-5 nuclear extract (INPUT), $pSer10p27^{Kip1}$ IP before and after λPPase treatment [95].

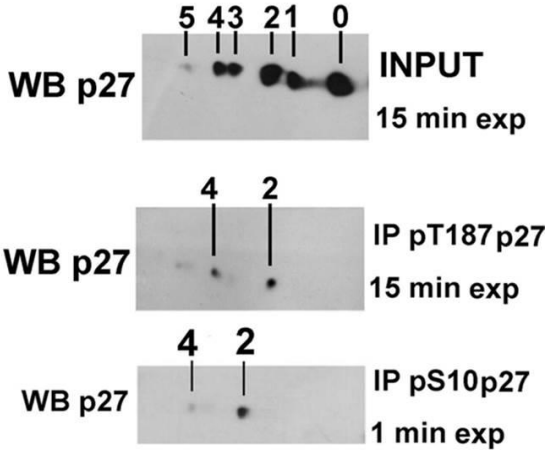

Fig. 13 pThr187p27^{Kip1} isoforms characterization. 2D/WB of Lan-5 nuclear extract (INPUT), Lan-5 nuclear extract immunoprecipitated (5mg) with anti-pThr187p27^{Kip1} rabbit polyclonal antibody, pSer10p27^{Kip1} IP (as in Fig. 12). All the samples were analyzed with anti-p27^{Kip1} mouse monoclonal antibody[95].

4.2 pSer10p27^{Kip1} metabolism

As definitely reported in Literature, pSer10p27^{Kip1} represents more than 60-70% of all p27^{Kip1} phosphoisoforms, and remarkably increases the CKI stability[87]. Thus, we decided: i) to verify pSer10 function(s) in neuroblastoma model; ii) to clarify its increase mechanism(s), and iii) to investigate nuclear p27^{Kip1} phosphoisoforms metabolism. At first, we verified pSer10p27^{Kip1} stability exposing Lan-5 cell line to the protein translation inhibitor cycloheximide (CHX) for 8 hours at the concentration of 36µM. 2D/WB analysis of Lan-5 nuclear extracts showed that after treatment, isoform 2 increases compared to isoform 0, suggesting that it is more stable than the unmodified protein (Fig.14). The obtained isoforms pattern was also analyzed with anti-pSer10p27^{Kip1} antibody revealing that p27^{Kip1} phosphorylated on Ser10 residue corresponds to the degradation resistant isoforms (Fig. 14).

p27^{Kip1} nuclear proteolysis is mainly regulated by SCF-Skp2 dependent mechanism that requires, in its initial step the phosphorylation on Thr187. To verify if the higher pSer10p27^{Kip1} nuclear stability was due to a reduction in SCF-Skp2 ubiquitination, we down-regulated Skp2 levels by transfecting Lan-5 with Skp2-siRNA for 48 hours. As shown in Fig. 15A, after siRNA treatment Skp2 levels are reduced, p27^{Kip1} is strongly up-regulated, but there are not any variations in pSer10p27^{Kip1} amount. These data show that p27^{Kip1} phosphorylated in Ser10 is not susceptible to SCF-Skp2 dependent proteolysis. Subsequently we verified the possibility that nuclear pSer10p27^{Kip1} levels were regulated by other degradation mechanisms Skp2-independent. Thus,

Lan-5 cells were cultured in the presence of two proteasome inhibitors: Mg132 (used at the concentration of 1µM) and epoxomicin (Epox, used at the concentration of 5µM) (Fig. 15B). To better evaluate phosphorylated isoforms, cells nuclear extracts were immunoprecipitated with anti-pSer10p27^{Kip1} antiserum (Fig. 15D). Data showed that after treatment there is an increase in p27^{Kip1} levels, but not in pSer10p27^{Kip1} (Fig. 15B and Fig. 15D). We hypothesized that p27^{Kip1} elevation in the presence of proteasome inhibitors could be due to the modification on Thr187. Therefore, we immunoprecipitated Lan-5 nuclear extracts with an anti-Thr187p27^{Kip1} antibody. WB analysis confirmed that pThr187p27^{Kip1} isoform is responsible of p27^{Kip1} increase after Mg132 and Epox treatment (Fig. 15C). To understand if there were variations in p27^{Kip1} isoforms pattern after Mg132 treatment, 2D/WB analysis of pThr187-depleted Lan-5 nuclear extract was performed (namely, pThr187p27^{Kip1} was preliminarly immunoprecipitated from treated nuclear extracts and, then, IP supernatant was analyzed by 2D/WB analysis). The experiment showed that p27^{Kip1} phosphorylated isoforms up-regulated by Mg132 contain high amount of pThr187p27^{Kip1} (Fig. 16A). 2D membranes were then incubated with anti-pSer10p27^{Kip1} antibody and, as shown in Fig. 16B, no change was detect in spot 4, suggesting that phosphorylation on Ser10 does not co-exist with phosphorylation on Thr187. These data confirm the evidence that pSer10p27^{Kip1} metabolism is not influenced by pThr187-dependent proteolysis mechanisms.

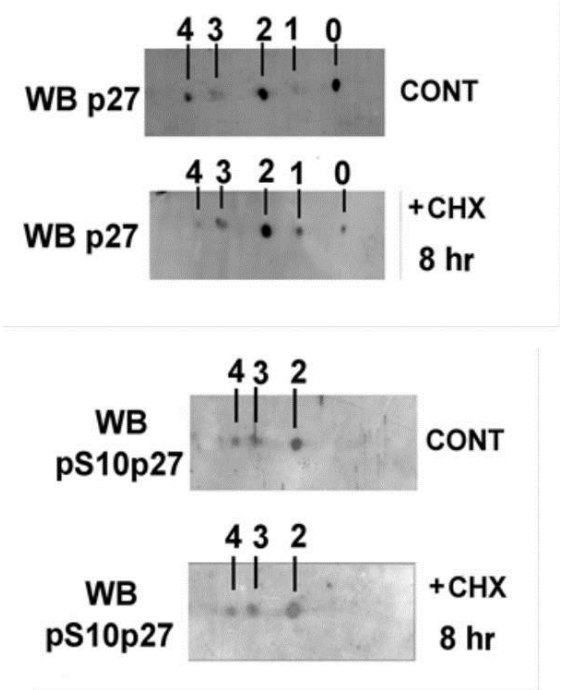

Fig. 14 Analysis of pSer10p27^{Kip1} stability. 2D/WB analysis of Lan-5 nuclear extracts before and after 8h of treatment with CHX 36µM. 2D membranes were incubated with anti-p27^{Kip1} mouse monoclonal antibody and with anti-pSer10p27^{Kip1} rabbit polyclonal antibodies.

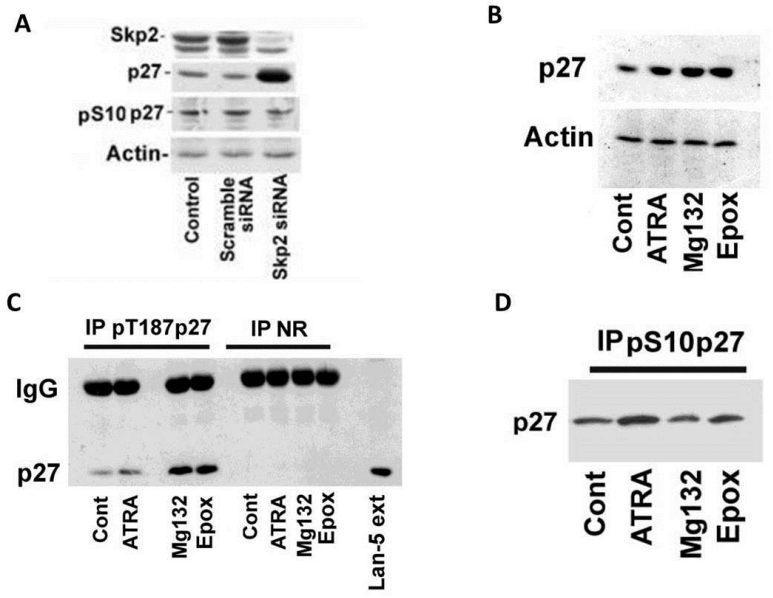

Fig. 15A-D Analysis of pSer10p27^{Kip1} metabolism. A) WB analysis of Lan-5 extracts control, transfected with Skp2 siRNA and scramble siRNA. All the samples were analyzed with anti-p27^{Kip1} monoclonal antibody, anti-Skp2, anti-pSer10p27^{Kip1} and anti-actin rabbit polyclonal antibodies. B) WB analysis of Lan-5 extracts control, Lan-5 extracts after treatments with ATRA 5µM, Mg132 1µM and Epox 5µM. all the samples were analyzed with anti-p27^{Kip1} mouse monoclonal antibody and with anti-actin rabbit polyclonal antibody. C) WB analysis of Lan-5 extracts described in B) immunoprecipitated (1mg) with anti-pThr187p27^{Kip1} polyclonal antibody and rabbit Not Related (NR) antibodies. All the samples were analyzed with anti-p27^{Kip1} mouse monoclonal antibody. D) WB analysis of Lan-5 extracts described in B) immunoprecipitated (1mg) with anti-pSer10p27^{Kip1} rabbit polyclonal antibody. All the samples were analyzed with anti-p27^{Kip1} mouse monoclonal antibody.

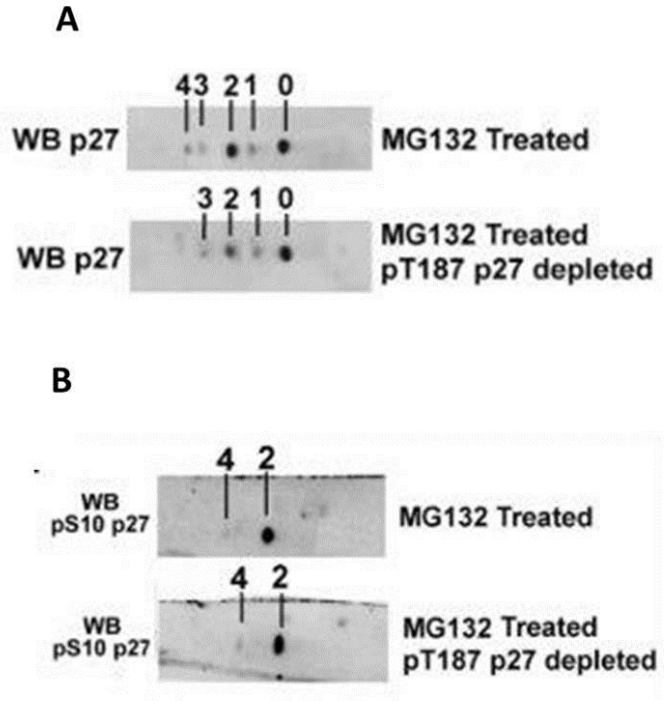

Fig. 16A-B Analysis of pSer10p27^{Kip1} and pThr187p27^{Kip1} isoforms after proteasome inhibition. A) 2D/WB of Lan-5 nuclear extracts treated for 8h with Mg132 1µM before and after pThr187p27^{Kip1} depletion (Lan-5 nuclear extracts treated with Mg132 were at first immunoprecipitated with anti-pTh187p27^{Kip1} rabbit polyclonal antibody and then IP supernatant was analyzed with 2D/WB). Membranes were analyzed with anti-p27^{Kip1} mouse monoclonal antibody. B) 2D/WB analysis of Lan-5 extract and IP supernatant described in A) analyzed with anti-pSer10p27^{Kip1} rabbit polyclonal antibody.

4.3 pSer10p27^{Kip1} biphosphorylated isoform identification

2D/WB analysis of Lan-5 pThr187p27^{Kip1}-depleted nuclear extract (Fig. 16B) showed the existence of a p27^{Kip1} biphosphorylated isoform constituted by pSer10 and another phosphorylation which is clearly not located on Thr187 residue. We decided, so, to clarify which should be the other modification compatible with pSer10 in cell nucleus. We focalized our attention in particular on pThr198, although this phosphorylation have been reported to occur mainly in cytoplasm. At first we verified if pThr198p27^{Kip1} could have a nuclear localization. WB analysis of Lan-5 nuclear extract immunoprecipitated with anti-pThr198p27^{Kip1} antibody confirmed that this modification occurs also in the nucleus and not only in cytosol (Fig. 17). Then we performed 2D/WB analysis on the same samples to examine pThr198p27^{Kip1} phosphorylation pattern. Fig. 18A shows that this modification appears as mono- and as biphosphorylated signals in the cytoplasm and also in the nucleus. 2D/WB analysis of Lan-5 nuclear pThr198p27^{Kip1} IP depleted of pSer10p27^{Kip1} (we made a subsequent IP with anti-pThr198p27^{Kip1} antibody from pSer10p27^{Kip1}IP supernatant) revealed that phosphorylation on Thr198 co-exists with phosphorylation on Ser10. As shown in Fig. 18B, in fact, spot 4 disappears when pSer10 is absent, so the biphosphorylated isoform of pSer10p27^{Kip1} is pSer10pThr198p27^{Kip1}.

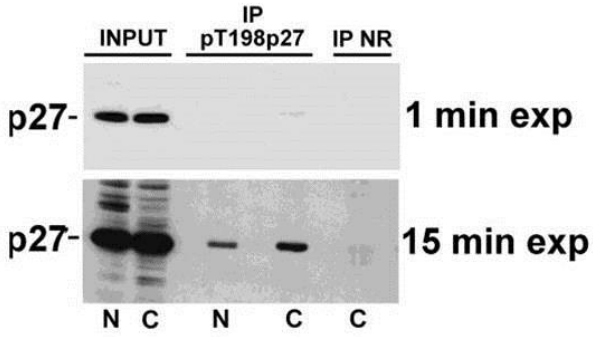

Fig. 17 pThr198p27^{Kip1} localization analysis. WB analysis of Lan-5 nuclear and cytoplasmic extracts immunoprecipitated (500µg) with anti-pThr198p27^{Kip1} rabbit polyclonal antibody and rabbit NR antibody. All the samples were analyzed with anti-p27^{Kip1} mouse monoclonal antibody and at different time of exposure.

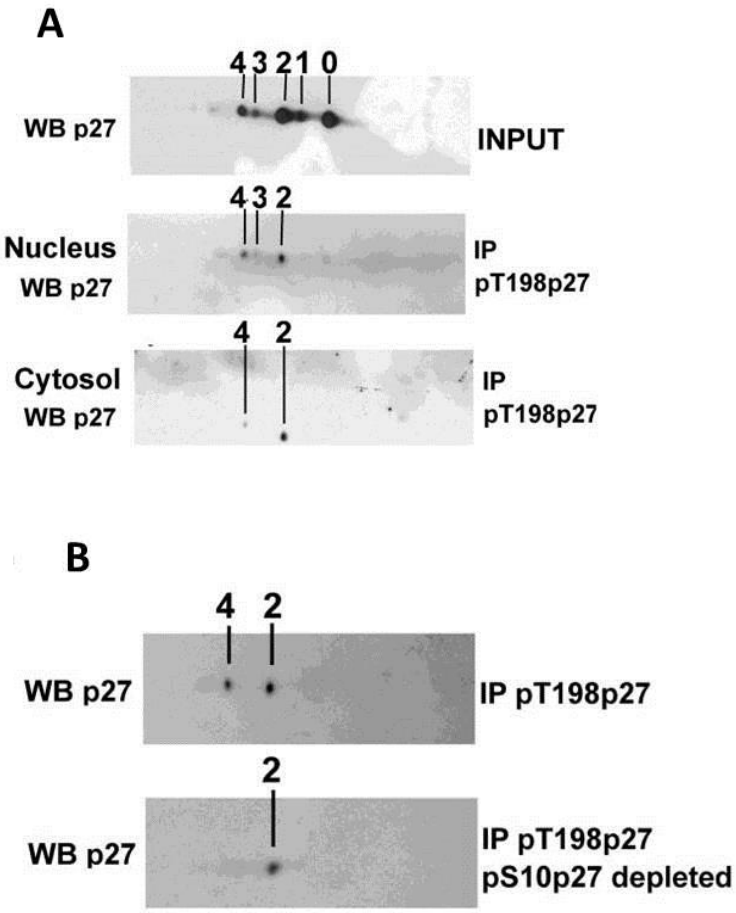

Fig. 18A-B pThr198p27^{Kip1} phosphorylation pattern analysis. A) 2D/WB analysis of Lan-5 nuclear and cytoplasmic extracts immunoprecipitated (1mg) with anti-pThr198p27^{Kip1} rabbit polyclonal antibody and analyzed with anti-p27^{Kip1} mouse monoclonal antibody. B) 2D/WB analysis of Lan-5 nuclear pThr198p27^{Kip1} IP, as in A), before and after pSer10p27^{Kip1} depletion analyzed with anti-p27^{Kip1} mouse monoclonal antibody.

4.4 p27^{Kip1} isoforms in cell cycle phases

The data reported in the previous sections demonstrated that phosphorylation on Ser10 confers more stability to p27^{Kip1} (probably by allowing the CKI to escape Skp2 and proteasome dependent degradation) and suggested that this isoform mostly occurs in cell nucleus as mono- and biphosphorylated forms. Therefore, we decided to investigate whether pSer10p27^{Kip1} could have a role not only in p27^{Kip1} cytoplasmic translocation but also into the CDK regulation along the cell division cycle. Preliminarly, we studied p27^{Kip1} phosphoisoforms distribution in the various cell cycle phases. Lan-5 nuclear and cytoplasmic extracts were prepared synchronizing cells in G0, G1, S and G2/M phases. To obtain cells enriched in G0, Lan-5 were starved with serum free culture medium for 72 hours; to obtain cells in G1 we cultured starved Lan-5 with

10% FBS medium for 3 hours and then we harvested them; cells in S phase were obtained treating Lan-5 with 5mM thymidine for 48h; finally G2 cells were obtained removing thymidine from Lan-5 blocked in S phase and maintaining them with a complete culture medium for 15 hours. Lan-5 synchronization was confirmed with FACS analysis and WB analysis of cyclins expression pattern (data not shown). Fig. 19 shows total $p27^{Kip1}$ levels in cell cycle phases: in G0 CKI levels are maximal, followed by a small decrease in G1; in S phase $p27^{Kip1}$ levels are significantly reduced and in G2/M they increase again, especially in the nucleus. To examine $p27^{Kip1}$ phosphoisoforms nuclear distribution in cell cycle phases we immunoprecipitated 1 mg of synchronized Lan-5 nuclear extracts with anti-$p27^{Kip1}$, anti-pSer10$p27^{Kip1}$, anti-pThr187$p27^{Kip1}$ and not related antibodies and analyzed them through WB. As shown in Fig. 20, $p27^{Kip1}$ IP confirms the distribution observed in previous experiment; pSer10$p27^{Kip1}$ IP occurred at significant high levels in G0 and then declined in G1. The phosphoisoform abruptly decreased in S phase while statistically increased in G2/M. In this experiment pThr187$p27^{Kip1}$ is not detected. To examine pThr187$p27^{Kip1}$ it was necessary immunoprecipitate 2 mg of Lan-5 nuclear extract (instead of 1mg) and expose the filter for a prolonged time. pThr187$p27^{Kip1}$ analyses revealed that the isoform was weakly detectable in G1, it increased in S phase and was expressed at maximal levels in G2/M (Fig. 21). Synchronized Lan-5 nuclear extracts pSer10$p27^{Kip1}$-depleted and their inputs were analyzed by 2D/WB to obtain a clear evaluation of $p27^{Kip1}$ phosphoisoforms distribution in cell cycle phases. pSer10$p27^{Kip1}$-depleted extracts were used to investigate pSer10$p27^{Kip1}$ contribution to phosphoisoforms amount. In G0 the most representative isoforms are monophosphorylated and biphosphorylated and, as demonstrated by pSer10$p27^{Kip1}$ depletion, a large percentage of them was pSer10$p27^{Kip1}$ (Fig. 22A). In G1, a reduction in both mono- and biphosphorylated isoforms was observed, however pSer10$p27^{Kip1}$ remained the most representative (Fig. 22B). During S phase biphosphorylated isoforms were not present and monophosphorylated CKI was weakly detectable. As shown in Fig. 22C, pSer10$p27^{Kip1}$ represented almost the totality of $p27^{Kip1}$ monophosphorylated isoforms. In G2/M phases, monophosphorylated isoforms were more detectable then in S phase and a small quantity of biphosphorylated was also observed. Also in these phases, pSer10$p27^{Kip1}$ is a significant percentage of all $p27^{Kip1}$ phosphoisoforms (Fig. 22D). In brief, our data demonstrate that pSer10$p27^{Kip1}$ amount varies during cell cycle, at least in part, independently on total $p27^{Kip1}$.

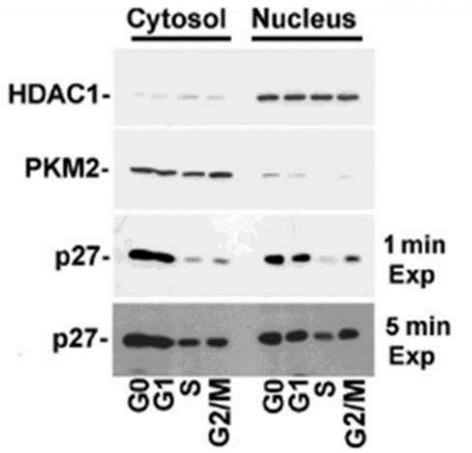

Fig. 19 p27^{Kip1} distribution in cell cycle phases. WB analysis of Lan-5 synchronized cytoplasmic and nuclear extracts analyzed with anti-p27^{Kip1} mouse monoclonal antibody at different time of exposure. Analysis with anti-HDAC1 rabbit polyclonal antibody and anti-PKM2 mouse monoclonal antibody were used as nuclear and cytoplasmic loading control.

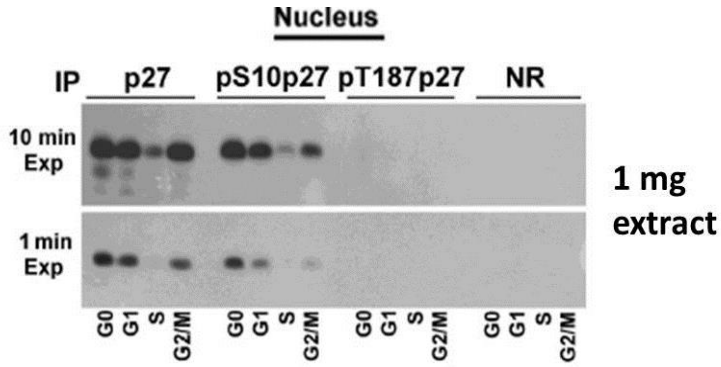

Fig. 20 p27^{Kip1} phosphoisoforms distribution in cell cycle phases. WB analysis of synchronized Lan-5 nuclear extracts immunoprecipitated (1mg) with anti-p27^{Kip1}, anti-pSer10p27^{Kip1}, anti-Thr187p27^{Kip1} and NR rabbit polyclonal antibodies, all analyzed with anti-p27^{Kip1} mouse monoclonal antibody at different time of exposure.

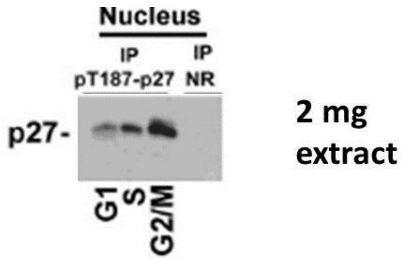

Fig. 21 pThr187p27^{Kip1} distribution in cell cycle phases. WB analysis of synchronized Lan-5 nuclear extracts immunoprecipitated (2mg) with anti-pThr187p27^{Kip1} and NR rabbit polyclonal antibodies, all analyzed with anti-p27^{Kip1} mouse monoclonal antibody.

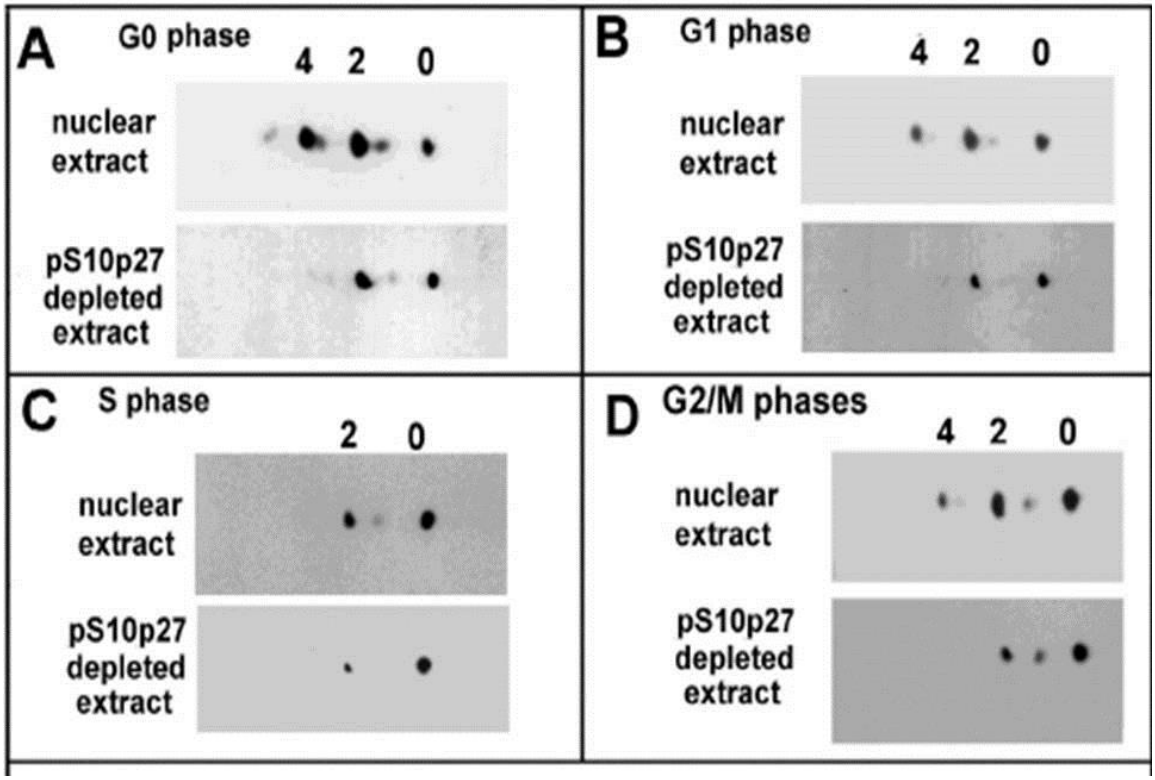

Fig. 22A-D. pSer10p27^{Kip1} distribution in cell cycle phases. 2D/WB analysis of synchronized Lan-5 nuclear extracts and synchronized Lan-5 nuclear extract pSer10p27^{Kip1}-depleted analyzed with anti-p27^{Kip1} mouse monoclonal antibody. Different amounts of Lan-5 extracts of each phase were used because of differences in p27^{Kip1} content: in G0 and G1 500µg; in S 4mg; in G2/M 2mg.

4.5 pSer10p27^{Kip1} interaction with CDKs

Subsequently to the characterization of pSer10p27^{Kip1} nuclear distribution in cell cycle phases, we examined its interaction with different CDKs, in particular CDK4, CDK2, CDK1, CDK5 and CDK7, in asynchronous Lan-5. WB analysis of p27^{Kip1} and pSer10p27^{Kip1} IP revealed that unmodified p27^{Kip1} interacts (although differently) with all these CDKs, except CDK7. pSer10p27^{Kip1}, instead, interacts only with CDK4 and CDK2 (Fig. 23A-B). We also verified pThr187p27^{Kip1} association with CDK2 and CDK1 in synchronized Lan-5. As shown in Fig. 24, this isoform in G1 is scarce and it does not associate with CDK2 and CDK1, in S and G2/M phases pThr187p27^{Kip1} levels increases and the isoform interacts with both the CDKs. To confirm our data on pSer10p27^{Kip1} association with CDKs, we performed 2D/WB analysis of CDK4 and CDK2 IPs. In Fig. 25A are shown 2D/WB analyses of CDK4 IP and CDK4 IP pSer10p27^{Kip1}-depleted, and, as evident by spot 2 reduction, pSer10p27^{Kip1} represents a significant proportion of p27^{Kip1} monophosphorylated isoform bound to CDK4. Fig. 25B demonstrates that pSer10p27^{Kip1} is able to bind CDK2 both in its monophosphorylated and

biphosphorylated forms and that it also represents a large percentage of p27^{Kip1} phosphoisoforms bound to CDK2.

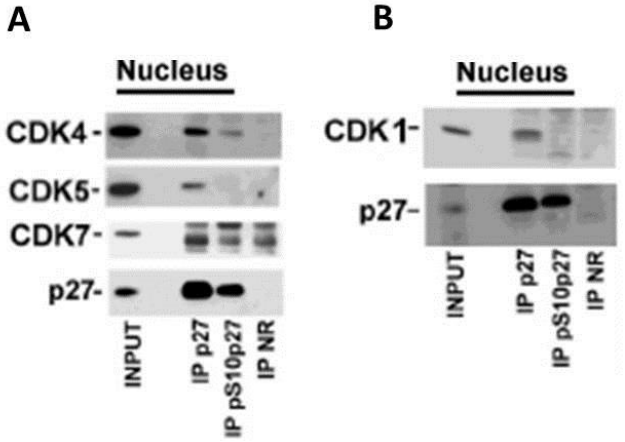

Fig. 23A-B p27^{Kip1} and pSer10p27^{Kip1} interaction with CDKs. WB analysis of Lan-5 nuclear extract (INPUT) and Lan-5 nuclear extracts immunoprecipitated (500µg) with anti-p27^{Kip1}, anti-pSer10p27^{Kip1} and NR rabbit polyclonal antibodies, all analyzed with anti-p27^{Kip1} mouse monoclonal antibody, anti-CDK4, anti-CKD5, anti-CDK7 and anti-CDK1 rabbit polyclonal antibodies.

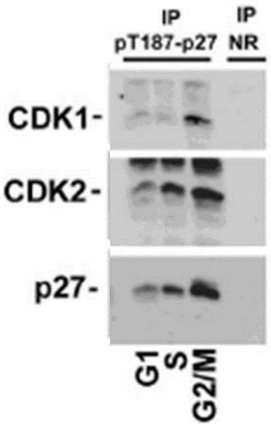

Fig. 24 pThr187p27^{Kip1} interaction with CDKs. WB analysis of synchronized (G1, S, G2/M) Lan-5 nuclear extracts immunoprecipitated (2mg) with anti-pThr187p27^{Kip1} and NR rabbit polyclonal antibodies, all analyzed with anti-p27^{Kip1}, anti-CDK1 and anti-CDK2 mouse monoclonal antibodies.

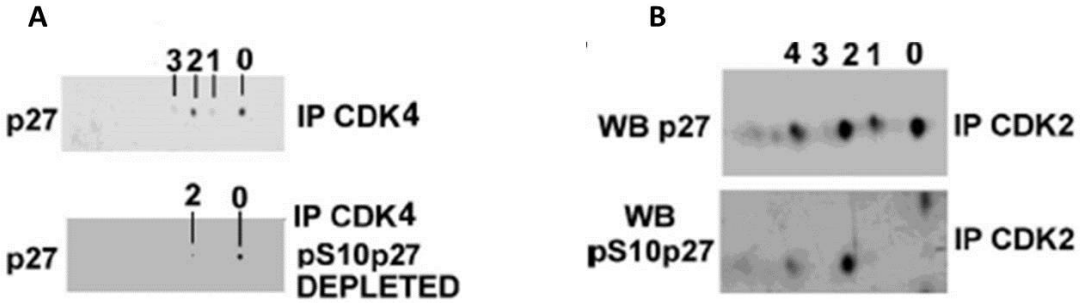

Fig. 25A-B pSer10p27^{Kip1} interaction with CDK4 and CDK2. A) 2D/WB analysis of Lan-5 nuclear extracts immunoprecipitated (2mg) with anti-CDK4 rabbit polyclonal antibody and CDK4 IP pSer10p27^{Kip1}-depleted. Both the membranes were analyzed with anti-p27^{Kip1} mouse monoclonal antibody. B) 2D/WB analysis of Lan-5 nuclear extracts immunoprecipitated (2mg) with anti-CDK2 rabbit polyclonal antibody and analyzed with anti-p27^{Kip1} mouse monoclonal and pSer10p27^{Kip1} rabbit polyclonalantibody.

4.6 pSer10p27^{Kip1} interaction with cyclins.

Since pSer10p27^{Kip1} interacts with CDK2 and CDK4, we planned to characterize its association with different cyclins, in particular cyclin E, cyclin A and cyclin B. As reported in literature, cyclins levels changes during cell cycle phases: cyclin E occurs mostly in S phase, cyclin A in S and G2/M phases and cyclin B in G2/M phases. Thus, we examined p27^{Kip1} and pSer10p27^{Kip1} association with these cyclins in the phases in which they are abundant. We verified p27^{kip1} and pSer10p27^{Kip1} interaction with cyclin E and cyclin A in S phase with mono- and bidimensional electrophoresis/WB. We observed with WB analysis of p27^{Kip1} and pSer10p27^{Kip1} IPs that total p27^{kip1} binds both the cyclins, instead, pSer10p27^{Kip1} interacts only with cyclin E (Fig. 26). 2D/WB analysis of cyclin E IP analyzed with anti-p27^{Kip1} antibody confirms that this cyclin binds preferentially p27^{Kip1} monophosphorylated isoform than the unmodified CKI (Fig. 27). Successively, we performed 2D/WB analysis of cyclin A IP: this experiment, however, was more complex than the previous because of cyclin A capability to form complexes with both CDK2 and CDK1. Thus, to identified p27^{Kip1} isoforms associated complexes (cyclin A/CDK2 or cyclin A/CDK1), we analyzed cyclin A IP CDK1-depleted and cyclin A IP CDK2-depleted (Fig. 28A-B). These experiments confirmed that both cyclin A complexes are preferentially associated with unmodified p27^{Kip1}; as shown in Fig. 28A-B, spot 0 is the most intense signal. However is also present a weaker p27^{Kip1} monophosphorylated isoforms interaction with cyclin A/CDK2 complex, but not with cyclin A/CDK1. Finally Fig. 29 shows that only unmodified p27^{Kip1} associates with cyclin B.

In conclusion, we found that p27^{Kip1} monophosphorylated isoforms and specifically pSer10p27^{Kip1}, bind preferentially to cyclin E/CDK2 complex. These data and the absence of biphosphorylated isoform pSer10pThr187p27^{Kip1} suggest that pSer10p27^{Kip1} does not act as cyclin E/CDK2 substrate, but more probably functions as inhibitor. In turn, we hypothesize that the phosphorylation on Ser10 contributes to p27^{Kip1} antiproliferative function(s).

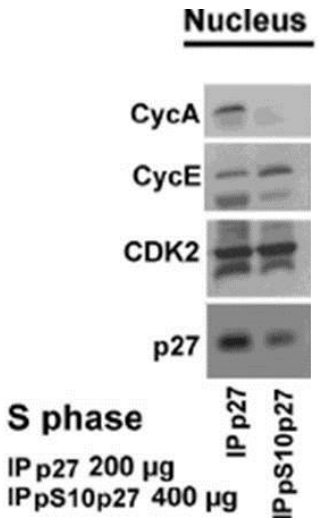

Fig. 26 pSer10p27^{Kip1} interaction with cyclins. WB analysis of p27^{Kip1} and pSer10p27^{Kip1} IPs (200µg and 400µg of synchronized Lan-5 nuclear extracts were immunoprecipitated respectively for p27^{Kip1} and pSer10p27^{Kip1} IPs) analyzed with anti-cyclin A, anti-cyclin E, anti-CDK2 and anti-p27^{Kip1} mouse monoclonal antibodies).

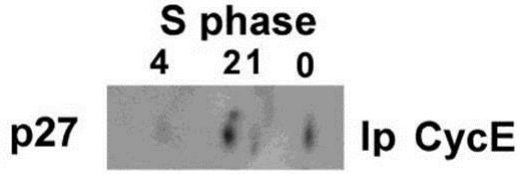

Fig. 27 p27^{Kip1} phopshoisoforms interaction with cyclin E. 2D/WB analysis of cyclin E IP (4mg of synchronized Lan-5 nuclear extract were immunoprecipitated with anti-cyclin E mouse monoclonal antibody) revealed with anti-p27^{Kip1} mouse monoclonal antibody.

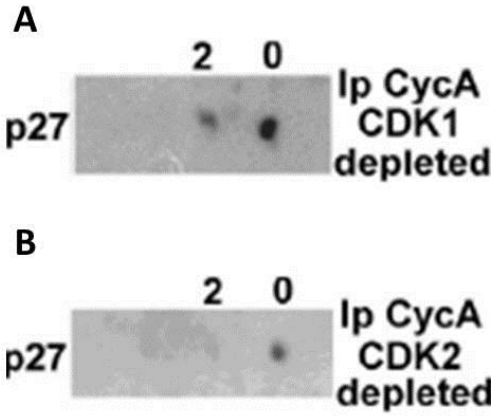

Fig. 28A-B. p27^{Kip1} phopshoisoforms interaction with cyclin A complexes. A) 2D/WB analysis of cyclin A IP depleted of CDK1 (Lan-5 synchronized in S+G2/M phases were previously immunoprecipitated with anti-CDK1 rabbit polyclonal antibody, successively IP supernatant was immunoprecipitated with anti-cyclin A rabbit polyclonal antibody) and then revealed with anti-p27^{Kip1} mouse monoclonal antibody. B) 2D/WB analysis of cyclin A IP depleted of CDK2 (Lan-5 synchronized in S+G2/M phases were previously immunoprecipitated with anti-CDK2 rabbit polyclonal antibody; successively IP supernatant was immunoprecipitated with anti-cyclin A rabbit polyclonal antibody) and then revealed with anti-p27^{Kip1} mouse monoclonal antibody.

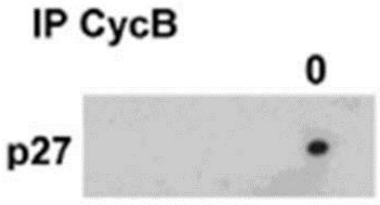

Fig. 29 p27^{Kip1} phosphoisoforms interaction with cyclin B. 2D/WB analysis of cyclin B IP (G2/M synchronized Lan-5 nuclear extract was immunoprecipitated with anti-cyclin B mouse monoclonal antibody) revealed with anti-p27^{Kip1} mouse monoclonal antibody.

4.7 p27^{Kip1}G9R mutation localization analysis

Recent studies reported the relevance of *CDKN1B* mutations in several human tumors development including neuroendocrine tumors[117;118], breast cancer[120] and hairy cell leukemia[121]. In particular, Costa-Guida and co-workers identified in a patient affected by non-familiar parathyroid adenoma, a missense mutation (c.25G<A, exon 1, CDKN1B gene) that results in Gly9Arg substitution in the translated protein[116]. Gly in position 9 of p27^{Kip1} is highly conserved across species, and its substitution to Arg might affect the phosphorylation of the adjacent Ser10 residue[116]. Given the role of Ser10 phosphorylation in the CKI activity, we were interested in investigating the effect of G9R mutation on Ser10 modification.

Thus, we transfected $p27^{Kip1}$ and $p27^{Kip1}$G9R encoding vectors in mouse (Mouse Embryo Fibroblasts, MEF, and NIH3T3) and human (EPN and K562) cell lines. In Literature, $p27^{Kip1}$ oncogenic activity was often associated with its cytoplasmic localization and was usually attributed to CKI C-terminal domain. Thus, we previously analyzed $p27^{Kip1}$G9R nuclear and cytoplasmic localization in EPN and MEF cell lines with WB and immunofluorescence approaches. Contrary to our expectations, WB analysis of transfected EPN revealed a higher nuclear localization of $p27^{Kip1}$G9R compared to $p27^{Kip1}$WT (Fig. 30). These data were confirmed by immunofluorescence (IF) experiments in MEF (Fig. 31A-B).

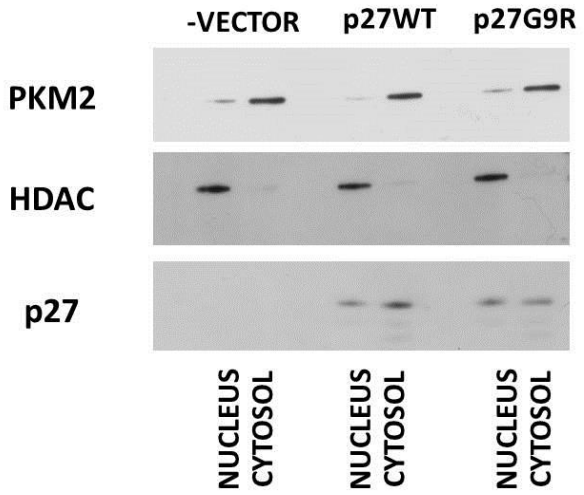

Fig 30 $p27^{Kip1}$G9R localization. WB analysis of transfected EPN nuclear and cytoplasmic extracts. EPN were transfected with $p27^{Kip1}$WT and $p27^{Kip1}$G9R pcDNA3 vectors and after 24h of transfection, were harvested and lysed to extract nuclear and cytoplasmic proteins. –VECTOR is transfection negative control. All the proteins were analyzed with anti-$p27^{Kip1}$ mouse monoclonal antibody, with anti-HDAC rabbit polyclonal antibody as nuclear loading control and with anti-PKM2 mouse monoclonal antibody as cytoplasmic loading control.

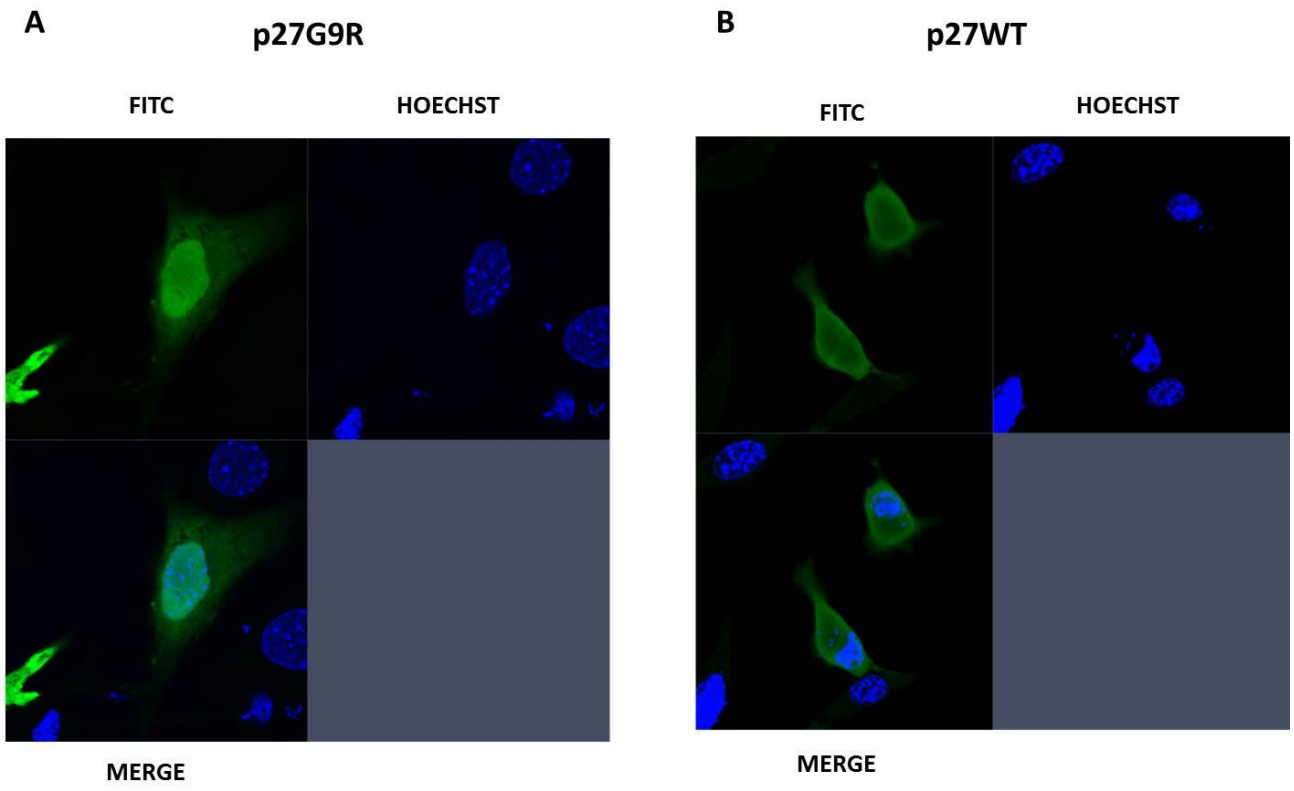

Fig. 31A-B p27^{Kip1}G9R and p27^{Kip1}WT localization. A) IF analysis of MEF cell line transfected with p27^{Kip1}G9R pcDNA3 vector. IF stain was performed with anti-p27^{Kip1} mouse monoclonal antibody and anti-mouse FITC-conjugated antibody. Cell nuclei were stained with Hoechst. B) IF analysis of MEF cell line transfected with p27^{Kip1}WT pcDNA3 vector. IF stain was performed with anti-p27^{Kip1} mouse monoclonal antibody and anti-mouse FITC-conjugated antibody. Cell nuclei were stained with Hoechst.

4.8 p27^{Kip1}G9R metabolism and phosphoisoforms pattern

To understand if the mutation can affect CKI stability, we examined p27^{Kip1}G9R half-life. Transfected K562 were treated with CHX 36µM for 6 hours and then analyzed with 2D/WB approach. Indeed, as shown in Fig. 32, p27^{Kip1}G9R presents some differences in its isoelectric point compared to p27^{Kip1}WT. In particular, glycine substitution with a positive charged arginine residue caused a lowering of isoelectric point. Thus, in 2D/WB analysis, we can see p27^{Kip1}G9R isoforms shift towards more acid isoelectric point (Fig. 32). Furthermore, mutated protein appears more phosphorylated then p27^{Kip1}WT: as shown, spot 2 and spot 4 are much more intense then spot 0 in p27^{Kip1}G9R, instead in p27^{Kip1}WT the unmodified is the most representative isoform (Fig. 32). CHX treatment does not indicate any differences in p27^{Kip1}G9R isoforms stability compared to p27^{Kip1}WT isoforms half-life (Fig. 33A-B). To understand if p27^{Kip1}G9R metabolism is influenced by a proteasome-dependent degradation, we treated transfected EPN with Mg132 1µM for 2 hours, after 6 hours of CHX 36µM treatment. As

shown in Fig. 34 $p27^{Kip1}$G9R levels increase after Mg132 treatment, suggesting a proteasome-dependent proteolysis, as for $p27^{Kip1}$WT.

Considered $p27^{Kip1}$G9R nuclear localization and its peculiar pattern of phosphorylation, we decided to clarify in detail its phosphoisoforms pattern, specifically pSer10 content, in different mouse and human cell models: MEF, NIH3T3, EPN and K562. To evaluate pSer10$p27^{Kip1}$G9R we used a site-directed mutagenesis approach, because we found that the available anti-pSer10$p27^{Kip1}$ antibodies are not able to recognize pSer10 residue in the mutated protein, probably due to Arg steric hindrance (data not shown). We mutagenized $p27^{Kip1}$G9R on residue 10 creating a Ser to Ala substitution and transfected NIH3T3 cell line with $p27^{Kip1}$G9RS10A pcDNA3 vector. As shown in Fig. 35 pSer10 absence determines an extreme biphosphorylated isoforms reduction and consequently an increase in unmodified isoform; monophosphorylated isoforms, instead, are only weakly decreased. These data suggest that Ser10, not only is phosphorylated, but it is one of the two residues modified in the totality of $p27^{Kip1}$G9R biphosphorylated isoforms (these findings were also confirmed in EPN cell lines, see below). We also confirmed these data treating transfected cells with λ phosphatase (Fig. 36A-B). However, the data reported in Fig. 35 demonstrated that a large amount of monophosphorylated forms is not constituted by pSer10 since the 2D/WB analysis of $p27^{Kip1}$G9RS10A mutant still showed an abundant phosphorylation.

Thus, we planned to identify this second quantitatively relevant phosphorylation site.

Initially, we evaluated the possibility that pThr198 could represent a large part of $p27^{Kip1}$G9R monophosphorylated isoforms and pSer10$p27^{Kip1}$G9R second phosphorylation. Therefore, we substituted threonine 198 with a valine. Fig. 37 shows that in NIH3T3 transfected with $p27^{Kip1}$G9RT198V phosphoisoforms variations are minimal: monophosphorylated isoforms levels are weakly decreased and also biphosphorylated isoforms are slightly reduced when pThr198 is absent. Successively we investigated Thr187phosphorylation in $p27^{Kip1}$G9R mutant, also for these experiments we used a site-directed mutagenesis approach creating a Thr to Ala substitution in $p27^{Kip1}$G9R. We analyzed $p27^{Kip1}$G9R phosphoisoforms pattern missing Thr187 phosphorylation, transfecting NIH3T3 cell line with $p27^{Kip1}$G9R, $p27^{Kip1}$G9RT187A and $p27^{Kip1}$G9RS10AT187A. As shown in Fig. 38, 2D/WB analysis revealed that $p27^{Kip1}$G9RT187A transfected cells present a weakly reduced ratio between unmodified and monophosphorylated isoforms compaired to $p27^{Kip1}$G9R, and a reduction in biphosphorylated content. NIH3T3 were then transfected with a pCDNA3 vector modified into both Ser10 and Thr187. 2D/WB analysis shows that spot 0 and spot 2 present equal levels and their ratio does not show any variations

compared to p27^{Kip1}G9RS10A (Fig. 38). Thus, pThr187p27^{Kip1}G9R represents a modest percentage of p27^{Kip1}G9R phosphorylation and it is not compatible with pSer10.

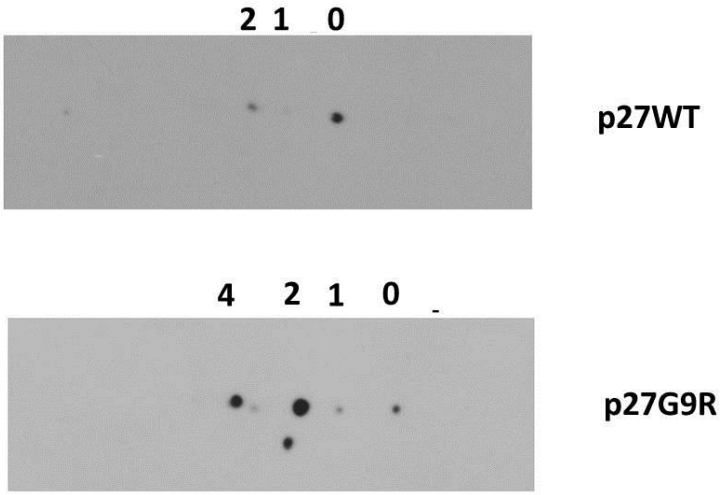

Fig. 32 p27^{Kip1}G9R phosphoisoforms characterization. 2D/WB analysis of K562 cells transfected with p27^{Kip1}WT and p27^{Kip1}G9R pCDNA3 vectors. Both extracts were analyzed with anti-p27^{Kip1} mouse monoclonal antibody.

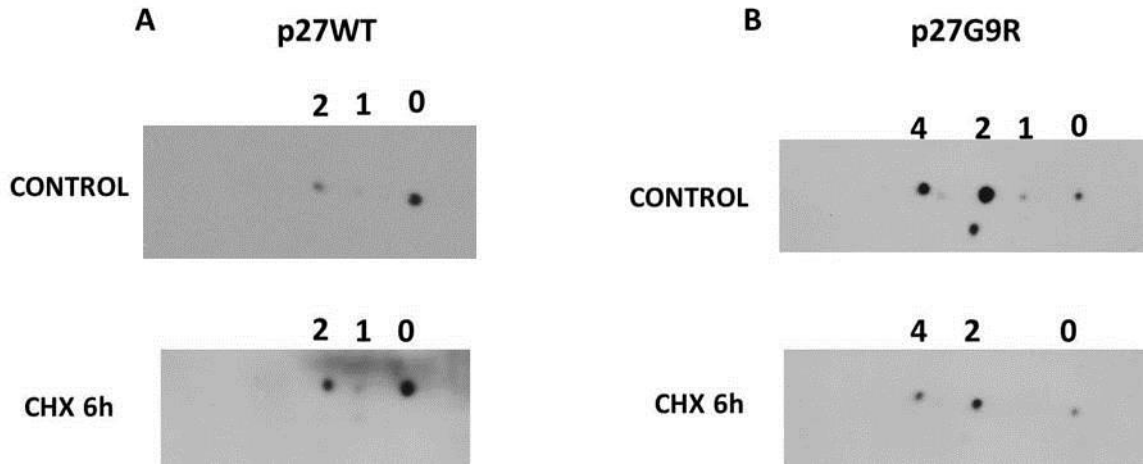

Fig. 33A-B p27^{Kip1}G9R half-life analysis. A) 2D/WB analysis of K562 transfected with p27^{Kip1}WT pCDNA3 vector before and after treatment with CHX 36µM for 6h. K562 extracts were analyzed with anti-p27^{Kip1} mouse monoclonal antibody. B) 2D/WB analysis of K562 transfected with p27^{Kip1}G9R pCDNA3 vector before and after treatment with CHX 36µM for 6h. K562 extracts were analyzed with anti-p27^{Kip1} mouse monoclonal antibody

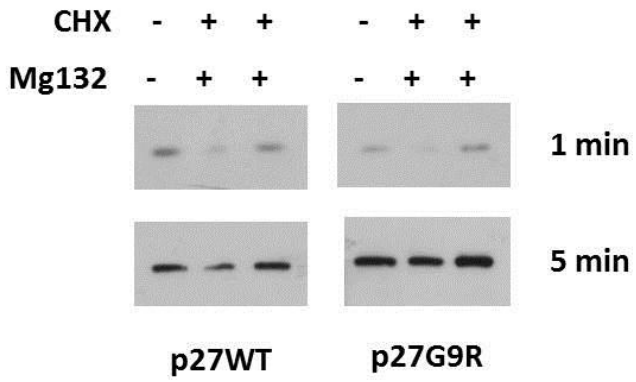

Fig. 34 p27^{Kip1}G9R metabolism. WB analysis of EPN transfected with p27^{Kip1}WT and p27^{Kip1}G9R pcDNA3 vectors. Transfected cells were treated with CHX 36µM for 6h and then treated with Mg132 1µM for 2h. Extracts were analyzed with anti-p27^{Kip1} mouse monoclonal antibody at different time of exposure.

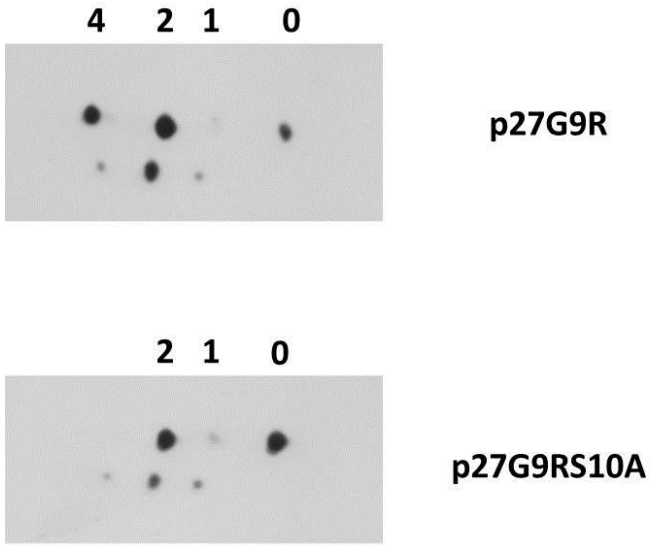

Fig. 35 pSer10p27^{Kip1}G9R isoforms analysis. 2D/WB analysis of NIH3T3 transfected with p27^{Kip1}G9R and p27^{Kip1}G9RS10A pCDNA3 vectors. NIH3T3 extracts were analyzed with anti-p27^{Kip1} mouse monoclonal antibody.

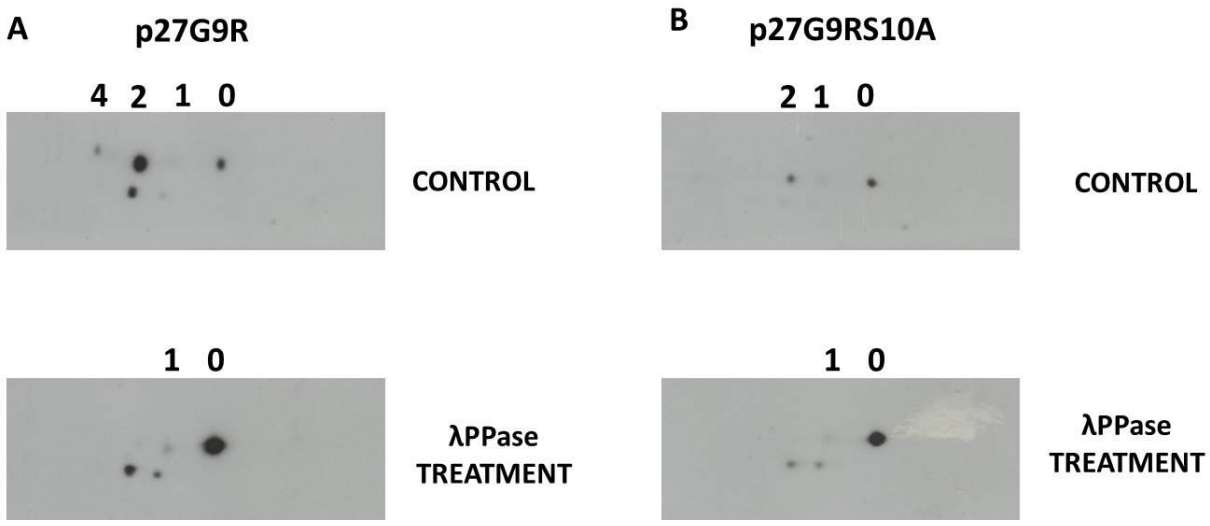

Fig. 36A-B p27^{Kip1}G9R and p27^{Kip1}G9RS10A λ phosphatase treatment. A) 2D/WB analysis of NIH3T3 transfected with p27^{Kip1}G9R pCDNA3 vector, before and after λ phosphatase (λPPase) treatment. NIH3T3 extracts were analyzed with anti-p27^{Kip1} mouse monoclonal antibody. B) 2D/WB analysis of NIH3T3 transfected with of p27^{Kip1}G9RS10A pCDNA3 vector, before and after λ phosphatase (λPPase) treatment. NIH3T3 extracts were analyzed with anti-p27^{Kip1} mouse monoclonal antibody.

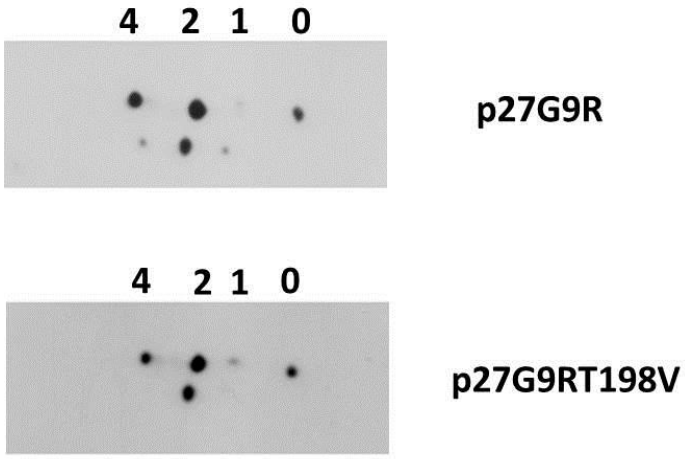

Fig 37 p27^{Kip1}G9R phosphoisoforms analysis. 2D/WB analysis of NIH3T3 transfected with p27^{Kip1}G9R and p27^{Kip1}G9RT198V pCNDA3 vectors. NIH3T3 extracts were analyzed with anti-p27^{Kip1} mouse monoclonal antibody.

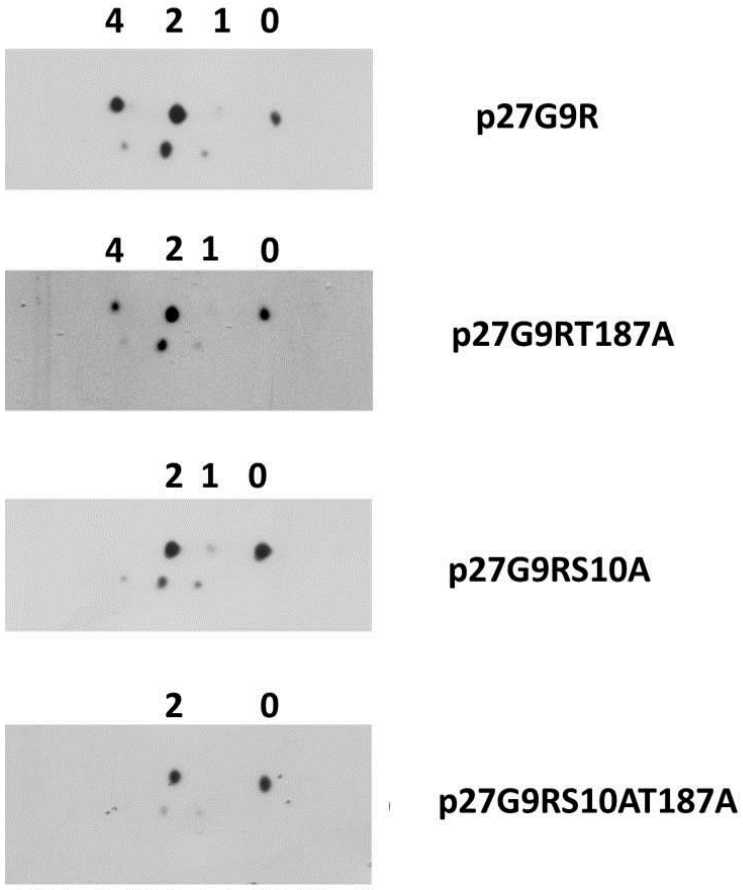

Fig. 38 p27^{Kip1}G9R phosphoisoforms analysis. 2D/WB analysis of NIH3T3 transfected with p27^{Kip1}G9R, p27^{Kip1}G9RS10A, p27^{Kip1}G9RT187A, p27^{Kip1}G9RS10AT187A pCDNA3 vectors. NIH3T3 extracts were analyzed with anti-p27^{Kip1} mouse monoclonal antibody.

4.9 p27^{Kip1}G9R interaction with CDKs

Until now, we found that p27^{Kip1} oncogenic mutant p27^{Kip1}G9R is principally located in cell nucleus and presents an enriched content in phosphorylated isoforms, especially in pSer10 amount. To investigate which characteristics make this mutant oncogenic, we decided to explore its functions starting from the cell compartment in which it is mainly situated, the nucleus. We began examining the possibility that p27^{Kip1}G9R could interact with CDKs differently from p27^{Kip1}WT. EPN were transfected with p27^{Kip1}WT, p27^{Kip1}G9R and p27^{Kip1}G9RS10A pCDNA3 vectors and then CDK2 and CDK1 were immunoprecipitated from nuclei. CDKs IPs were analyzed with WB and 2D/WB techniques. Fig. 39A shows that all the proteins interact with CDK2, and that p27^{Kip1}G9R binds to the kinase a little more than p27^{Kip1}WT and p27^{Kip1}G9RS10A. Instead, p27^{Kip1}G9R interacts less than the others with CDK1 (Fig. 40), it is possible to think that the large pSer10 content present in the mutated CKI, could have a role in

this weaker interaction, in fact when pSer10 is absent, mutant association to CDK1 is reverted to $p27^{Kip1}$WT level (Fig. 40). 2D/WB analysis of CDK2 IPs revealed that $p27^{Kip1}$WT interacts with the kinase as previously shown for endogenous protein in Lan-5 cell line. Instead $p27^{Kip1}$G9R interacts mainly with its monophosphorylated isoforms, however on CDK2 the ratio between unmodified and biphosphorylated isoforms is reverted, suggesting that probably there is not a pSer10 essential role in this association. In fact, in $p27^{Kip1}$G9RS10A transfected extracts, is observed only a weak variation in unmodified/monophosphorylated isoforms ratio in CDK2 IP compared to control extract (Fig. 41). These data suggest that in $p27^{Kip1}$G9R, pSer10 is not CDK2 main interactor and that probably another modification is responsible of this association. 2D/WB analysis of CDK1 IPs confirms that pSer10 content can cause $p27^{Kip1}$G9R reduced interaction with CDK1: as shown in Fig. 42, just a minimal percentage of biphosphorylated isoforms binds to the kinase. As previously described, almost the totality of $p27^{Kip1}$G9R biphosphorylated isoforms is composed by pSer10 and, as demonstrated in Lan-5 cell line, this post translational modification does not interact with CDK1. However a large part of mutated CKI monophosphorylated isoforms binds to CDK1, it is possible to think that some phosphorylation not well characterized could favorite this association.

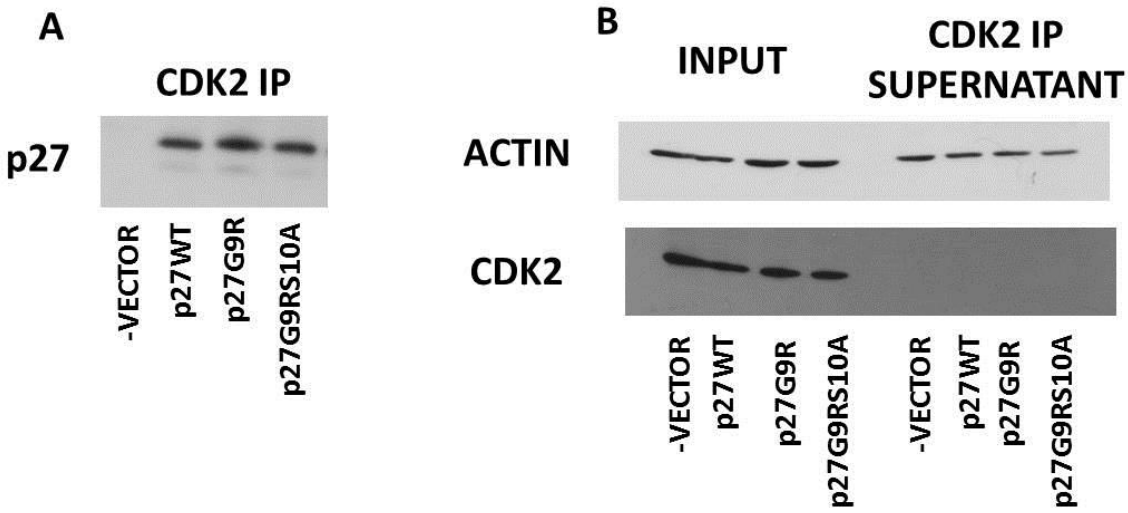

Fig. 39A-B $p27^{Kip1}$G9R interaction with CDK2. A) WB analysis of EPN nuclear extracts immunoprecipitated (50µg) with anti-CDK2 rabbit polyclonal antibody and analyzed with anti-$p27^{Kip1}$ mouse monoclonal antibody. EPN were previously transfected with $p27^{Kip1}$WT, $p27^{Kip1}$G9R and $p27^{Kip1}$G9RS10A pCDNA3 vectors (-VECTOR is transfection negative control). B) WB analysis of EPN nuclear extracts transfected as in A) and CDK2 IPs supernatans. All the samples were analyzed with anti-CDK2 and anti-Actin mouse monoclonal antibodies.

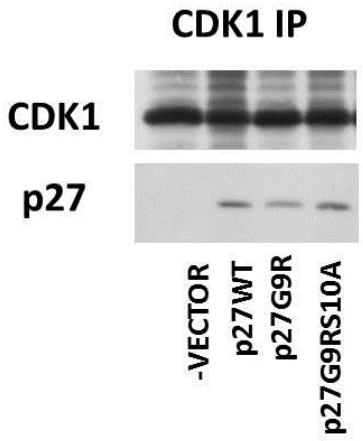

Fig. 40 p27^{Kip1}G9R interaction with CDK1. WB analysis of EPN nuclear extracts immunoprecipitated (50µg) with anti-CDK1 rabbit polyclonal antibody and analyzed with anti-p27^{Kip1} and anti-CDK1 mouse monoclonal antibodies. EPN were previously transfected with p27^{Kip1}WT, p27^{Kip1}G9R and p27^{Kip1}G9RS10A pCDNA3 vectors (-VECTOR is transfection negative control).

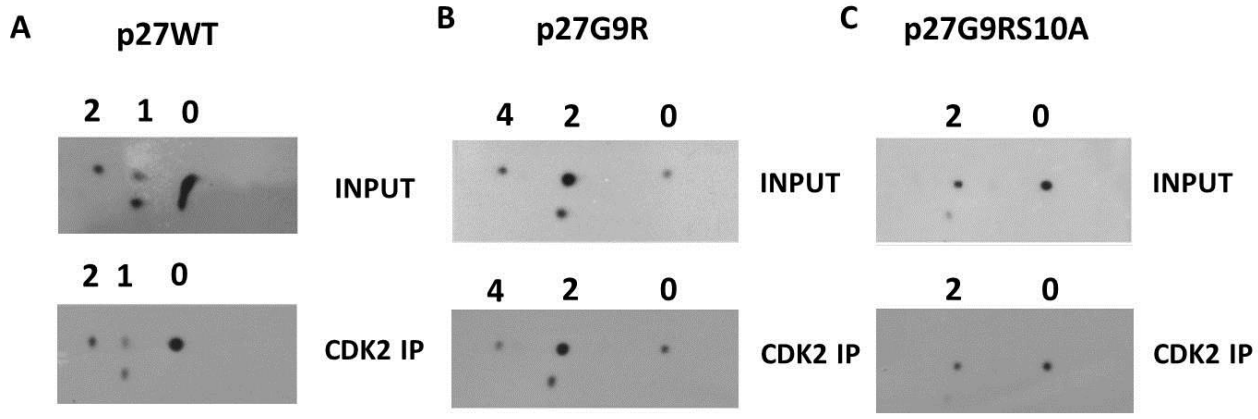

Fig. 41 A-C p27^{Kip1}G9R phosphoisoforms interaction with CDK2. A) 2D/WB analysis of EPN nuclear extracts immunoprecipitated (300µg) with anti-CDK2 rabbit polyclonal antibody and their INPUTs and analyzed with anti-p27^{Kip1} mouse monoclonal antibody. EPN were previously transfected with p27^{Kip1}WT pCDNA3 vector. B) 2D/WB analysis of EPN nuclear extracts immunoprecipitated (300µg) with anti-CDK2 rabbit polyclonal antibody and their INPUTs and analyzed with anti-p27^{Kip1} mouse monoclonal antibody. EPN were previously transfected with p27^{Kip1}G9R pCDNA3 vector. C) 2D/WB analysis of EPN nuclear extracts immunoprecipitated (300µg) with anti-CDK2 rabbit polyclonal antibody and their INPUTs and analyzed with anti-p27^{Kip1} mouse monoclonal antibody. EPN were previously transfected with p27^{Kip1}G9RS10A pCDNA3 vector.

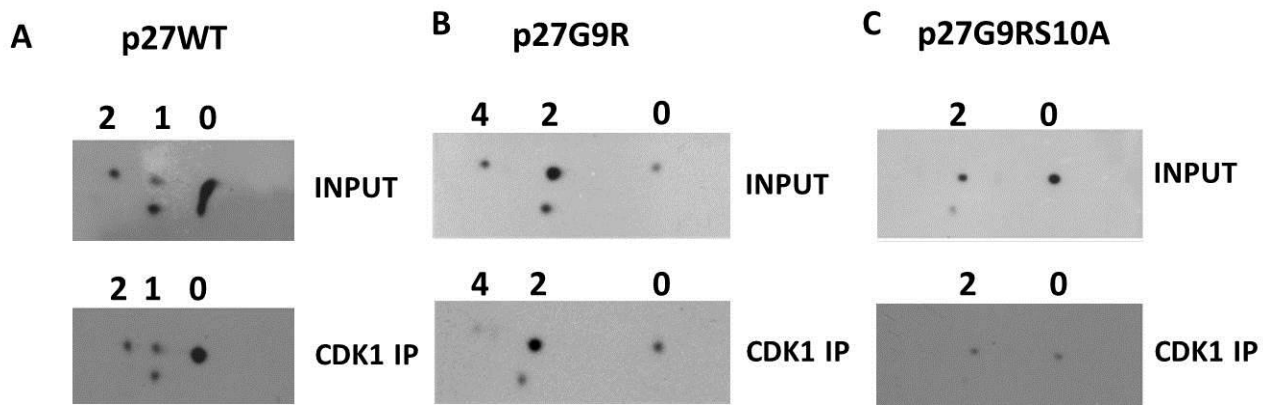

Fig. 42 A-C p27^{Kip1}G9R phosphoisoforms interaction with CDK1. A) 2D/WB analysis of EPN nuclear extracts immunoprecipitated (300µg) with anti-CDK1 rabbit polyclonal antibody and their INPUTs and analyzed with anti-p27^{Kip1} mouse monoclonal antibody. EPN were previously transfected with p27^{Kip1}WT pCDNA3 vector. B) 2D/WB analysis of EPN nuclear extracts immunoprecipitated (300µg) with anti-CDK1 rabbit polyclonal antibody and their INPUTs and analyzed with anti-p27^{Kip1} mouse monoclonal antibody. EPN were previously transfected with p27^{Kip1}G9R pCDNA3 vector. C) 2D/WB analysis of EPN nuclear extracts immunoprecipitated (300µg) with anti-CDK1 rabbit polyclonal antibody and their INPUTs and analyzed with anti-p27^{Kip1} mouse monoclonal antibody. EPN were previously transfected with p27^{Kip1}G9RS10A pCDNA3 vector.

4.10 p27^{Kip1}G9R functions in cell motility

Data obtained from CDK1 interaction analysis suggested to us that p27^{Kip1}G9R association to the kinase could contribute, at least in part, to mutant p27^{Kip1} oncogenic activity. Although p27^{Kip1}G9R is especially located in cell nucleus, we also decided to evaluate if its cytoplasmic content has some functions in tumor progression. In particular we focused our attention on p27^{Kip1}G9R possible involvement in cell motility increase. As widely documented in literature, one of the main p27^{Kip1} cytoplasmic molecular targets is RhoA-GTPase. Cytosolic p27^{Kip1} is able to bind to RhoA and prevent its activation by GEFs leading to enhanced cell motility[108].

At first we transfected NIH3T3 cell line with p27^{Kip1}WT and p27^{Kip1}G9R pCDNA3 vectors and evaluated their capability to migrate and to close the "healing" made on starved and confluent plates. NIH3T3 were photographed at time 0, monitored during successive hours and photographed again after 42 hours, when results were more appreciable. As shown in Fig. 43, there are not significant differences between migration induced by p27^{Kip1}WT and by p27^{Kip1}G9R. We confirm these data investigating RhoA signaling through RhoA immunoprecipitation and p-Cofilin WB analysis. EPN cell line were transfected with p27^{Kip1}WT and p27^{Kip1}G9R pCDNA3 vectors and then cytoplasmic extracts were immunoprecipitated with anti-RhoA antibody and analyzed with WB. Fig. 44A shows that p27^{Kip1}G9R is less associated to RhoA than p27^{Kip1}WT suggesting a GTPase lower inhibition by p27^{Kip1} mutant. phospho-Cofilin

levels were also examined from EPN cytoplasmic extracts, and as shown in Fig. 45 and in RhoA IP, $p27^{Kip1}$G9R does not determines a reduction in Cofilin phosphorylation, confirming the absence of an increased RhoA inactivation and the lack of an enhanced migration stimulus in $p27^{Kip1}$G9R transfected cells. Therefore, we suggest that $p27^{Kip1}$G9R does not seem favor cell motility.

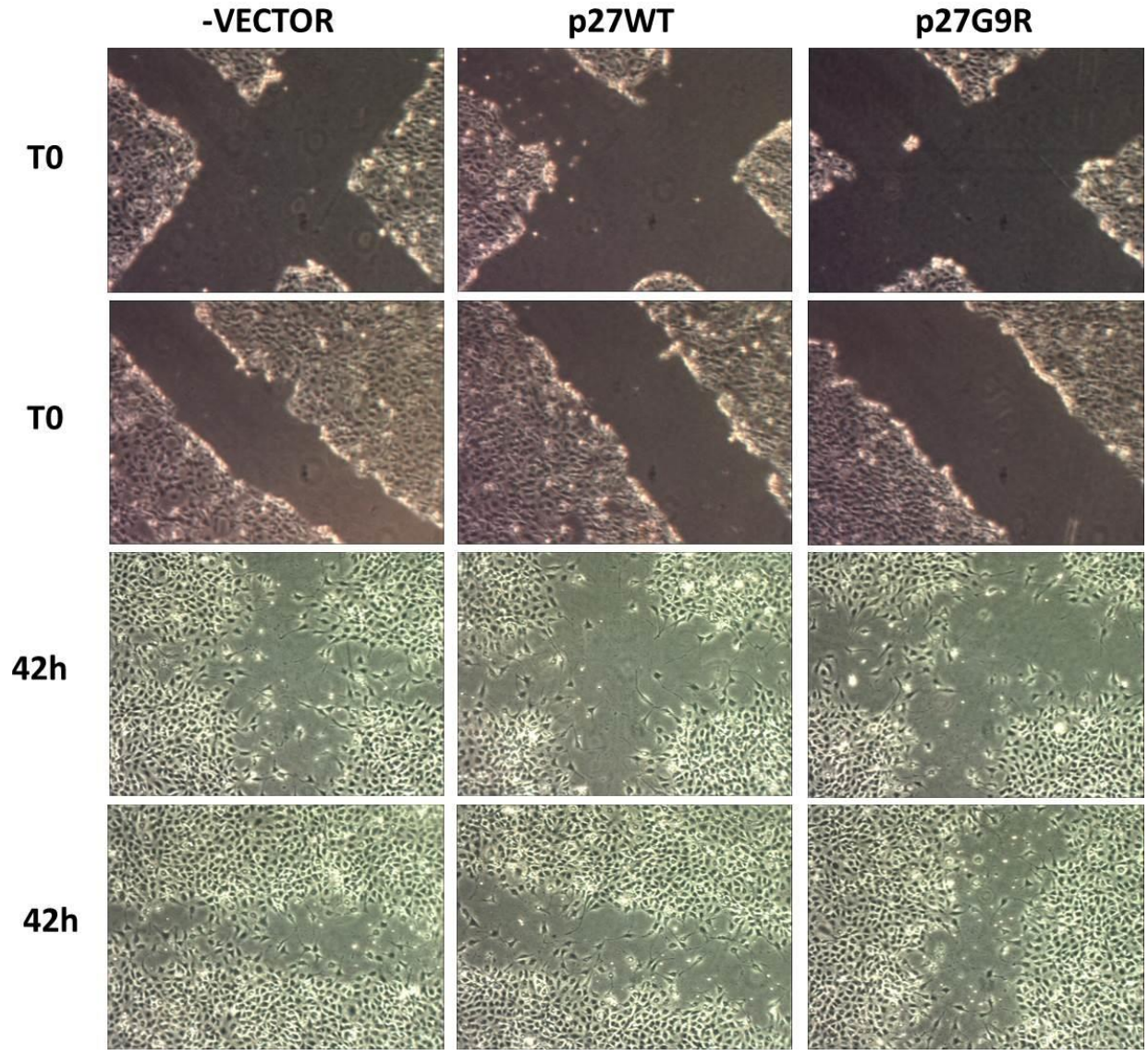

Fig. 43 $p27^{Kip1}$G9R and cell migration capability. "Wound Healing" assay of NIH3T3 transfected with $p27^{Kip1}$WT and $p27^{Kip1}$G9R pCDNA3 vectors (-VECTOR is transfection negative control). Cells were photographed at time 0 and after 42h.

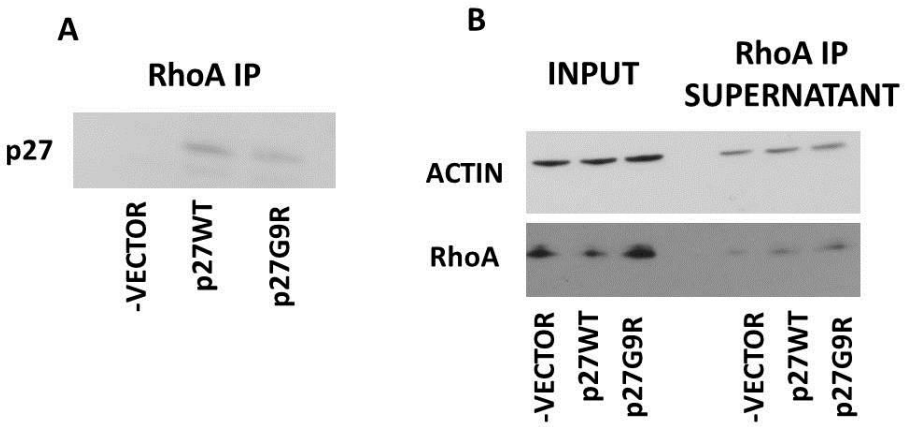

Fig. 44A-B A) p27^{Kip1}G9R interaction with RhoA. WB analysis of EPN cytoplasmic extracts immunoprecipitated (100µg) with anti-RhoA rabbit polyclonal antibody and analyzed with anti-p27^{Kip1} mouse monoclonal antibody. EPN were previously transfected with p27^{Kip1}WT and p27^{Kip1}G9R pCDNA3 vectors (-VECTOR is transfection negative control). B) WB analysis of RhoA IP supernatants and EPN cytoplasmic extracts transfected as in A), analyzed with anti-RhoA rabbit polyclonal antibody and anti-p27^{Kip1} mouse monoclonal antibody.

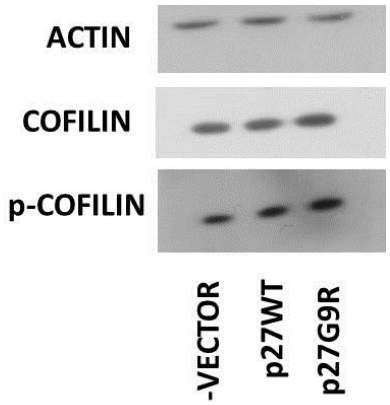

Fig. 45 p27^{Kip1}G9R effects on RhoA signaling. WB analysis of EPN cytoplasmic extracts analyzed with anti-Cofilin rabbit polyclonal antibody, anti-p-Cofilin, and anti-Actin mouse monoclonal antibodies. EPN were previously transfected with p27^{Kip1}WT and p27^{Kip1}G9R pCDNA3 vectors (-VECTOR is transfection negative control).

5. DISCUSSION AND PERSPECTIVES

$p27^{Kip1}$ is an atypical tumor suppressor. The protein has been initially considered only as a key regulator of cell proliferation, which explicates its activity mainly interfering with cyclin/CDK complexes. On this basis, since many tumors show CKI decreased levels, $p27^{Kip1}$ expression has been suggested to have a prognostic and therapeutic importance. On the other hand, in the last decade, $p27^{Kip1}$ has also emerged as a potential oncogene, because of its capability, when localized in the cytosol, to favor processes as cell migration and motility and to activate mitogenic pathway (for example, the PI3K signaling pathway). More recently, additional studies have demonstrated that mutations of *CDKN1B* gene can be implicated in the oncogenesis of some human tumors. Thus, a precise characterization of $p27^{Kip1}$ involvement in tumor progression is particularly intricate, and the protein has been defined as "Dr. Jekyll and Mr. Hyde"[100].

In addition, a number of $p27^{Kip1}$ features (the lack of secondary structure, its ability to bind to different targets and the occurrence of several post translational modifications), make remarkably difficult to investigate its roles. Particularly, the occurrence of numerous post-translational modifications (PTMs) strongly affects $p27^{Kip1}$ interactions. Thus, the knowledge of $p27^{Kip1}$ PTMs constitutes a fundamental step to unravel the CKI roles. In this study, we have focused our attention on the phosphorylation on serine 10. It has been reported that the Ser10 residue modification is responsible of $p27^{Kip1}$ increased stability. It has also been associated to some CKI oncogenic characteristics as cytoplasmic mislocalization and cyclin D/CDKs complexes assembly and nuclear translocation. $pSer10p27^{Kip1}$ represents the mayor $p27^{Kip1}$ nuclear phosphoisoform[87] and, as reported by Borriello et al in neuroblastoma cell model, its levels increase in response to ATRA treatment[95]. Thus, we decided to investigate the mechanisms by which this modification confers more stability to $p27^{Kip1}$ and to explore the possibility that pSer10 could have a role in cell nucleus focusing on cyclin/CDKs complexes regulation.

Examination of pSer10 Skp2-dependent ubiquitination revealed that the high nuclear content of this CKI isoform might be due, at least in part, to a reduced proteasome-dependent removal that it is known to represent the principal mechanism of $p27^{Kip1}$ nuclear degradation. This view is strengthened by the lack of a $p27^{Kip1}$ isoform phosphorylated contemporaneously on Ser10 and Thr187 residues. In fact, as demonstrated by 2D/WB of Lan-5 nuclear extracts treated with Mg132 and analyzed with anti-$pSer10p27^{Kip1}$ antibody, no differences have been observed in $pSer10p27^{Kip1}$ biphosphorylated amount after pThr187 depletion. Thus, we concluded that serine

10 phosphorylation might confer more stability to the CKI by allowing the escape from the proteasome-dependent degradation mechanism.

A detailed analysis of CKI biphosphorylated isoforms revealed that the second modification compatible with phosphorylation on Ser10 residue is pThr198. As known this modification is involved in some $p27^{Kip1}$ oncogenic properties: it can inhibit the CKI nuclear translocation, determining its cytoplasmic accumulation, it promotes cyclin D/CDKs complexes assembly and it can favor RhoA interaction and inhibition[84;85]. Although, $pThr198p27^{Kip1}$ is known to be localized principally in cytoplasm, our IP and WB experiments revealed that it also exists as nuclear isoform. Moreover, although 2D/WB experiments demonstrated that in Lan-5 cell nucleus exists a $pSer10pThr198p27^{Kip1}$ biphosphorylated isoform, its functions need to be further clarified.

As a subsequent step of this study, we have evaluated a possible $pSer10p27^{Kip1}$ function in cell cycle regulation. Particularly, we analyzed the phosphoisoform distribution in different cell division phases and its interaction with CDKs and cyclins. We observed that $pSer10p27^{Kip1}$ presents a pattern similar to total $p27^{Kip1}$, in that both are expressed at maximum levels in G0 phase and their expression decreases slightly in G1 phase. In S phase the total CKI and its phosphoserine 10 derivative are scarcely detectable and, finally, their content increases again in G2/M. These similarities in cell cycle distribution between $pSer10p27^{Kip1}$ and total $p27^{Kip1}$ could not be coincidental or foregone. $pThr187p27^{Kip1}$, for example, presents a different distribution. It is weakly detectable in G1, increases in S phase and is expressed at maximal levels in G2/M. What emerged from CDKs immunoprecipitation experiments is that $pSer10p27^{Kip1}$ binds to CDK4 and CDK2 (as unmodified $p27^{Kip1}$), but not to CDK1 (differently from unmodified $p27^{Kip1}$).

As previously discussed, we ruled out the existence of a contemporary phosphorylation on Ser10 and Thr187 residues. Thus, it is difficult to suggest that $pSer10p27^{Kip1}$ is a substrate of active CDK. It is more probable that $pSer10p27^{Kip1}$ associated to CDK2 mostly functions as kinase inhibitor. Although CDK2 can form complexes with both cyclin E and cyclin A, we found that cyclin E/CDK2 interacts preferentially with $pSer10p27^{Kip1}$ isoform while cyclin A/CDK2 associates with unmodified protein. These data suggest that $pSer10p27^{Kip1}$ binds to and inhibits cyclin E/CDK2 complex, possible leading to a cell cycle block. In conclusion, we hypothesized that this serine 10 modification can contribute to $p27^{Kip1}$ canonical antiproliferative activity while we ruled out that it presents oncogenic properties.

Recently, it has been hypothesized that mutant forms of $p27^{Kip1}$ are involved in human carcinogenesis since mutations of *CDKN1B* have been found in several cancers. Variations have been found in endocrine tumors[117;118] as NETs (especially siNET and pNET), MEN4, pituitary adenomas and parathyroid adenomas, and also in breast cancer[120] and hairy cell leukemia[121].

In this study, we have chosen to characterize a $p27^{Kip1}$ mutant identified by Costa-Guida et al, in a patient with non-familial parathyroid adenoma. This mutation (c.25G<A in *CDKN1B* exon 1) lead to a Gly substitution in position 9 with an Arg[116]. Considering that the site of mutation is strictly associated to serine 10, and that Ser10 phosphorylation plays an important role in the CKI anticancer functions, we decided to evaluate how Gly9Arg substitution is involved in tumorigenesis and, in particular, whether the mutation affects Ser10 phosphorylation.

We observed that $p27^{Kip1}$G9R presents some peculiar and unexpected features. First, it is principally located in cell nucleus. Indeed, although $p27^{Kip1}$ mutated in G9R is associated with tumorigenesis, only a minor percentage of its amount is located is cytoplasm, where, conversely, $p27^{Kip1}$ generally explicates oncogenic activity. Second, it is characterized by an elevated phosphorylation degree, remarkably higher than the wild-type protein. In addition, by 2D/WB analysis we demonstrated that while unmodified Gly9Argp27^{Kip1} is scarcely present in cell extract of transfected cells, high levels of mono- and bi-phosphorylated isoforms are detectable.

Thus we analyzed $p27^{Kip1}$G9R phosphoisoforms pattern and verified that almost the totality of mutant biphosphorylated isoforms and a part of monophosphorylated isoforms, includes serine 10 phosphorylation. An additional site of phosphorylation should be present in the protein, although we still failed in its identification. Subsequent CDKs association experiments evidenced that $p27^{Kip1}$G9R binds to CDK2 similarly to the wild-type protein, but binds to CDK1 with a minor affinity compared to $p27^{Kip1}$. This reduced interaction with CDK1 is possibly linked to pSer10 hyperphosphorylation. As demonstrated by 2D/WB analysis, $p27^{Kip1}$G9R associates to the kinase with its unmodified isoforms, with a percentage of its monophosphorylated isoforms, but not with biphosphorylated. These data allowed us to hypothesize that part of $p27^{Kip1}$G9R oncogenic action could be explicated through a reduction in CDK1 complexes inhibition. In this case it seems that the high phosphorylation grade on Ser10 confers oncogenic properties to mutant $p27^{Kip1}$.

We finally evaluated the possibility that the minimal $p27^{Kip1}$G9R cytoplasmic amount could be involved in cell migration increase. However neither “wound healing” experiments, and neither RhoA interaction and signaling analysis, suggested an augmented cell motility.

What emerges from this work is the high complexity and difficulty of studying the function(s) of intrinsically unstructured proteins and how in these studies are important the precise characterization of the PTMs. As a matter of facts, these modifications might significantly affect protein localization, metabolism and functions. Although we provides novel insights in pSer10p27^{Kip1} nuclear metabolism and functions, several aspects of this modification, as its role in p27^{Kip1} oncogenic mutants, need to be clarified to obtain more information on the role of this protein in carcinogenesis and to develop novel therapies based on the handling of CKIs level and activities.

6. REFERENCES

1) Li J. M. and Brooks G. “Cell cycle regulatory molecules (cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors) and the cardiovascular system; potential targets for therapy?” European Heart Journal (1999) 20, 406–420

2) Guan KL, Jenkins CW, Li Y, Nichols MA, Wu X, O'Keefe CL, Matera AG, Xiong Y. “Growth suppression by p18, a p16INK4/MTS1- and p 14INK4B/MTS2- related CDK6 inhibitor, correlates with wild-type pRb function”. Genes & Development (1994) 8:2939-2952.

3) Sherr CJ, Roberts JM “Inhibitors of mammalian G 1 cyclin-dependent kinases” Genes & Development (1995) 9; 1149-1163.

4) Elledge SJ “Cell cycle checkpoints: preventing an identity crisis”. Science (1996) 274; 1664-72.

5) Hannon GJ, Beach D “p15INK4B is a potential effector of TGF-β-induced cell cycle arrest.” Nature (1994) 371; 257-61.

6) Serrano M, Hannon GJ, Beach D “A new regulatory motif of cell-cycle control causing specific inhibition of cyclin D/CDK4”. Nature (1993) 366; 704-7.

7) Chan FK, Zhang J, Cheng L, Shapiro DN, Winoto A “Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16INK4”. Molecular and Cellular Biology (1995) 2682–2688.

8) Della Ragione F, Takabayashi K, Mastropietro S, Mercurio C, Oliva A, Russo GL, Della Pietra V, Borriello A, Nobori T, Carson DA, Zappia V “Purification and characterization of

recombinant human 5'-methylthioadenosine phosphorylase: definite identification of coding cDNA." Biochemical and Biophysical Research Communications (1996) 223; 514-519.

9) Gu Y, Turck CW, Morgan DO "Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit". Nature (1993) 366; 707-10.

10) Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A "p27 Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-13 and contact inhibition to cell cycle arrest". Genes & Development (1994) 8; 9-22

11) Lee MH, Reynisdóttir I, Massagué J "Cloning of p57Kip2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution". Genes & Development (1995) 9; 639-649.

12) Ou L, Ferreira AM, Otieno S, Xiao L, Bashford D, Kriwacki RW "Incomplete Folding upon Binding Mediates Cdk4/Cyclin D Complex Activation by Tyrosine Phosphorylation of Inhibitor p27 Protein." The Journal of Biological Chemistry (2011) 286; 30142–30151.

13) LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow E. "New functional activities for the p21 family of CDK inhibitors". Genes & Development (1997) 11;847-62.

14) Cheng M, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM, Sherr CJ. "The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts". The EMBO Journal (1999) 18; 1571–1583.

15) Cheng M, Sexl V, Sherr CJ, Roussel MF. "Assembly of cyclin D-dependent kinase and titration of p27Kip1 regulated by mitogen-activated protein kinase kinase (MEK1)". Proc Natl Acad Sci U S A. (1998) 95;1091-6.

16) Starostina NG, Kipreos ET. “Multiple degradation pathways regulate versatile CIP/Kip CDK inhibitors”. Trends Cell Biology (2012) 1; 33-41

17) Pérez-Sayáns M, Suárez-Peñaranda JM, Gayoso-Diz P, Barros-Angueira F, Gándara-Rey JM, García-García A. “The role of p21Waf1/CIP1 as a Cip/Kip type cell-cycle regulator in oral squamous cell carcinoma”. Med Oral Patol Oral Cir Bucal. (2013) 18; 219-25.

18) Coats S, Flanagan WM, Nourse J, Roberts JM. “Requirement of p27Kip1 for restriction point control of the fibroblast cell cycle. Science (!996) 272; 877-80.

19) Li G, Domenico J, Lucas JJ, Gelfand EW. “Identification of multiple cell cycle regulatory functions of p57Kip2 in human T lymphocytes”. The Journal of Immunology (2004) 173;2383-91.

20) Besson A, Dowdy SF, Roberts JM. “CDK inhibitors: cell cycle regulators and beyond”. Developmental Cell (2008) 2; 159-69.

21) Lacy ER, Filippov I, Lewis WS, Otieno S, Xiao L, Weiss S, Hengst L, Kriwacki RW. “p27 binds cyclin-CDK complexes through a sequential mechanism involving binding-induced protein folding”. Nature Structural & Molecular Biology (2004) 4;358-64.

22) Kriwacki RW, Hengst L, Tennant L, Reed SI, Wright PE. “Structural studies of p21Waf1/Cip1/Sdi1 in the free and Cdk2-bound state: conformational disorder mediates binding diversity”. Proc Natl Acad Sci U S A (1996) 93; 11504-9.

23) Bienkiewicz EA, Adkins JN, Lumb KJ. “Functional consequences of preorganized helical structure in the intrinsically disordered cell-cycle inhibitor p27(Kip1).” Biochemistry (2002) 41; 752-9.

24) Adkins JN, Lumb KJ. “Intrinsic structural disorder and sequence features of the cell cycle inhibitor p57Kip2”. Proteins (2002) 46;1-7.

25) Goubin F, Ducommun B. “Identification of binding domains on the p21Cip1 cyclin-dependent kinase inhibitor”. Oncogene (1995) 10;2281-7.

26) Waga S, Hannon GJ, Beach D, Stillman B. “The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA”. Nature (1994) 369;574–578.

27) Watanabe H, Pan ZQ, Schreiber-Agus N, DePinho RA, Hurwitz J, Xiong Y. “Suppression of cell transformation by the cyclin-dependent kinase inhibitor p57KIP2 requires binding to proliferating cell nuclear antigen”. Proc Natl Acad Sci U S A (1998) 95;1392–1397.

28) Montagnoli A, Fiore F, Eytan E, Carrano AC, Draetta GF, Hershko A, Pagano M. “Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation”. Genes &Development (1999) 13;1181–1189.

29) Nguyen H, Gitig DM, Koff A. “Cell-free degradation of p27(kip1), a G1 cyclin-dependent kinase inhibitor, is dependent on CDK2 activity and the proteasome”. Molecular Cell Biology (1999) 19;1190–1201.

30) Kamura T, Hara T, Kotoshiba S, Yada M, Ishida N, Imaki H, Hatakeyama S, Nakayama K, Nakayama KI. “Degradation of p57Kip2 mediated by SCFSkp2-dependent ubiquitylation”. Proc Natl Acad Sci USA (2003) 100;10231–10236.

31) Galea CA, Wang Y, Sivakolundu SG, Kriwacki RW. “Regulation of cell division by intrinsically unstructured proteins: intrinsic flexibility, modularity, and signaling conduits”. Biochemistry (2008) 47;7598–7609.

32) Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevsky SA, Dowdy SF. “Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration”. Nature Medicine (1998) 4; 1449-52.

33) McAllister SS, Becker-Hapak M, Pintucci G, Pagano M, Dowdy SF. “Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions”. Molecular and Cellular Biology (2003) 1;216-228.

34) Yokoo T, Toyoshima H, Miura M, Wang Y, Iida KT, Suzuki H, Sone H, Shimano H, Gotoda T, Nishimori S, Tanaka K, Yamada N. “p57Kip2 regulates actin dynamics by binding and translocating LIM-kinase 1 to the nucleus”. Journal of Biological Chemistry (2003) 278;52919-23.

35) Borriello A, Bencivenga D, Criscuolo M, Caldarelli I, Cucciolla V, Tramontano A, Borgia A, Spina A, Oliva A, Naviglio S, Della Ragione F. “Targeting p27Kip1 protein: its relevance in the therapy of human cancer”. Expert Opin Ther Targets (2011) 15;677-93.

36) Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. “Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress”. Nature (2002) 419;316-21.

37) Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. “Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor”. Cell (1999) 96;857-68.

38) Tran PT, Erdeniz N, Dudley S, Liskay RM. “Characterization of nuclease-dependent functions of Exo1p in Saccharomyces cerevisiae”. DNA Repair (2002) 1;895-912.

39) Burgering BM, Kops GJ. “Cell cycle and death control: long live Forkheads”. Trends in Biochemical Science (2002) 27;352-60.

40) Chu IM, Hengst L, Slingerland JM. “The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy”. Nature Reviews Cancer (2008) 8;253-267.

41) Karnik SK, Hughes CM, Gu X, Rozenblatt-Rosen O, McLean GW, Xiong Y, Meyerson M, Kim SK. “Menin regulates pancreatic islet growth by promoting histone methylation and expression of genes encoding p27Kip1 and p18INK4c”. Proc Natl Acad Sci USA. (2005) 102;14659-64.

42) Fontanière S, Casse H, Bertolino P, Zhang CX. “Analysis of p27(Kip1) expression in insulinomas developed in pancreatic beta-cell specific Men1 mutant mice”. Familial Cancer (2006) 5;49-54.

43) Pellegata NS, Quintanilla-Martinez L, Siggelkow H, Samson E, Bink K, Höfler H, Fend F, Graw J, Atkinson MJ. "Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans". PNAS (2006) 103;15558-63.

44) Wang C, Hou X, Mohapatra S, Ma Y, Cress WD, Pledger WJ, Chen J. "Activation of p27Kip1 Expression by E2F1. A negative feedback mechanism". Journal of Biological Chemistry (2005) 280;12339-43.

45) Murata K, Hattori M, Hirai N, Shinozuka Y, Hirata H, Kageyama R, Sakai T, Minato N. "Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1". Molecular and Cellular Biology (2005) 25; 4262-71.

46) Huang YC, Chen JY, Hung WC. "Vitamin D3 receptor/Sp1 complex is required for the induction of p27Kip1 expression by vitamin D3". Oncogene (2005) 23; 4856-61.

47) Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafrè SA, Farace MG. "miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1". Rhe Journal of Biological Chemistry (2007) 282; 23716-24.

48) Visone R, Russo L, Pallante P, De Martino I, Ferraro A, Leone V, Borbone E, Petrocca F, Alder H, Croce CM, Fusco A. "MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle". Endocrine Related Cancer (2007) 14;791-8.

49) Fu X, Wang Q, Chen J, Huang X, Chen X, Cao L, Tan H, Li W, Zhang L, Bi J, Su Q, Chen L. "Clinical significance of miR-221 and its inverse correlation with p27Kip¹ in hepatocellular carcinoma". Mol Biol Rep. (2011) 38;3029-35.

50) Frenquelli M, Muzio M, Scielzo C, Fazi C, Scarfò L, Rossi C, Ferrari G, Ghia P, Caligaris-Cappio F. “MicroRNA and proliferation control in chronic lymphocytic leukemia: functional relationship between miR-221/222 cluster and p27”. Blood (2010) 115; 3949-59.

51) Wurz K, Garcia RL, Goff BA, Mitchell PS, Lee JH, Tewari M, Swisher EM. “MiR-221 and MiR-222 alterations in sporadic ovarian carcinoma: Relationship to CDKN1B, CDKNIC and overall survival”. Genes Chromosomes Cancer (2010) 49;577-84.

52) Russo AA, Jeffrey PD, Patten AK, Massagué J, Pavletich NP. “Crystal structure of the p27Kip1 cyclin-dependent-kinase inhibitor bound to the cyclin A-Cdk2 complex”. Nature (1996) 382;325-31.

53) Yoon MK, Mitrea DM, Ou L, Kriwacki RW. “Cell cycle regulation by the intrinsically disordered proteins p21 and p27”. Biochemical Society Transactions (2012) 40;981-88.

54) Borriello A, Cucciolla V, Oliva A, Zappia V, Della Ragione F. “p27Kip1 metabolism: a fascinating labyrinth”. Cell Cycle (2007) 6;1053-61.

55) Besson A, Gurian-West M, Schmidt A, Hall A, Roberts JM. “p27Kip1 modulates cell migration through the regulation of RhoA activation”. Genes & Development (2004) 18;862-76.

56) McAllister SS, Becker-Hapak M, Pintucci G, Pagano M, Dowdy SF. “Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions”. Molecular Cell Biology (2003) 23;216-28.

57) Baldassarre G, Belletti B, Nicoloso MS, Schiappacassi M, Vecchione A, Spessotto P, Morrione A, Canzonieri V, Colombatti A. “p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion”. Cancer Cell (2005) 7;51-63.

58) Moeller SJ, Head ED, Sheaff RJ. “p27Kip1 inhibition of GRB2-SOS formation can regulate Ras activation”. Molecular Cell Biology (2003) 23;3735-52.

59) Fujita N, Sato S, Katayama K, Tsuruo T. “Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization”. Journal of biological chemistry (2002) 277;28706-13.

60) Zeng Y, Hirano K, Hirano M, Nishimura J, Kanaide H. “Minimal requirements for the nuclear localization of p27(Kip1), a cyclin-dependent kinase inhibitor”. Biochem Biophys Res Commun. (2000) 274;37-42.

61) Wang Y, Fisher JC, Mathew R, Ou L, Otieno S, Sublet J, Xiao L, Chen J, Roussel MF, Kriwacki RW. “Intrinsic disorder mediates the diverse regulatory functions of the Cdk inhibitor p21”. Nature Chemical Biology (2011) 7; 214-21

62) Besson A, Gurian-West M, Chen X, Kelly-Spratt KS, Kemp CJ, Roberts JM. “A pathway in quiescent cells that controls p27Kip1 stability, subcellular localization, and tumor suppression”. Genes & Development (2006) 20;47-64.

63) Nakayama K, Nagahama H, Minamishima YA, Miyake S, Ishida N, Hatakeyama S, Kitagawa M, Iemura S, Natsume T, Nakayama KI. “Skp2-mediated degradation of p27 regulates progression into mitosis”. Developmental Cell (2004) 6;661-72.

64) See WL, Miller JP, Squatrito M, Holland E, Resh MD, Koff A. “Defective DNA double-strand break repair underlies enhanced tumorigenesis and chromosomal instability in p27-

deficient mice with growth factor-induced oligodendrogliomas". Oncogene (2010) 29;1720-31.

65) Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai LH, Broudy V, Perlmutter RM, Kaushansky K, Roberts JM. "A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice". Cell (1996) 85;733-44.

66) Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, Horii I, Loh DY, Nakayama K. "Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors". Cell (1996) 85;707-20.

67) Kardinal C, Dangers M, Kardinal A, Koch A, Brandt DT, Tamura T, Welte K. "Tyrosine phosphorylation modulates binding preference to cyclin-dependent kinases and subcellular localization of p27Kip1 in the acute promyelocytic leukemia cell line NB4". Blood (2006) 107;1133-40.

68) Grimmler M, Wang Y, Mund T, Cilensek Z, Keidel EM, Waddell MB, Jäkel H, Kullmann M, Kriwacki RW, Hengst L. "Cdk-inhibitory activity and stability of p27Kip1 are directly regulated by oncogenic tyrosine kinases". Cell (2007) 128; 269-80.

69) Chu I, Sun J, Arnaout A, Kahn H, Hanna W, Narod S, Sun P, Tan CK, Hengst L, Slingerland J. "p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2". Cell (2007) 128;281-94.

70) Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. "Cyclin E-CDK2 is a regulator of p27Kip1". Genes & Development (1997) 11;1464-78.

71) Vlach J, Hennecke S, Amati B. “Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27”. (1997) 16;5334-44.

72) Nakayama KI, Nakayama K. “Ubiquitin ligases: cell-cycle control and cancer”. Nature Review Cancer (2006) 6;369-81.

73) Bloom J, Pagano M “Deregulated degradation of the cdk inhibitor p27 and malignant transformation”. Seminars in Cancer Biology (2003) 13;41-7.

74) Malek NP, Sundberg H, McGrew S, Nakayama K, Kyriakides TR, Roberts JM. “A mouse knock-in model exposes sequential proteolytic pathways that regulate p27Kip1 in G1 and S phase”. Nature (2001) 413;323-7.

75) Bondar T, Kalinina A, Khair L, Kopanja D, Nag A, Bagchi S, Raychaudhuri P. “Cul4A and DDB1 associate with Skp2 to target p27Kip1 for proteolysis involving the COP9 signalosome”. Molecular and Cellular Biology (2006) 26;2531-9.

76) Li B, Jia N, Kapur R, Chun KT. “Cul4A targets p27 for degradation and regulates proliferation, cell cycle exit, and differentiation during erythropoiesis”. Blood (2006) 107;4291-9.

77) Ishida N, Hara T, Kamura T, Yoshida M, Nakayama K, Nakayama KI. “Phosphorylation of p27Kip1 on serine 10 is required for its binding to CRM1 and nuclear export”. The Journal of Biological Chemistry (2002) 277;14355-8.

78) Connor MK, Kotchetkov R, Cariou S, Resch A, Lupetti R, Beniston RG, Melchior F, Hengst L, Slingerland JM. “CRM1/Ran-mediated nuclear export of p27(Kip1) involves a

nuclear export signal and links p27 export and proteolysis". Molecular Biology of the Cell (2003) 14;201-13.

79) Delmas C, Aragou N, Poussard S, Cottin P, Darbon JM, Manenti S. "MAP kinase-dependent degradation of p27Kip1 by calpains in choroidal melanoma cells. Requirement of p27Kip1 nuclear export". Journal of Biological Chemistry (2003) 278;12443-51.

80) Akashiba H, Matsuki N, Nishiyama N. "Calpain activation is required for glutamate-induced p27 down-regulation in cultured cortical neurons". Journal of Neurochemistry (2006) 99;733-44.

81) Fuster JJ, González JM, Edo MD, Viana R, Boya P, Cervera J, Verges M, Rivera J, Andrés V. "Tumor suppressor p27(Kip1) undergoes endolysosomal degradation through its interaction with sorting nexin 6". FASEB J. (2010) 24;2998-3009

82) Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E, Slingerland JM. "PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest". Nature Medicine (2002) 8;1153-60.

83) Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, Fusco A, Santoro M. "Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer". Nature Medicine (2002) 8;1136-44.

84) Fujita N, Sato S, Tsuruo T. "Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization". Journal of Biological Chemistry (2003) 278;49254-60.

85) De Vita F, Giuliani F, Silvestris N, Rossetti S, Pizzolorusso A, Santabarbara G, Galizia G, Colucci G, Ciardiello F, Orditura M. “Current status of targeted therapies in advanced gastric cancer”. Expert Opinion on Therapeutic Targets (2012) 16;S29-34.

86) Motti ML, De Marco C, Califano D, Fusco A, Viglietto G. “Akt-dependent T198 phosphorylation of cyclin-dependent kinase inhibitor p27kip1 in breast cancer”. Cell Cycle (2004) 3;1074-80

87) Ishida N, Kitagawa M, Hatakeyama S, Nakayama K. “Phosphorylation at serine 10, a major phosphorylation site of p27(Kip1), increases its protein stability”. The Journal of Biological Chemistry (2000) 275;25146-54.

88) Alessandrini A, Chiaur DS, Pagano M. “Regulation of the cyclin-dependent kinase inhibitor p27 by degradation and phosphorylation”. Leukemia (1997) 11;342-5.

89) Boehm M, Yoshimoto T, Crook MF, Nallamshetty S, True A, Nabel GJ, Nabel EG. “A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression”. EMBO (2002) 21; 3390-401.

90) Deng X, Mercer SE, Shah S, Ewton DZ, Friedman E. “The cyclin-dependent kinase inhibitor p27Kip1 is stabilized in G(0) by Mirk/dyrk1B kinase”. The Journal of Biological Chemistry (2004) 279; 22498-504.

91) Kawauchi T, Chihama K, Nabeshima Y, Hoshino M. “Cdk5 phosphorylates and stabilizes p27kip1 contributing to actin organization and cortical neuronal migration”. Nature Cell Biology (2006) 8;17-26.

92) Kajihara R, Fukushige S, Shioda N, Tanabe K, Fukunaga K, Inui S. “CaMKII phosphorylates serine 10 of p27 and confers apoptosis resistance to HeLa cells”. Biochem Biophys Res Commun. (2010) 401;350-5.

93) Shin I, Rotty J, Wu FY, Arteaga CL. “Phosphorylation of p27Kip1 at Thr-157 interferes with its association with importin alpha during G1 and prevents nuclear re-entry”. The Journal of Biological Chemistry (2005) 280;6055-63.

94) Rodier G, Montagnoli A, Di Marcotullio L, Coulombe P, Draetta GF, Pagano M, Meloche S. “p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis”. EMBO (2001) 20;6672-82.

95) Borriello A, Cucciolla V, Criscuolo M, Indaco S, Oliva A, Giovane A, Bencivenga D, Iolascon A, Zappia V, Della Ragione F. “Retinoic acid induces p27Kip1 nuclear accumulation by modulating its phosphorylation”. Cancer Research (2006) 66;4240-8.

96) Tsai LH, Takahashi T, Caviness VS Jr, Harlow E. “Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system”. Development (1993) 119;1029-40.

97) Lee MH, Nikolic M, Baptista CA, Lai E, Tsai LH, Massagué J. “The brain-specific activator p35 allows Cdk5 to escape inhibition by p27Kip1 in neurons”. PNAS (1996) 93;3259-63.

98) Brinkkoetter PT, Olivier P, Wu JS, Henderson S, Krofft RD, Pippin JW, Hockenbery D, Roberts JM, Shankland SJ. “Cyclin I activates Cdk5 and regulates expression of Bcl-2 and Bcl-XL in postmitotic mouse cells”. The Journal of Clinical Investigation (2009) 119;3089-101.

99) Su SC, Tsai LH. “Cyclin-dependent kinases in brain development and disease”. Annu Rev Cell Dev Biol. (2011) 27;465-91.

100) Larrea MD, Wander SA, Slingerland JM. “p27 as Jekyll and Hyde: regulation of cell cycle and cell motility”. Cell Cycle (2009) 8;3455-61.

101) Tsihlias J, Kapusta L, Slingerland J. “The prognostic significance of altered cyclin-dependent kinase inhibitors in human cancer”. Annu Rev Med (1999) 50;401-23.

102) Slingerland J, Pagano M. “Regulation of the cdk inhibitor p27 and its deregulation in cancer”. Journal of Cellular Physiology (2000) 183;10-7.

103) le Sage C, Nagel R, Agami R. “Diverse ways to control p27Kip1 function: miRNAs come into play”. Cell Cycle (2007) 6;2742-9.

104) le Sage C, Nagel R, Egan DA, Schrier M, Mesman E, Mangiola A, Anile C, Maira G, Mercatelli N, Ciafrè SA, Farace MG, Agami R. “Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation”. EMBO (2007) 26; 3699-708.

105) Hershko DD. “Oncogenic properties and prognostic implications of the ubiquitin ligase Skp2 in cancer”. Cancer (2008) 112; 1415-24.

106) Susaki E, Nakayama K, Nakayama KI. “Cyclin D2 translocates p27 out of the nucleus and promotes its degradation at the G0-G1 transition”. Molecular and Cellular Biology (2007) 27; 4626-40.

107) Bar-Sagi D, Hall A. “Ras and Rho GTPases: a family reunion”. Cell (2000) 103;227-38.

108) Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, Becker-Hapak M, Ezhevsky SA, Dowdy SF. “Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration”. Nature Medicine (1998) 4;1449-52.

109) Wu FY, Wang SE, Sanders ME, Shin I, Rojo F, Baselga J, Arteaga CL. “Reduction of cytosolic p27(Kip1) inhibits cancer cell motility, survival, and tumorigenicity”. Cancer Research (2006) 7;51-63.

110) Denicourt C, Saenz CC, Datnow B, Cui XS, Dowdy SF. “Relocalized p27Kip1 tumor suppressor functions as a cytoplasmic metastatic oncogene in melanoma”. Cancer Research (2007) 67;9238-43.

111) Larrea MD, Hong F, Wander SA, da Silva TG, Helfman D, Lannigan D, Smith JA, Slingerland JM. “RSK1 drives p27Kip1 phosphorylation at T198 to promote RhoA inhibition and increase cell motility”. PNAS (2009) 106;9268-73

112) Schiappacassi M, Lovat F, Canzonieri V, Belletti B, Berton S, Di Stefano D, Vecchione A, Colombatti A, Baldassarre G. “p27Kip1 expression inhibits glioblastoma growth, invasion, and tumor-induced neoangiogenesis”. Molecular Cancer Therapeutics (2008) 7;1164-75.

113) Belletti B, Pellizzari I, Berton S, Fabris L, Wolf K, Lovat F, Schiappacassi M, D'Andrea S, Nicoloso MS, Lovisa S, Sonego M, Defilippi P, Vecchione A, Colombatti A, Friedl P, Baldassarre G. “p27kip1 controls cell morphology and motility by regulating

microtubule-dependent lipid raft recycling". Molecular and Cellular Biology (2010). 30;2229-40.

114) Borriello A, Bencivenga D, Della Ragione F. "The unpredictable consequences of CDKN1B/p27Kip1 mutations in cancer". Cell Cycle (2015) 14;1-2.

115) Fero ML, Randel E, Gurley KE, Roberts JM, Kemp CJ. "The murine gene p27Kip1 is haplo-insufficient for tumour suppression". Nature (1998) 396;177-80.

116) Costa-Guda J, Marinoni I, Molatore S, Pellegata NS, Arnold A. "Somatic mutation and germline sequence abnormalities in CDKN1B, encoding p27Kip1, in sporadic parathyroid adenomas". Journal of Clinical Endocrinology and Metabolism (2011) 96;E701-E706.

117) Marinoni I, Pellegata NS. "p27kip1: a new multiple endocrine neoplasia gene?" Neuroendocrinology (2011) 93;19-28.

118) Francis JM, Kiezun A, Ramos AH, Serra S, Pedamallu CS, Qian ZR, Banck MS, Kanwar R, Kulkarni AA, Karpathakis A, Manzo V, Contractor T, Philips J, Nickerson E, Pho N, Hooshmand SM, Brais LK, Lawrence MS, Pugh T, McKenna A, Sivachenko A, Cibulskis K, Carter SL, Ojesina AI, Freeman S, Jones RT, Voet D, Saksena G, Auclair D, Onofrio R, Shefler E, Sougnez C, Grimsby J, Green L, Lennon N, Meyer T, Caplin M, Chung DC, Beutler AS, Ogino S, Thirlwell C, Shivdasani R, Asa SL, Harris CR, Getz G, Kulke M, Meyerson M. "Somatic mutation of CDKN1B in small intestine neuroendocrine tumors". Nature Genetics (2012) 45;1483-6.

119) Maxwell JE, Sherman SK, Li G, Choi AB, Bellizzi AM, O'Dorisio TM, Howe JR. “Somatic alterations of CDKN1B are associated with small bowel neuroendocrine tumors”. Cancer Genetics (2015) 208;564-570.

120) Stephens PJ, Tarpey PS, Davies H, Van Loo P, Greenman C, Wedge DC, Nik-Zainal S, Martin S, Varela I, Bignell GR, Yates LR, Papaemmanuil E, Beare D, Butler A, Cheverton A, Gamble J, Hinton J, Jia M, Jayakumar A, Jones D, Latimer C, Lau KW, McLaren S, McBride DJ, Menzies A, Mudie L, Raine K, Rad R, Chapman MS, Teague J, Easton D, Langerød A; Oslo Breast Cancer Consortium (OSBREAC), Lee MT, Shen CY, Tee BT, Huimin BW, Broeks A, Vargas AC, Turashvili G, Martens J, Fatima A, Miron P, Chin SF, Thomas G, Boyault S, Mariani O, Lakhani SR, van de Vijver M, van 't Veer L, Foekens J, Desmedt C, Sotiriou C, Tutt A, Caldas C, Reis-Filho JS, Aparicio SA, Salomon AV, Børresen-Dale AL, Richardson AL, Campbell PJ, Futreal PA, Stratton MR. “The landscape of cancer genes and mutational processes in breast cancer”. Nature (2012) 486;400-4.

121) Dietrich S, Hüllein J, Lee SC, Hutter B, Gonzalez D, Jayne S, Dyer MJ, Oleś M, Else M, Liu X, Słabicki M, Wu B, Troussard X, Dürig J, Andrulis M, Dearden C, von Kalle C, Granzow M, Jauch A, Fröhling S, Huber W, Meggendorfer M, Haferlach T, Ho AD, Richter D, Brors B, Glimm H, Matutes E, Abdel Wahab O, Zenz T. “Recurrent CDKN1B (p27) mutations in hairy cell leukemia”. Blood (2015) 126;1005-8.

122) Pellegata NS. “MENX and MEN4”. Clinics (2012) 67;13-18.

123) Pellegata NS, Quintanilla-Martinez L, Siggelkow H, Samson E, Bink K, Höfler H, Fend F, Graw J, Atkinson MJ. “Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans”. PNAS (2006) 103;15558-63.

124) Molatore S, Kiermaier E, Jung CB, Lee M, Pulz E, Höfler H, Atkinson MJ, Pellegata NS. Characterization of a naturally-occurring p27 mutation predisposing to multiple endocrine tumors". Molecular Cancer (2010) 9;116.

125) Georgitsi M, Raitila A, Karhu A, van der Luijt RB, Aalfs CM, Sane T, Vierimaa O, Mäkinen MJ, Tuppurainen K, Paschke R, Gimm O, Koch CA, Gündogdu S, Lucassen A, Tischkowitz M, Izatt L, Aylwin S, Bano G, Hodgson S, De Menis E, Launonen V, Vahteristo P, Aaltonen LA. "Germline CDKN1B/p27Kip1 mutation in multiple endocrine neoplasia". The Journal of Clinical Endocrinology and Metabolism (2007) 92;3321-5.

126) Agarwal SK, Mateo CM, Marx SJ. "Rare germline mutations in cyclin-dependent kinase inhibitor genes in multiple endocrine neoplasia type 1 and related states". The journal of Clinical Endocrinology and Metabolism (2009) 94;1826-34.

127) Molatore S, Marinoni I, Lee M, Pulz E, Ambrosio MR, degli Uberti EC, Zatelli MC, Pellegata NS. "A novel germline CDKN1B mutation causing multiple endocrine tumors: clinical, genetic and functional characterization". Human Mutation (2010) 31; E1825-35.

128) Crona J, Gustavsson T, Norlén O, Edfeldt K, Åkerström T, Westin G, Hellman P, Björklund P, Stålberg P. "Somatic Mutations and Genetic Heterogeneity at the CDKN1B Locus in Small Intestinal Neuroendocrine Tumors". Annals of Surgical Oncology (2015).

129) Lee M, Pellegata NS. "Multiple endocrine neoplasia syndromes associated with mutation of p27". The Journal of Endocrinological Investigation (2013) 36;781-7.

130) Tichomirowa MA, Lee M, Barlier A, Daly AF, Marinoni I, Jaffrain-Rea ML, Naves LA, Rodien P, Rohmer V, Faucz FR, Caron P, Estour B, Lecomte P, Borson-Chazot F,

Penfornis A, Yaneva M, Guitelman M, Castermans E, Verhaege C, Wémeau JL, Tabarin A, Fajardo Montañana C, Delemer B, Kerlan V, Sadoul JL, Cortet Rudelli C, Archambeaud F, Zacharieva S, Theodoropoulou M, Brue T, Enjalbert A, Bours V, Pellegata NS, Beckers A. "Cyclin-dependent kinase inhibitor 1B (CDKN1B) gene variants in AIP mutation-negative familial isolated pituitary adenoma kindreds". Endocrine Related Cencer (2012) 19;233-41.