Université de Montréal

Study of the subcellular localization of cell cycle regulator Cks1 and its impact on cancer

par
Yipeng Yao

Département de pharmacologie
Faculté de médecine

Thèse présenté à la Faculté des études supérieures
en vue de l'obtention du grade de maîtrise
en pharmacologie

Décembre 2012

Université de Montréal
Faculté des études supérieures et postdoctorales

Cette thèse intitulée:

Study of the subcellular localization of cell cycle regulator Cks1 and its impact on cancer

Présenté par :
Yipeng Yao

a été évalué par un jury composé des personnes suivantes :

Dr. Richard L. Momparler, président-rapporteur
Dr. Sylvain Meloche, directeur de recherche
Dr. Jean-François Gauchat, membre du jury

Résumé

La progression dans le cycle cellulaire est contrôlée par de vagues oscillantes de cyclines et des kinases cycline-dépendantes (Cdk). Ces kinases sont régulées positivement par l'association des sous-unités cyclines régulatrices et négativement en se liant aux inhibiteurs de Cdk. Parmi ces derniers, p27 inhibe tous les complexes cycline-Cdk quelle que soit la phase cellulaire et agit en tant que régulateur négatif principal de la prolifération cellulaire dans une variété de cellules et de tissus. Intrinsèquement, p27 phosphorylé est ubiquitiné et dégradé par le complexe SCF^{Skp2}-Cks1. Des études génétiques de la souris, ainsi que des examens cliniques chez l'homme, ont montré que p27 est un important suppresseur de tumeur. Le gène est rarement muté. Cependant, p27 est fréquemment réprimé dans les cancers humains en raison d'une augmentation de l'expression de Skp2 et de Cks1 dans le noyau, ce qui est généralement associée à un mauvais pronostic. La localisation subcellulaire de Cks1 est donc d'une importance primordiale dans le contrôle de la prolifération cellulaire. Les résultats récents de notre laboratoire ont montré une interaction entre Cks1 et les protéines de transport nucléaire importine α1 et β3. Aussi, l'analyse de la séquence primaire de Cks1 a également révélé un signal de localisation nucléaire classique (NLS) à son extrémité C-terminale. Des mutations ont été effectuées sur le NLS suspect pour déterminer si oui ou non l'import nucléaire de Cks1 était contrôlé par cette séquence. Un inhibiteur synthétique de l'importine β a également été utilisé pour étudier l'import de Cks1 dans le noyau. Les résultats indiquent que l'extrémité C-terminale

de Cks1 est en effet un NLS puisque les mutations de Cks1 et l'inhibition de l'importine β conduisent, tous deux, à l'accumulation de Cks1 dans le cytoplasme. Ces résultats ont été utiles pour mieux comprendre le mécanisme régulant la localisation de Cks1. Toutefois, des travaux futurs sont nécessaires pour mieux comprendre l'impact de la séquestration cytoplasmique de Cks1 sur le cancer et ainsi espérer aboutir à l'identification de nouvelles cibles pharmacologiques impliqués dans la prolifération cellulaire.

Mots-clés: cycle cellulaire, cycline, Cdk, p27, dégradation, Cks1, NLS, localisation

Abstract

Progression through the cell cycle is controlled by oscillating waves of cyclins and cyclin-dependent kinases (Cdk). These kinases are regulated positively by association with cyclin regulatory subunits and negatively by binding of Cdk inhibitors. Among the latter, p27 inhibits all cyclin-Cdk complexes regardless of the cell cycle phase and acts as a primary negative regulator of cell proliferation in a variety of cell types and tissues. Intrinsically, phosphorylated p27 is ubiquitinated and degraded by the SCF^{Skp2}-Cks1 complex. Mouse genetic studies and human clinical investigations have shown p27 as an important tumor suppressor, which gene is rarely mutated. However, p27 is frequently downregulated in human cancers due to an increased expression of nuclear Cks1 and this is usually associated with a poor prognosis. The subcellular localization of Cks1 is thus of primordial importance in the control of cell proliferation. Recent results from our laboratory have shown an interaction between Cks1 and nuclear transport proteins α1 and β3 importin. Analysis of the primary sequence of Cks1 also revealed a classic nuclear localization signal (NLS) at its C-terminal. Mutations have been done on the suspected NLS to determine whether or not Cks1's nuclear import is regulated by this motif. A synthetic inhibitor of β importin has also been used to study the mechanism of Cks1 import. Results indicated that the C-terminal end of Cks1 is indeed a NLS since mutations of Cks1 and inhibition of β importin both lead to accumulation of Cks1 in the cytoplasm. These outcomes were helpful to better understand the mechanism regulating Cks1 localization.

However, future works are required to further understand the impact of cytoplasmic sequestration of Cks1 on cancer and hopefully lead to the identification of novel pharmacological targets involved in cell proliferation.

Keywords: cell cycle, cyclin, Cdk, p27, degradation, Cks1, NLS, localization

Table of Contents

List of figures

List of Abbreviations

α-imp : α importin

APC/C : anaphase-promoting complex/cyclosome

ATF1 : activating transcription factor 1

β-imp : β importin

BP : bait protein

β-TRCP : β-transducin repeat-containing protein

CAK : cdk-activating kinase

$CaPO_4$: calcium phosphate

CCRK : cell cycle related kinase

Cdc : cell division cycle protein

CDC2L : Cdc2-like protein

CDE : cell cycle dependent element

Cdk : cyclin depedent kinase

CHR : cell cycle gene homology region

Cks1 : cyclin dependent kinase regulatory subunit 1

CksHs1 : human Cks1

Cki : cdk inhibitor

Co-Ip : coimmuno-precipitation

COPS5 : COP9 constitutive photomorphogenic homolog subunit 5

DMSO : dimethyl solfoxide

DNA : deoxyribonucleic acid

FBS : fetal bovine serum

FBW7 : F-box and WD-40 domain protein 7

FoxO : forkhead box class O family

GEF : guanine nucleotide exchange factor

Grb2 : growth factor receptor-bound protein 2

hKIS : human kinase interacting stathmin

Il-8 : interleukin 8

IRES : internal ribosome entry site

kDa : kilo-dalton

KID : kinase inhibitory domain

KPC : Kip1 ubiquitination-promoting complex

Leu : leupeptin

LRR : leucine rich repeat

LS/MS : liquid chromatography mass spectrometry

Mad2 : mitotic arrest deficient 2

MAPK : nitogen-activating protein kinase

MPF : mitosis promoting factor

N>C : nuclear accumulation of specific protein

N<C : cytoplasmic accumulation of specific protein

N=C : even distribution throughtout cell of specific protein

NES : nuclear export signal

NF-γ : nuclear factor γ

NLS : nuclear localization signal

PA : pepstatin A

PCNA : proliferating cell nuclear antigen

PFA : paraformaldehyde

PI3K : phosphatidylinositol-3-kinase

PKB/Akt : protein kinase B

PMSF : phenylmethylsulfonyl fluoride

RanGEF : Ran guanine nucleotide exchange factor

RanGTP : Ran GTPase activating protein

Rb : retinoblastoma protein

RBX1 : ring box protein 1

RNA : ribonucleic acid

ROCK : rho-kinase

RT : room temperature

SB : sample buffer

SCF : Skp, Cullin, F-box complex

shRNA : short hairpin RNA

Skp: S-phase kinase associated protein

TAP : tandem affinity purification

TGF-β : transforming growth factor β

TPR : tetratricopeptide repeat

Ub : ubiquitin

UTR : untranslated region

Van : sodium orthovanadate

À mes grands-parents, dont trois ont été enlevés par le cancer,

à mes chers parents

et aux héros en blouse de laboratoire, travaillant jour et nuit pour guérir le cancer.

Thanks

- *To Dr. Sylvain Meloche for giving me the opportunity to attain my masters' degree under his supervision and guidance.*
- *To Laure Voisin and Benoit Pelletier for their guidance and help throughout my project*
- *To Edith Giasson, Paul Deleris, Pierre-Luc Tanguay, Stéphanie Duhamel , Christophe Fremin, Marc Saba El Leil, and Justine Rousseau for their experimental support and helpful advices.*
- *To Dr. Ricahrd L. Momparler and Dr. Jean-François Gauchat for taking time to read and correct my thesis.*
- *To Christian Charbonneau for his help with microscopes*
- *To Sylvie Caron for her help with my thesis deposit.*
- *To all other members of the lab*
- *To my parents Jia and Jianhua for their unconditional love and support*
- *To Fan dan, love of my life, for her love and support*

1. Introduction

1.1. Cell cycle

Cell division is a process by which one parent cell undergoes different stages in order to become two identical daughter cells each with the same genetic material as the parent cell. Most of the living organisms will divide once or many times during their life. For unicellular creatures, it serves as means of replication. For multicellular organisms, it serves many functions: growth, repair, and replacement. Prokaryotic cells replicate via either binary fission or budding. However, eukaryotic cells must go through different stepwise phases, each of which will start only after the full termination of the previous one. All of these steps are called the cell cycle.

In human, the cell cycle consists mainly of 5 phases (see Fig.1A). [1-3] The first phase or Gap 1 (G_1) is where most of the normal adult cells are. They either become quiescent (G_0) and differentiate into non-dividing cells with specific functions or, upon stimulation by mitogenic factors, activate proteins that will drive the cells into cell division. The ensuing synthesis phase (S) encompasses the important step of deoxyribonucleic acid (DNA) replication. Once past the step of G_1/S transition, cells will be committed to cell division. All chromosomes duplicate and form pairs of chromatids. The completion of DNA duplication is followed by a second gap phase (G_2). The cell further increases in size, synthesizes proteins, and assembles the necessary cellular structures to prepare for cell separation. Mitosis or M phase occurs when the nuclear envelop breaks apart and the sister chromatids separate via a system of microtubules, each attached to the kinetochore of a

chromatid and to one of the polar bodies located on opposite side of the cell. Cell cleavage or cytokinesis occurs and two identical daughter cells are created. Both cells are now in the G_1 phase and the cycle starts again.

1.2. Regulation

As all biological activities are, the cell cycle is tightly regulated to prevent production of genetically damaged daughter cells and uncontrolled cell division that would ultimately lead to cancer. At G_1, the restriction checkpoint first decide, according to environmental factors, whether the cell differentiate or re-enter cell division. [4] Then, the retinoblastoma proteins (Rb), inhibitor of the transcription factor E2F, must be inactivated via phosphorylation for the cell to transit from G_1 to S phase. The post-replication DNA damage checkpoint at the G_2/M transition ensures that no damaged DNA will be passed on to daughter cells.[5] Finally, the spindle or mitotic checkpoint monitors the even segregation of chromosomes via kinetochore attachments and microtubule tensions. [6]

Cell cycle regulations mainly occur via two post-translational mechanisms: phosphorylation and proteolysis. Phosphorylation is mediated by two families of regulatory proteins: cyclins and cyclin-dependent kinases (Cdk). [1, 2, 7] The effect can be either activator or inhibitory depending on the function of the substrate or the site of phosphorylation. Proteolysis remove proteins no longer required or in excess, whether they are negative or positive regulators. [8] Ubiquitination of proteins targeted for proteolysis is marked by two ubiquitin-dependent complexes: the anaphase-promoting complex/cyclosome (APC/C) [9, 10] and the Skp, Cullin, F-box containing (SCF) complex. [11] The ubiquitinated protein is then directed to the 26S proteasome for degradation. [12]

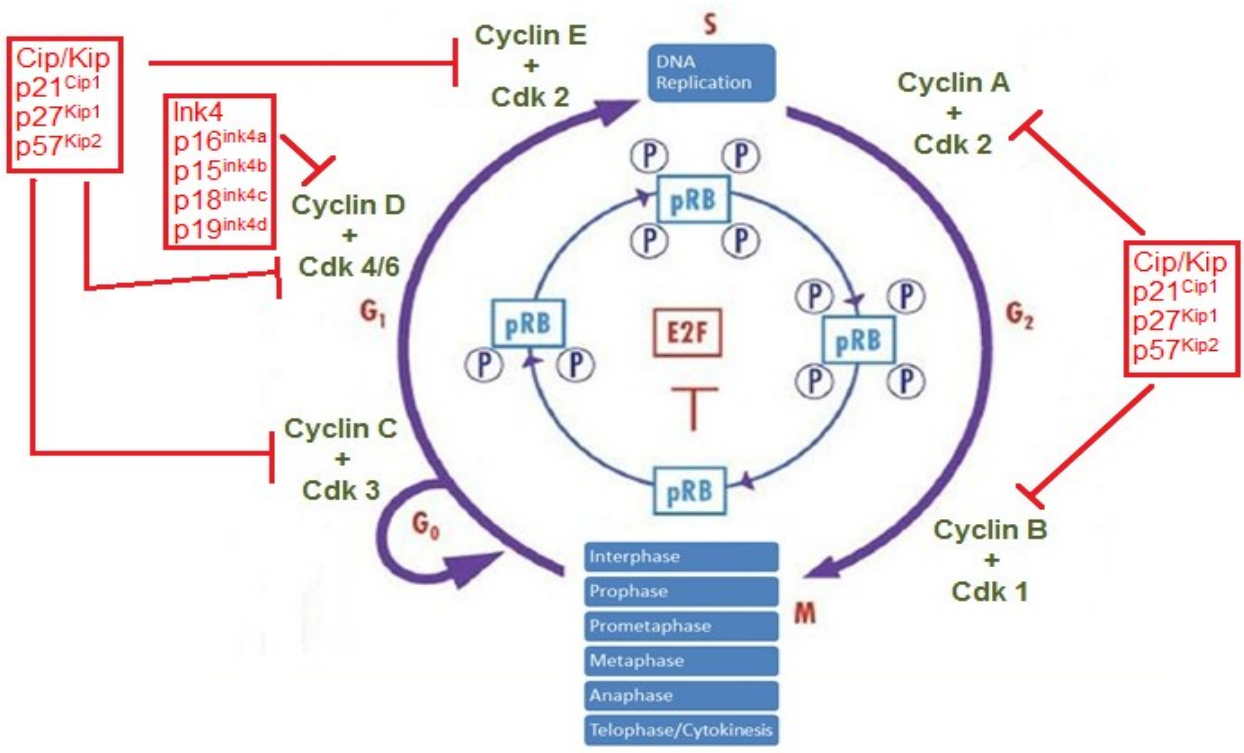

Figure 1 Cell cycle overview (adapted from *{Viglietto et al. Cell Cycle 2002;1(6)}*)

A) Overview: Eukaryotic cells must undergo specific steps in order to produce two identical daughter cells. The mammalian cell cycle can be separated into 5 phases. Differentiated or quiescent cells are at G_0. Normal adult cells are at the G_1-phase. In presence of mitogens, such as growth hormones, inhibitory phosphorylation of retinoblastoma protein occurs, which release the transcription factor E2F, leading to cell growth and replication of its DNA at the S-phase. Once past the G_1/S transition, the cell is committed to finish the cell cycle. At the second gap phase G_2, the cell grows further in size and produce proteins required for mitosis. Mitosis or M-phase is where the cell cleaves into 2 identical daughter calls each containing one copy of the duplicated chromosomes. Mitosis is further separated into six steps, each describing a step of DNA separation. Finally, cytokinesis is the separation of cell cytoplasm via membrane cleavage.

B) Cyclin/Cdks: regulatory cyclins and Cdks form holoenzymes that regulate the progression of cell cycle. Each cyclin bind to specific Cdks and each cyclin/Cdk complex modulate a specific part of the cell cycle. Cyclin C/Cdk3 promote quiescence exit in presence of mitogens. Cyclin D/Cdk4 and Cyclin D/Cdk6 up-regulate transcription of cell cycle required genes. Cyclin E/Cdk2 mediate the G_1/S transition via formation of prereplication complex and other proteins required for DNA replication. Cyclin B/Cdk1 is the mitotic cyclin complexes that drive the cell to enter into mitosis.

C) Cyclin/Cdk inhibitors: Inhibition of cell cycle is mediated by two families of cyclin/Cdk inhibitors, the Ink4 and Cip/Kip families. Ink4 inhibitors are named due to their abilities to inhibit specifically Cdk4 associated complexes. Its members are p16, p15, p18 and p19. Cip/Kip inhibitors, however, are involved in regulating all cyclin/Cdk complexes. Cip/Kip members are p21, p27 and p57.

1.2.1. Phosphorylation

Phosphorylation refers to the addition of one or more phosphate group (PO_4^{3-}) to a protein. Phosphates are sterically bulky and negatively charged moiety. Therefore, their addition can alter a protein's biochemical properties as well as its structure and activities. The conformational and property changes can then create docking sites to mediate protein-protein interactions, modify signal sequences on proteins to regulate their subcellular localization, and activate enzymes by bringing their active sites into proper position. [13] Phosphorylation can activate or inhibit a protein depending on its function and phosphorylation site. Effects are often amplified due to pathway mechanisms. Thus, reversible phosphorylation is an important mechanism of control of cellular enzymatic

activities. Protein kinases phosphorylate while protein phosphatases dephosphorylate. In the cell cycle, most proteins, required for the activation of genes involved in DNA synthesis and replication, are regulated by those mechanisms. The Rb protein is an example of a protein whose activity is inactivated by phosphorylation.

1.2.1.1. Cyclins

Cyclins are a family of proteins first discovered by Evans et al. when studying sea urchin eggs' cell division. [14] They are so named because of the cyclic variation in their concentration during the cell cycle. These regulatory proteins are furthermore grouped into different classes on the basis of expression timing, amino acid sequences, and structure. [15] However, all cyclins share a region of homology called the cyclin box present up to twice on the protein depending on its class. [1, 16] This conserved sequence of about 100 amino acids long is responsible for the binding and activation of Cdks. Cyclins are the regulatory subunits of Cdk-holoenzymes. [17] Therefore, most cyclins must associate with specific catalytic cofactors (Cdks) to perform checkpoint regulatory functions. [18] Several cyclins have been found to take parts in other regulatory complexes, such as cyclin F which compose the F-box protein of the SCF complex. [19]

There are in total 12 classes of cyclins [16] : A, B, C, D, E, F, G, H, I, J, K, and L. However, only the cyclins A, B, C, D, and E are involved in regulating the cell cycle phase transition via cyclin/Cdk complexes. (see Fig.1B) During G_0, cyclin C is transcriptionally

activated in response to mitogens. [2] By binding and activating Cdk3, the cyclin C/Cdk3 complex will then mediate the cell cycle re-entry of quiescent cells. [20, 21] At G_1, Cyclin D acivation is mitogen-induced. [17] The formation of cyclin D/Cdk4 and cyclin D/Cdk6 complexes will in turn phosphorylate Rb, their main substrate, which acts on centrosome duplication, mitochondrial function, and cytoskeletal modeling. [22] Cyclin D has also been shown to promote transcriptions, independent of Cdks, by acting on promoter regions. [23] Cyclin D/Cdk4/6 also activate indirectly cyclin E by sequestering cyclin/Cdk inhibitor p21 and p27. During G_1, Cyclin E is known to primarily bind and activate Cdk2, but interaction with Cdk1 and Cdk3 have also been demonstrated. [24] The cyclin E/Cdk2 holoenzyme is essential for G_1/S phase transition due to its role in E2F activation, prereplication complex formation, centrosome duplication, histone biosynthesis, and endoreplication. [25] Recent studies by Geng et al. revealed cyclin E's role in loading of the minichromosome maintenance helicase into the DNA replication complex in a cyclin-independent manner. [24] A- and B-types cyclins are called mitotic cyclins due to their role in S and M phases. [26] Cyclin A concentration accumulates in late G_1 phase, through S and G_2 phase, and rapidly decreases in M phase. [26] Once activated, it forms complexes with Cdk2, and drives DNA synthesis by interacting with a subunit of DNA polymerase δ, the proliferating cell nuclear antigen. [27, 28] B cyclins are synthesised during S phase and are retained in the cytoplasm by binding to the nuclear export protein Crm1 via its nuclear export signal. [29] (for mechanism of nuclear import/export, see Fig.2) Binding of the cyclin to Cdk1 forms the M-phase-promoting factor (MPF), essential for the triggering of mitosis. [30] During mitosis, MPF,

activated by cyclin A, enters the nuclear and mediate the dissolution of nuclear envelope and the separation of chromatids. [1, 31]

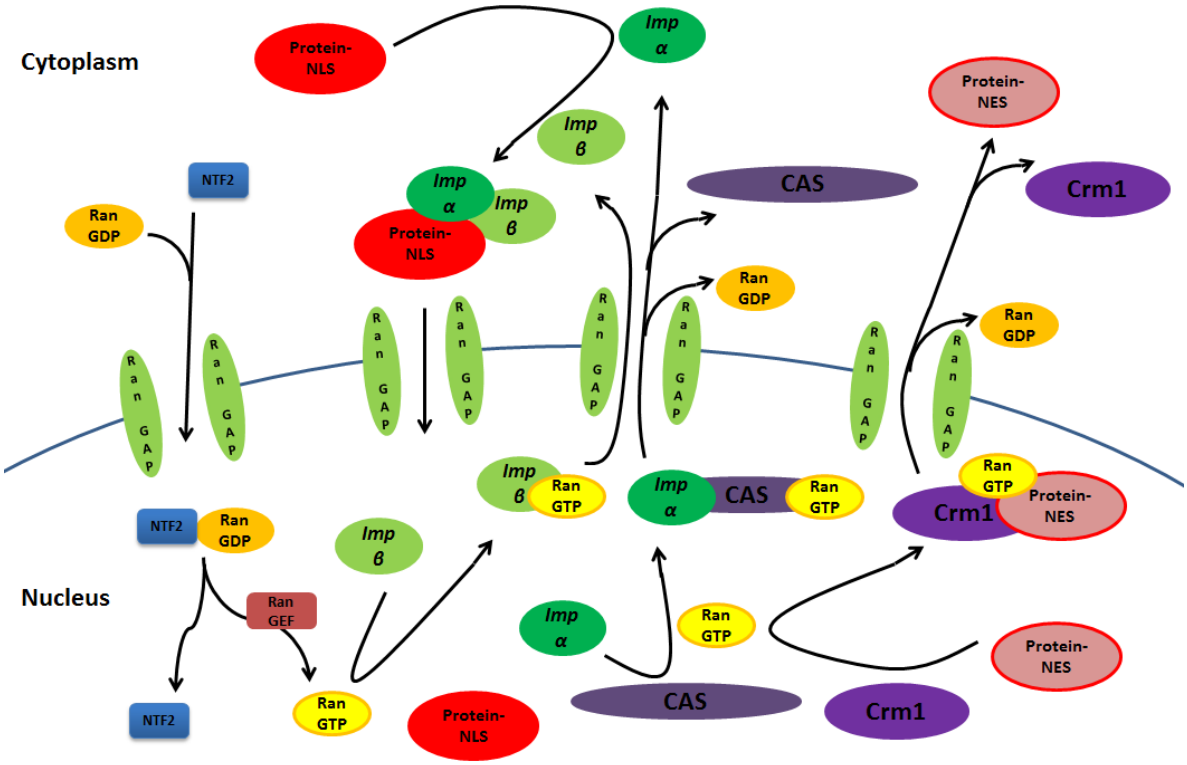

Figure 2 Mechanisms of nuclear import and export (adapted from *{Pemberton and Paschal, Traffic 2005; 6}*)

Nuclear import: cytoplasmic proteins containing the nuclear localization signal are recognized by specific α importin or karyopheryn (Imp-α) and β importin (imp-β). The heterodimerization of imp-α and imp-β on the NLS site of cargo protein mediate its entry into the nucleus through nuclear pore complexes.

Nuclear export: nuclear proteins containing the nuclear export signal (NES) is recognized by the exportin or karyopherin Crm1 (Crm1), either directly or indirectly via adaptor protein. Crm1 mediate the nuclear export of cargo proteins in cooperation with RanGTP.

Ran is a guanosine triphosphate (GTP) binding protein involved in release of importins from cargo proteins after import, nuclear export of unbound imp-β, export of unbound imp-α in cooperation with exportin CAS and export of Crm1-bound proteins. The energy, released from hydrolysis of Ran-bond GTP to guanosine diphosphate (GDP) by the GTPase-activating protein (RanGTP), is utilized in transport across the nuclear membrane. Inactive RanGDP is reactivated by Ran Guanine nucleotide Exchange Factors (RanGEF).

1.2.1.2. Cyclin-dependent kinases

Cyclin-dependent kinases are a family of small proline directed serine/threonine kinases, all ranging from 34-40 kiloDalton (kDa). [32, 33] This kinase family is highly conserved through evolution. In an experiment, Lee and Nurse replaced the yeast's *Cdc2* gene (a Cdk1 homologue in fission yeast) with human *Cdk1* gene. The yeast was able to progress through the cell cycle. [34] Also, recent studies have shown a high complementarity between the Cdks. In mammalian cells, due to the numerous compensatory mechanisms, Cdk1 alone with its' partner cyclin A and B is able to drive the cell cycle. [35]

In humans, there are up to twenty different Cdks (1-20) classified to date. [2] These include 11 classical CDKs (Cdk1–11), two recently studied kinases (Cdk12 and 13), several proteins named based on the presence of a cyclin-binding element (PFTAIRE and PCTAIRE proteins) and a few Cdks-likes with similar sequences, such as CDC2-like kinases (CDC2L) or cell cycle-related kinases (CCRK). [36] Among them, only Cdk1, 2, 3,

4, and 6 are involved in the regulation of cell cycle progression at different checkpoints. The Cdks accomplish a variety of functions in numerous mechanisms such as DNA transcription and modification (Cdk11), mRNA processing, telomere elongation (Cdk1), activation of other Cdks (Cdk7), cell differentiation, senescence (Cdk5), etc. [2, 32, 37]

Crystallography revealed that Cdks form a tertiary structure containing a small amino-terminal lobe and a larger carboxyl-terminal lobe between which ATP bind to donate its γ-phosphate during phosphorylation. However, a large, flexible T-loop or activation loop blocking the binding of protein substrate at the entrance of the active-site cleft and incorrectly positioned amino acid side chains prevent Cdk activity. [33] Indeed, as mentioned above, most Cdks are inactive on their own. For activation, binding of its cyclin partner and phosphorylation of a threonine near the active site by a Cdk-activating kinase are both required. [33, 38] Thus, the availability of the specific corresponding cyclin is by itself a regulation mechanism. Upon holoenzyme formation, 2 alpha helices on the Cdk, the PSTAIRE helix and the L12 helix, interact with the bound Cdk and change position and structure for the reconfiguration of the active site to allow binding of ATP. Once activated, the cyclin/Cdk complexes will phosphorylate their substrates on a specific consensus sequence of 4 amino acids long: [S/T*]PX[K/R]. [39] "S/T" is the serine or threonine on which the phosphate group attaches. "P" stands for proline. "X" is any polar amino acid. "K" is for lysine. "R" is for arginine.

Cdk1 is the first Cdk studied, also known in yeast as either Cdc28 or Cdc2. [40, 41] There are numerous alternatively spliced isoforms produced for the same gene. This kinase

forms the catalytic subunit of the highly conserved MPF complex along with cyclin B. MPF activates proteins needed for mitosis. A-cyclins also associate with Cdk1 and regulate its activity. The phosphatase Cdc25 is responsible for removal of the inhibitory phosphates on Thr(14) and Tyr(15), while Wee 1 is responsible for their phosphorylation. [42] Cdk2 has two alternatively spliced variants and binds to both cyclin E and A. [43] Cdk2 binds to G_1-phase cyclin E to mediate G_1/S transition. Activated Cdk2 also interacts with Smads, which are signal transduction proteins activated in response to transforming growth factor β (TGF-β), to inhibit cell cycle arrest. [44] In the S phase its association with cyclin A recognizes DNA damage and initiate DNA repair during DNA synthesis. The main function of cyclin C-associated Cdk3 is to promote cell cycle reentry via Rb phosphorylation. [20] Cdk3 also works downstream of Rb by activating directly E2F to promote S-phase entry. [45] Recent studies by Zheng et al. demonstrated Cdk3's non-cell cycle regulatory function in cell transformation, via its interaction with the activating transcription factor 1 (ATF 1). [46] Type 4 Cdks bind with D-cyclins to regulate the G_1/S progression. Along with Cdk2, it regulates Smads during G_1/S transition. [44] Cdk4 is also involved in controlling the subcellular localization of BRCA1, a nuclear phosphoprotein that function as tumor suppressors by activating apoptosis. [47] Cdk 6 binds to D-cyclins in mid G_1 phase, prior to Cdk2 activities, and drives the cell into S phase. [48] The main function of cyclin D/Cdk6 is in the phosphorylation of Rb protein. Slomiany et al. observed that primary mouse astrocytes infected with retroviral RCAS-Cdk6 resulted in drastic morphological changes. [49] When further investigated, changes in patterns of gene

expression, changes in the actin cytoskeleton and enhanced motility have been noted. These changes are all part of the process of cellular differentiation.

1.2.2. Proteolysis

Proteolysis is defined by the directed degradation or digestion of proteins by cellular enzymes into simpler, more soluble substances such as peptides and amino acids. This degradation is performed by either a simple protease or the complex proteasome machinery. Cell cycle regulating proteolysis is mediated by ubiquitination enzymes that label the degradation-targeted protein with multiple ubiquitins (Ub). [12, 50] The tagged protein is then directed to the 26S proteasome, which will catalyze the breakdown of peptide bonds.

Ubiquitins are small proteins (~8 kDa) that target proteins for degradation. First, an E1 ubiquitin-activating enzyme activates Ub in an ATP-dependent way. The activated Ub is then transferred to an E2 ubiquitin-conjugating enzyme. The latter one, attached to an E3 ubiquitin-ligase, mediates the covalent bounding of ubiquitin's carboxyl terminal to a lysine residue on the target protein via an isopeptide bond. This step is repeated multiple times leading to the formation of a polyubiquitin chain in which each ubiquitin is linked to a specific lysine of the previous Ub. [12, 50] The number of ubiquitins forming the chain determines the affinity of the modified substrate protein for the proteasome and also the timing of degradation. Studies have shown that 4 Ubs is the minimal targeting signal. [51]

Once the marked protein binds to the proteasome, unfolding and deubiquitination occurs. [52] The substrate is then translocated to the core proteolytic sites, which cleaves the protein into short polypeptides by catalyzing nucleophilic attacks on threonine residues. [53] Upon peptides release, the Ubs are recycled for future ubiquitination. During the degradation of the cyclin/cdk comlplexes, the catalytic subunit cdk is also released, intact. [54]

Since the length of the polyUb chain is E3-ligase specific and can affect the affinity of proteasome binding, the type of E3 involved in the ubiquitination-dependent degradation pathway is of primordial importance. In humans, multiple E1, E2 and E3 enzymes have been identified, each have a specific subset of ubiquitination partners. E3-ligases are classified into 4 major groups on the basis of their subunit composition: HECT-types, RING-finger-type, U-box-type and PHD-finger-type. [50, 55] In cell cycle, two polymeric E3 ubiquitin-ligases are implicated in the proteolysis pathway, both of which are of the RING-type class. The SCF complex is active from late G_1 to early M phase; it is primarily involved in the G_1/S and G_2/M transitions. On the other hand, APC/C's expression is required from mid-M phase to S phase to induce progression and exit of mitosis. [9, 50]

SCF and APC/C complexes expression overlap and control each other in form of positive and negative feedbacks. (see Fig.3) At early mitosis, $SCF^{\beta\text{-}TRCP}$ ubiquitinates APC^{Cdc20} inhibitor Emi1/2; thus, activating the latter. Then in G_1, APC/C^{Cdh1} recognizes Skp2 and mediates its proteolysis, leading to low SCF level and accumulation of cyclin/cdk inhibitors. This results in an increase of cyclin/Cdk which phosphorylates Cdh1 and mediates its inhibition. [50]

1.2.2.1. Skp, Cullin, and F-box complex

The SCF complex is formed of 4 subunits: Ring Box protein 1 (RBX1), CUL1, S-phase kinase-associated protein (Skp1), and F-box protein. The RBX1 protein contains a zinc binding domain, called the RING finger, which binds to the E2 ubiquitin-conjugating enzyme. CUL1 is from the cullin protein family that functions as a scaffold protein linking RBX1 to Skp1. Skp1 is the adaptor protein that links the substrate receptor protein to the CUL1-RING complex. The receptor responsible for tagged substrate recognition is called an F-box protein. Consequently, Skp1 is also called an F-box binding protein. [56]

RBX1 and Skp1 are highly conserved. In some cases, they can be replaced by related proteins without affecting the activity rate of SCF. [33] In fact, the rate of ubiquitination by SCF is not controlled by the core RBX1-CUL1-Skp1 complex, but rather by the substrate's affinity for its corresponding F-box protein. The binding affinity is influenced by both the phosphorylation of target protein at specific site or sites and by the type of F-box receptor involved.

F-box proteins contain an approximately 40 amino acids long motif, called F-box due to its presence on cyclin F when discovered, which functions as a site of substrate recognition domain. [56-58] There are over seventy different F-box proteins identified in humans, each of which recognizes a specific subset of target proteins. [33, 59] They are divided into 3 classes, based on their protein-protein interaction domains. Fbw interacts with their WD-40 domains while Fbl uses leucine-rich repeats and Fbx contains various other protein-protein interaction domains (zinc-finger, proline-rich, carbohydrate-

interacting, Sec7, cyclin box, calponin homology and Traf-domain-like). [11, 60, 61] Only three of the F-box receptor proteins are implicated in SCF-dependent cell cycle regulation: S-phase kinase-associated protein 2 (Skp 2), F-box and WD-40 domain protein 7 (FBW7), and β-transducin repeat-containing protein (β-TRCP).

Skp 2 recognizes and promotes ubiquitination of various regulatory proteins such as the transcription factor E2F-1, the RNA elongation factor Cdk 9, and the negative cell cycle regulators, p27, p21 and p57; thus, promoting cell cycle progression. On the opposite, FBW7 promotes the degradation of positive regulators such as the growth factor receptor Notch 4, and the transcription factors Myc and c-Jun. Both Skp2 and FBW7 target cyclin E, one on the free form and the other one on the Cdk 2-bound form. β-TRCP has various functions during the cell cycle since it regulates IκB, the inhibitor of the transcription factor NF-κB, and β-catenin, involved in the Wnt signaling pathway; as well as Wee1, inhibitor of Cdk1, and Cdc25, activator of Cdks. [59]

1.2.2.2. Anaphase-promoting complex

The Anaphase-promoting complex or cyclosome is the largest and most complex E3 ligase known to date. It is a polymeric complex formed from more than 11 different proteins. [8, 62] These subunits include the scaffolding cullin-homolog Apc2, the WD-40 domain protein APC4 as well as the RING-H2 finger APC11. [54] Studies have shown that a complex formed of only the core subunits, Apc2 and Apc11, is able to catalyse protein

ubiquitylation, although in a non-specific manner. [62] Sequencing of the subunits APC3, APC6, APC7 and APC8 revealed the presence of multiple tandem repeats of a conserved 34-residues motif, dubbed tetratricopeptide repeat (TPR), which is involved in protein-protein interactions mediating a wide variety of cellular functions. [63]

As the name implies, the main function of APC/C is to trigger metaphase/anaphase transition and mitosis exit. Recent studies have also found a new role in genome replication checkpoint by destroying Rad17, a promoter of DNA damage repair, to terminate cell-cycle arrest after DNA have been repaired. [10] The activities of APC/C are highly regulated with two activator proteins: Cdc20 and Cdh1; as well as 4 inhibitors: Emi1 and mitotic checkpoint complex subunits, Mad2, Mad2b and BubR1. [54, 63] As with the F-box protein subunits of SCF, each activator recognizes a subset of substrates for specific protein degradation. The ubiquitination of target protein is mediated by a small, 9 or more residues long, amino-terminal motif known as the destruction box. The consensus sequence on B-cyclins was found to be RXALGXIXN. [63] However, neither the sequence nor the number of destruction-box is conserved between proteins, considerable differences are found even in A- and B-type cyclins.

Metaphase occurs when all duplicated chromosomes align at the metaphase plate. Anaphase is where the chromatids separate and move to opposite poles of the cell. Spindle assembly checkpoint regulatory proteins, mitotic arrest deficient 2 (Mad2) and serine/threonine kinase BubR1, delay chromosomal segregation until all chromosomes are aligned and attached at their kinetochore to the opposing poles. Free kinetochores link to and inhibit the APC/C activator Cdc20 via the Mad2 protein. [64] The regulatory cyclin

B/Cdk1 complex phosphorylates Cdc20, thus increasing its affinity for APC/C. Once activated, APC/C^{Cdc20} mediates the degradation of both mitotic A- and B-cyclins and securin. Securin inhibits the cysteine protease separase, which cleaves cohesin, a protein complex responsible for holding the sister chromatids together. [9]

The APC/C activator Cdh1 has much more versatile biological functions. Cdh1 is negatively regulated by phosphorylation. APC^{Cdc20}-degradation of cyclin B activate Cdh1 which in turn degrade various substrates including cyclin A, cyclin B and Cdc20. Expression of APC^{Cdh1} is required from mid-M phase to S phase. [10] In late mitosis, it participates in mitosis exit by degrading proteins such as kinesin Cin8, aurora kinases A, B and C, as well as polo-like kinases Plk1-4, for chromatids separation, centrosome formation and cytokinesis. [2, 10] In G_1, Cdh1 coordinates cell division and differentiation. APC^{Cdh1} can either degrade transcriptional suppressors of differentiating-licensing factors, Id1,2 and 4, for cellular differentiation or proceeds to degrade many proteins, Tome-1, Geminin, Cdc6, Skp2, Ets2, Rb protein, etc, to maintain high expression of cyclin/cdk inhibitors such as Wee1 and p27 and to assemble the pre-replication complex required in S-phase. [10, 65] S-phase Cdh1 let the cell progress to G_2-phase after DNA repair.

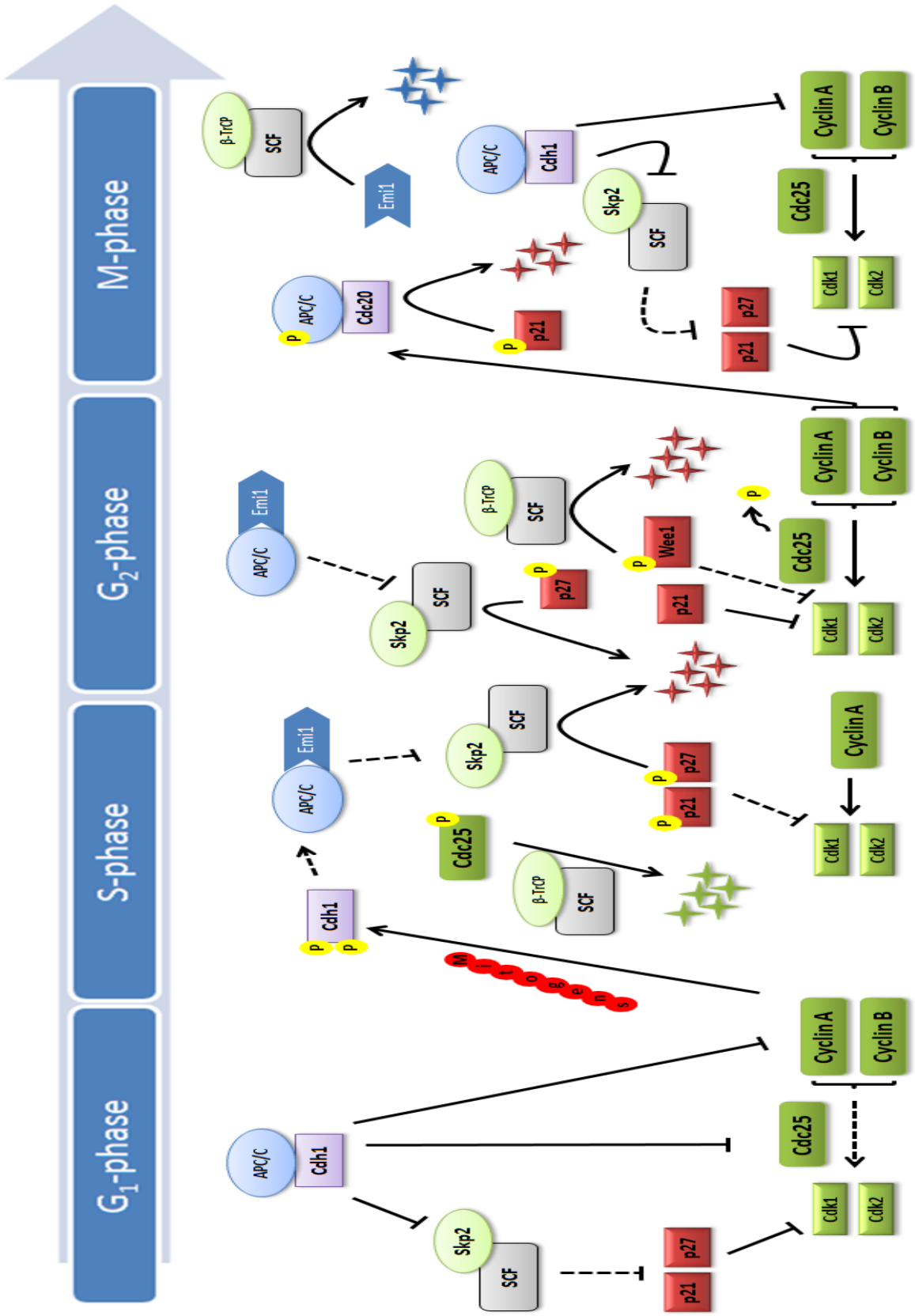
G1-phase
S-phase
G2-phase
M-phase
APC/C
Cdh1
Skp2
SCF
p27
p21
Cdk1
Cdk2
Cdc25
Cyclin A
Cyclin B
Emi1
β-TrCP
Cdc20
Wee1
Mitogens
P

Figure 3 SCF and APC/C complexes regulate each other through cell cycle (adapted from *{Frescas and Pagano, Nat Rev Cancer 2008; 8(6)}*)

The cross-regulation of SCF and APC/C complexes leads to progression of cell cycle. In G_1, the APC/C^{Cdh1} complex promotes degradation of cyclins and Skp2, resulting in inactive Cdks and cell cycle arrest. Mitogenic factors increase expression and activities of cyclin/Cdk complexes, which inhibit Cdh1 via its phosphorylation. In addition to the inactivation of Cdh1, the APC/C inhibitor Emi1 binds to APC/C and prevent further degradation of Skp2, resulting in the decrease p21 and p27 concentration and increase of cyclin/Cdk activities. On the other hand, activated $SCF^{\beta\text{-}TrCP}$ promotes the proteolysis of the phosphorylated Cdk activator Cdc25. In G_2, dephosphorylation of Cdc25 and phosphorylation of Cdk inhibitor Wee1 further contribute to activation of cyclin/Cdks. APC/C^{Cdc20} is activated by high cyclin expression and participate in a negative feedback of cyclin regulation. Phosphorylation of Emi1 during M-phase releases it from APC/C^{Cdh1}, which resumes Skp2 and mitotic cyclins degradation. Cell cycle arrests at the G_1-phase.

1.3. Cyclin/Cdk inhibitors

Cyclin/Cdk complexes are regulators of the cell cycle and are involved in many mechanisms. Therefore, their regulation is essential for cell survival. Control of cyclin/cdk expression occurs at multiple levels, from synthesis to degradation, through phosphorylation and dephosphorylation. As mentioned above, monomeric Cdks are endogenously inactive enzymes. Their activation depends on the specific binding of their partner cyclin as well as on the phosphorylation of the conserved threonine residues. Phosphorylation of T-loop by the Cdk-activating kinase (CAK), a multi-subunit complex containing cyclin H and Cdk7, activates Cdks. Wee1-mediated phosphorylation at the N-terminal inactivates Cdks. [38, 66] This activation/inactivation phosphorylation effect can be reversed by the phosphatase Cdc25. [67] Moreover, cyclin/cdk is negatively controlled via either binding of inhibitory proteins, the cdk inhibitors (Cki), or ubiquitin-dependent degradation by SCF and APC/C. Inhibition of cyclin/cdks are mediated by two families of CKIs working in coordination: the Ink4 family containing p15, p16, p18 and p19, and the Cip/Kip family with p21, p27 and p57. (see Fig.1C)

1.3.1. Ink 4 family

The Ink4 CKI family is composed of 4 structurally similar members: p16, also called ink4a, p15 or ink4b, p18 or ink4c and p19, also known as ink4d. They are named after their ability to specifically inhibit the D-cyclins dependent catalytic subunits Cdk4 and

Cdk6. [68, 69] Each ink4 inhibitory protein contains multiple ankyrin repeats, which are conserved sequences of 33 amino-acid long that form the protein-protein interaction modules [70], and are essential for recognizing only Cdk4 and 6 but not cyclin D. Ink 4 proteins are highly conserved through evolution, actually mouse ink4 inhibitors share ~90% homology with the corresponding human proteins. [71] Many cancers often carry mutation or deletion of one of the *Ink4* genes.

Outside the inhibition of Cdk4 and 6, $p16^{ink4a}$ also act as a tumor suppressor by interacting with c-Jun N-terminal kinases to prevent cell transformation via the c-Jun pathway [72] and by contributing to oxidative stress relief in a Rb-independent way. [73] An alternative spliced form of the $p16^{ink4a}$ gene, $p14^{arf}$, inhibits Mdm2, which is an inhibitor of the tumor suppressor p53. [74] The $p15^{ink4b}$ protein is an important part of the TGF-β-mediated anti-proliferative response pathway. [75] It is able to rescue cell-cycle inhibition in $p16^{ink4a}$-negative mice embryonic fibroblasts. [76] The third ink4 member, $p18^{ink4c}$, suppresses tumor formation by collaborating with the phosphatase Pten [77] while working independently of the Cip/Kip inhibitor p27 [78] and tumor suppressor p53 [79]. The $p19^{ink4d}$ is different from the other ink4 proteins as its expression fluctuates during cell cycle and is mediated by the transcription factor E2F1, [80] thus, regulating G_1 phase termination. Inactivation of p18ink4c and p19ink4d are found to impair fertility in male mouse during spermatogenesis. [81]

1.3.2. Cip/Kip family

The Cip/Kip family contains three members: p21 also called cip1, p27 or kip1 and p57 or kip2. Unlike the Ink4s, the Cip/Kip proteins have a much broader spectrum of inhibitory targets; they can modulate and inhibit the activities of all the cyclin D-, E-, A- and B-Cdk complexes. They all share a conserved amino-terminal motif which confers them the ability to bind to both cyclins and Cdks subunits. [69, 82] However, no homology was found in the rest of their sequences. This indicates that even though each Cip/kip preforms different functions, they are still regulated via the same mechanism. [83]

The importance of $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$ inhibition during cell cycle has been largely documented. Stress-caused DNA damage activates the tumor suppressor p53 protein, which will then bind, along with DEAD box RNA helicase p68 [84] , to a consensus sequence of the $p21^{cip1}$ promoter, leading to its transcription. [85] Other than to mediate cell cycle arrest, p21 also competes for binding with Fen1 to the proliferating cell nuclear antigen (PCNA) to inhibit DNA replication and DNA damage repair. [86] When not required, p21 as well as p27 are cleaved by the cysteine-aspartic acid protease caspase 3, which suggests their importance in the caspase-dependent apoptosis pathway. [87] p27 is highly expressed in quiescent cells and in normal cells in the absence of mitogenic signals. [66, 83] The main regulatory function of p27 is to prevent cell cycle entry in G_1 by inhibiting both cyclin D/Cdk4 and cyclin E/Cdk2. Once the cell enters the cell cycle, p27 is rapidly degraded and exported to the cytoplasm. [67] Unlike the other CKIs, p57 is pivotal for the regulation of embryonic development as it's the only CKI required during mousse

embryogenesis. In addition to cyclin/cdk binding, p57 can inhibit PCNA in similar way as p21. [88] Moreover, p57 is involved in various mechanisms: in cell signaling via Notch, in myogenesis via myoD, embryonic growth via the growth factors BMP2 and 6, and apoptosis via the p53-homolog p73. [83]

Most cell cycle inhibition actions of Cip/kip proteins are performed in the nucleus, but they also partake in various non-proliferative mechanisms in the cytoplasm. Indeed, all Cip/kip proteins have shown abilities to modulate cytoskeleton dynamics via the RhoA pathway, in which the Ras homolog GTPase RhoA is inhibited by p27. [83] While p21 is able to affect cell shape and movement by inhibiting the cytoskeleton regulator rho-kinase (ROCK) [89], p57 interacts with and translocate the serine/threonine kinase LIMK, resulting in actin fiber reorganization. [90] Loss of Cip/Kip inhibitors in cancer cell often results in increased cell invasion and metastasis.

1.4. Cyclin/Cdk inhibitor p27

Among the Cip/Kip inhibitors, one is of particular interest: the $p27^{kip1}$ inhibitor. In G_1 phase, it is the principal inhibitor responsive to proliferative and anti-proliferative signals to maintain the balance between cell differentiation and cell cycle entry. p27 deregulation has been observed in various types of cancers and often correlates with poor prognostic results. [91] In recent years, p27 has been the target of many anti-cancer strategies as it is a pivotal defence mechanism against uncontrolled cell division. Therefore, understanding p27's function and regulation mechanism is primordial.

1.4.1. Structure

$p27^{kip1}$, encoded by the *CDKN1B* gene, is a 198 amino acids long intrinsically unstructured protein containing two major functional domain. [92] On the amino-terminal, a kinase inhibitory domain (KID), conserved between all Cip/Kip, is responsible for separate binding to cyclin and Cdks, which confers more stability to binding of complexes rather than cyclin or cdk alone. [67, 91] The sequential binding of cyclin induces the formation of a $^{3}_{10}$ helix which then lead to binding of Cdk. The carboxyl-terminal end of $p27^{kip1}$ contains various domains responsible for non-cell cycle related functions in the cytoplasm. It mediates interactions with GTPases RhoA and Rac, microtubule-destabilizing protein Stathmin, adaptor protein Grb2 and regulatory protein 14-3-3. [91, 93]

There are also various regulatory domains and motifs in $p27^{kip1}$. The presence of a nuclear export signal (NES) is found near the amino-terminal end of p27. This NES helps to recruit the COP9 constitutive photomorphogenic homolog subunit 5 (COPS5) or Jab1 to the Jab1 binding domain, located around the center of the protein sequence. [67] A bipartite nuclear localisation signal is located between amino acid 152 and 168. α and β importins bind to p27 separately to mediate its nuclear import. [91] Multiple serine, threonine and tyrosine residues on p27 are sites of phosphorylation, which modulate p27 localisation, function, and degradation.

1.4.2. Function

The cell cycle inhibitor $p27^{kip1}$ is mainly expressed in G_0 and G_1 phases. In the absence of mitogens and/or presence of TGF-β, $p27^{kip1}$ binds to cyclin E/Cdk2 complex in a sequential manner and inhibits its activities. [92] This results in maintaining of quiescence and cell cycle arrest at the G_1 phase [94]. This arrest can be lifted when cyclin E/Cdk4/6 are produced in mid-G_1 and titrate p27 away from cyclin E/Cdk2. Then, as cell cycle progresses, p27 is phosphorylated and exported out of the nucleus. Interesting to note, during embryonic development, nuclear p27 was found to promote neuronal differentiation by stabilizing neurogenin 2 via its amino-terminal. [95]

A Cdk-independent function of p27, once transported into the cytoplasm, is to modulate cell shape and motility via actin cytoskeletal rearrangement. Exported p27 co-

localises with F-actin and binds to Rho GTPase RhoA and Rac1. [96] Rho-mediated signaling pathway leads in inhibition of the actin depolymerizing protein cofilin, resulting in stress fibers stabilization. Cytoplasmic p27 prevents Rho GTPase activation by the guanine-nucleotide exchange factor (GEF), thus promoting cell migration. [91] The group of Baldassarre also discovered that $p27^{kip1}$ inhibits the extracellular matrix mediated cell migration, but not cell-cell adhesion. [93] This inhibition is done by binding to the microtubule-destabilizing protein stathmin via its C-terminal.

Another inhibitory function of cytoplasmic $p27^{Kip1}$ is to prevent growth factor receptor-bound protein 2 (Grb2) activation by blocking its association with the guanine nucleotide exchange factor SOS. [97] Grb2 plays a role in the activation of the GTPase Ras, which is the activator of the mitogen-activating protein kinase (MAPK) signal pathway. MAPK cascade promotes transcription activation of a variety of genes involved in cell survival, mitosis, cell differentiation, etc.

1.4.3. Regulation

The regulation of p27 occurs at 3 levels: transcription regulation on the promoter region of p27, translational regulation of the mRNA and post-translational cytoplasmic retention and proteolysis. [67] In absence of growth or survival signals, protein kinase B (PKB/Akt) is inactivated. PKB/Akt is an inhibitor of transcription factors of the Forkhead box class O family (FoxO). Inhibition of PKB/Akt increases FoxO in the nucleus, which in

turn results in an increased transcription of the *CDKN1B* gene as well as p27 protein stability. [98] The oncogenic tyrosine kinase Bcr-Abl was found to interact with p27 inducers and prevent p27 up-regulation after growth factor deprivation or TGF- β treatment. [99] Bcr-Abl was also found to promote p27 degradation in phosphatidylinositol-3-kinase (PI3K)-dependent (i.e. PKB/Akt) and PI3K-independent ways. Furthermore, the presence of an internal ribosome entry site (IRES) in the 5' untranslated region (5'-UTR) of p27 mRNA facilitates its translation in presence of stress. However, a U-rich region of the 5'-UTR loop has been shown to interact with the RNA-binding protein HuR, previously characterized as an inhibitor of p27 translation and down-regulator of endogenous p27. [100]

Post-translational regulation often begins with phosphorylation of p27. In general, tyrosine phosphorylation inactivates the protein while phosphorylation of serine or threonine residues leads to delocalisation and/or degradation. [101] In response to mitogens, p27 cytoplasmic retention begins with phosphorylation of two sites. While the human kinase interacting stathmins (hKIS) phosphorylates serine 10 of p27, PKB/Akt proteins phosphorylates the threonine 157 residue. [67, 102] The phosphorylated p27 is then bound by Jab-1 at the NES/Jab 1 binding domain, which serves as an adaptor for binding of p27 to the shuttle protein, exportin CRM-1, to mediate nuclear export. [103] (for mechanism of export, see Fig.2)

The transport of p27 to the cytoplasm lifts the cell from the $p27^{kip1}$-induced G_1 arrest and pushes it toward cell cycle. Some p27 are retained in the cytoplasm to mediate cell motility regulation while most are degraded. The regulatory 14-3-3 protein was shown

to compete with α5-importin for binding to p27 NLS to suppress the nuclear transport of threonine 157-phosphorylated p27, thus promoting cytoplasmic accumulation of p27. [104]

Different mechanisms mediate the proteolysis of $p27^{Kip1}$ depending of its location (nuclear or cytoplasmic), state (free or bound) and/or phosphorylated sites. The degradation of cyclin-dependent kinase inhibitor $p27^{Kip1}$ at the G_1/S transition of the cell cycle by the ubiquitin–proteasome pathway is its predominant type of negative regulation. At the G_1/S transition, cyclin E/Cdk2 complexes phosphorylate nuclear p27 on threonine 187, making it susceptible for ubiquitination by the nuclear ubiquitin ligase (E3) SCF^{Skp2}. [105] (see Fig.4)

Studies revealed that the tyrosine kinase Src can phosphorylate p27 on both tyrosine 74 and 88, resulting in a decrease in Cdk2 inhibition of p27 but an increase in cyclin E/Cdk 2-mediated proteolysis. [106] However, if $p27^{Kip1}$ is exported from the nucleus, then the cytoplasmic Kip1 ubiquitination-promoting complex (KPC) will be the one promoting proteolysis of $p27^{Kip1}$. [107] The adaptor protein Grb2 can bind to exported p27 at its phosphor-tyrosine site and accelerate Jab1/CSN5-mediated degradation of p27. [108]

1.4.4. Relevance to cancer

$p27^{kip1}$ has been deemed a tumor suppressor protein because its functional impairment has been implicated in tumor development in humans. Unlike other well characterized tumor suppressors, mutation of the p27 *CDKN1B* gene is rarely observed. [67] Most alterations of p27 are caused by down-regulation of translation and transcription,

increased proteolysis rate, sequestration by cyclin D/Cdk 4/6 complexes and cytoplasmic retention. [91] Indeed, a drastic reduction of p27 level has been observed in about 50% of all cancer types, such as breast, colon, lung, etc., and is associated with poor outcome. [66, 67] Incidentally, an up-regulation of SCF^{Skp2} was observed in most of the p27 deregulation-mediated tumor formations. [109]

Two different tyrosine kinase receptors-activated Ras-mediated signaling pathways cause the deregulation of p27 in malignant cells. First, Ras activates PKB/Akt, which phosphorylates both p27 and transcription factor Afx, resulting in cytoplasmic retention of p27 and lower p27 transcription. Furthermore, Ras activates MAPK signaling, leading to increased expression of cyclin D/Cdk4, thus more free cyclin E/Cdk 2 and more nuclear p27 degradation. [67] The consequence of increased degradation of nuclear p27 is the absence of cyclin/cdk inhibition, thus absence of cell cycle arrest at G_1. Moreover, cytoplasmic p27 was observed in 70% of the invasive melanomas while none in the non-invasive ones. [110] Since cytoplasmic p27 are involved in cell motility, its delocalisation to the cytoplasm contributes to cancer cell metastasis. [111]

Many factors, such as tumour differentiation, grade, size, and stage, are often used as prognostics to predict severity of a cancer and outcome of a treatment. Levels of p27 are found to be of independent prognostic significance, since reduced p27 in cancer cells have a 1.82–5.94 fold increased risk of disease recurrence or death. [111] Therefore, in cancers originated from epithelials, central nervous system and lymphoid tissues, where p27 functions are the most affected, restoration of p27 levels and/or nuclear localization may predict treatment responses.

Various anti-cancer therapies have sought to restore p27's inhibitory functions. Studies found that a degradation-resistant form of p27 was able to induce growth arrest and apoptosis in breast cancer cells. [112] In recent years, a multitude of molecular therapies have been developed targeting growth factor receptors, including the epidermal and insulin-like growth factor families, signal transducer kinase PKB/Akt and MEK (also called MAP2K), and tyrosine kinase Bcr-Abl and Src; all of which regulate endogenous p27. [91, 111] Other therapies sought to reduce p27 degradation with use of specific proteasome inhibitors. However, to date, only the proteasome inhibitor argyrin A was shown to affect p27 selectively. [91] Other strategies are also aiming to reduce metastasis caused by cytoplasmic p27. MiRNA-mediated inhibition of p27 translation has emerged as a novel mechanism that can reduce p27 in some human cancers. [111] Two mi-RNAs (miRNA-221 and miRNA-222) have been found to down-regulate p27 translation via the 3'UTR of its mRNA. [91]

1.5. Skp2

1.5.1. Structure and Function

Skp2 protein is called an F-box protein due to the presence of the conserved F–box motif substrate recognition domain. [56] The structural F-box domain is responsible for its substrate specificity. Skp2 is a member of the 22 F-box proteins belonging to the Fbl sub-class in which 'Fb' stands for F-box and 'L' represents the leucine-rich repeat (LRR). In fact, Skp2 counts 10 tandem leucine-rich repeats forming an arc-shaped α-β-repeat-structured protein-protein interaction domain. [11]

Skp2 (also called p45) stands for S-phase kinase-associated protein 2, because human Skp1 and Skp2 were originally discovered in association with the cyclin A/Cdk2 complex and was later shown to promote entry into S-phase. [105, 113] Both Skp2 and cyclin A possess a non-canonical interaction motif specific for their mutual binding. This association serves to directly protect cyclin A-Cdk2 from p27 inhibition through competitive binding. [114] Most of Skp2's functions involve in triggering degradation when linked to the SCF ubiquitination complex.

1.5.2. SCF^{Skp2}-complex

The SCF^{Skp2} complex refers to the core Cul1 - Rbx1 - Skp1 linked to the substrate-recognition F-box protein Skp2. (see Fig. 4) Incorporation of Skp2 into the SCF complex confers the ability to induce ubiquitination of a variety of targets, namely the Cip/Kip cyclin/Cdk inhibitors $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, cyclins D and E, transcription factors c-Myc, Foxo1 and E2F-1, as well as the pocket protein p130 and various others. [11, 109, 115]

Mostly expressed at G_0, p130 form suppressor complexes with E2F factors and inhibit all the E2F-dependent transcriptions. In addition, p130 has the ability to directly inhibit Cdk2/cyclin E and A complexes. Therefore, poly-ubiquitination of S672-phosphorylated p130 by SCF^{Skp2} is required to remove cells from quiescence. [116] Furthermore, studies discovered that Skp2-containing SCF complexes were able to interact directly with E2F-1 and mediate its rapid degradation at the S/G_2 transition. [117] Skp2 also mediates the ubiquitination of c-Myc, resulting in cell cycle inhibition. [118] This inhibition was largely compensated by up-regulation of c-Myc promoter gene transcription by the complex Skp2-c-Myc.

The previous interactions were only a few examples of Skp2's role in cell cycle regulation. Despite all its other functions, ubiquitination of p27 by Skp2 is essential for cell cycle progression. At the G_1/S transition, SCF^{Skp2} targets specifically T187-phosphorylated p27 for proteolysis. [105] An interesting fact, unlike other known SCF substrates, the binding of p27 by SCF^{Skp2} requires the accessory protein Cks1. [119]

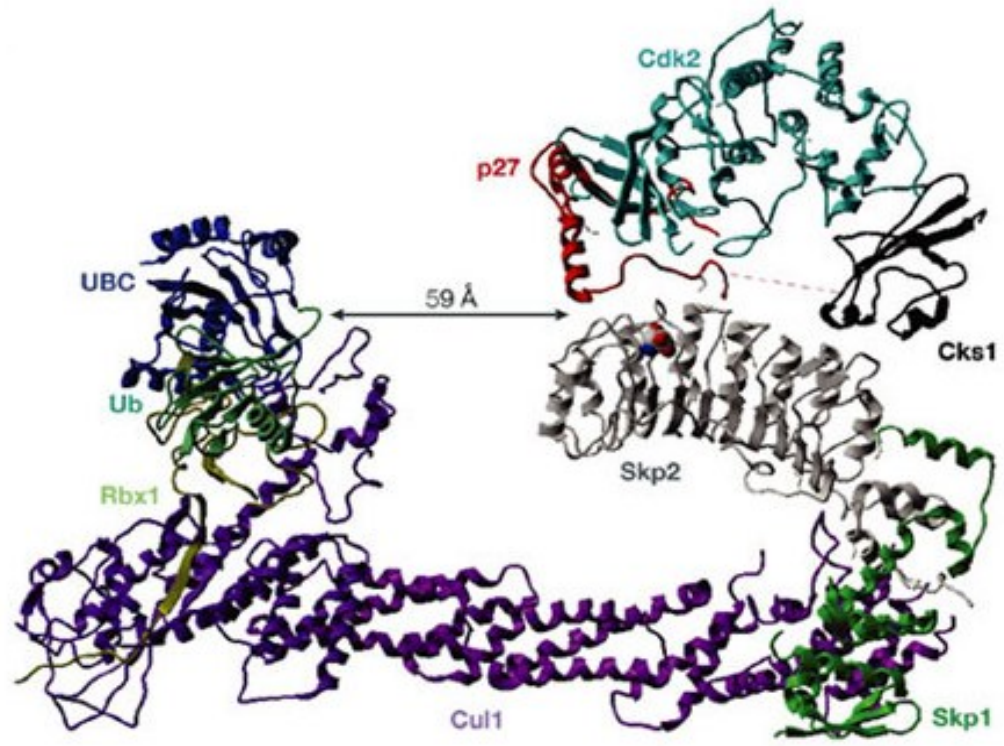

Figure 4 Structure of the SCF^{Skp2}–Cks1 complex linked to p27-Cdk2 (adapted from *{Cardozo and Pagano, Nat Rev Mol Cell Biol 2004; 5(9)}*)

The E3 ubiquitin ligase SCF^{Skp2} is composed of a Cul1 scaffolding protein with a Rbx1 E2 binding protein and a F-box binding protein Skp1 on each end. Rbx1 recognize and bind to a E2 ubiquitin conjugating enzyme while Skp1 binds to the Skp2-Cks1-p27 complex. Cks1 interacts with the F-box protein Skp2 and greatly increases its affinity for T187 phosphorylated p27. Cks1 further strengthen Skp2-p27 binding by associating with Cdk2, which is bound to p27 to mediate its phosphorylation on threonine 187. SCF^{Skp2} then will transfer the ubiquitin molecule from E2 to p27. A poly-ubiquitin chain will be formed. This poly-Ubs chain targets p27 to the 26S proteasome for proteolysis and increases the proteasome's affinity for the tagged p27.

1.5.3. Regulation and Cancer

Level of Skp1 and Cul-1 are constant throughout the cell cycle while Skp2 expression is regulated. Various transcription factors, E2F1, FoxM1, GABP, NFκB, SP1 and CBF1, all promote Skp2 gene transcription while Foxp3 has been shown to repress Skp2 expression. [115] Akt-mediated phosphorylation of Skp2 can further modulate its localisation as well as function. Skp2 accumulates from late G_1 and rapidly degrade before mitosis. [117] Poly-ubiquitination of Skp2 was found to be mediated by APC^{CDH1}, as a result of SCF and APC/C coordinated regulation of cell cycle. [120] Phosphorylation of Skp2 on serine 64 and 72 by Cdk2 and Akt respectively prevent Cdh1 binding. [115]

An increased level of Skp2 has been observed along with reduced levels of p27 as well as increased invasion and metastasis in many types of cancer. [113, 121] The level of Skp2 mRNA can be used to predict p27 level in tumors and correlate with the tumor stage. [122] Thus, SCF^{Skp2} inhibitors have been considered as a novel class of antitumor agents. Ji et al. discovered that knock-down of Skp2 alone in cancer cells only had mild anti-tumor effects. On the other hand, disruption of Skp2-cyclin A interaction by a blocking peptide was able to induce selective cancer cell killing. [123] Further studies permitted Chen et al. to find a compound, dubbed compound A, that could prevent the incorporation of Skp2 into the SCF complex. This resulted in G_1/S cell-cycle arrest as well as SCF^{Skp2}- and p27-dependent, caspase-independent, programmed cell death via autophagy. [109]

Recent works by Wu et al have identified small molecular inhibitors specific to SCF-Skp2 activity using both in silico and in vitro assays. [124] These compounds

selectively blocked SCF^{Skp2}-dependent degradation of p27 by acting on the targeted pocket formed by Skp2-Cks1. Effective cell cycle arrest at G_1 or G_2/M phases has been observed in cancer cells treated with these inhibitors. Once again, this discovery underlines the importance of the Skp2-Cks1-dependent p27 degradation mechanism in cancers.

1.6. Cks1

The human Cdc28 protein kinase regulatory subunit 1 (Cks1) is encoded by the *Cks1B* gene on the chromosome 1q. [125, 126] It belongs to the cell cycle regulatory Suc1 (suppressor of Cdc2 mutation)/Cks family. First discovered in yeasts, they were identified as genetic suppressors of Cdk mutations. [119, 127] Functional domain sequences are largely conserved between species. Two homologous Cks1 proteins are found in human: Cks1 (CksHs1) and Cks2 (CksHs2). They share ~81% of sequence homology, yet differ in respective folding. [128] All Cks proteins are characterized by their ability to bind to a Cdk catalytic subunit; however, only human Cks1 possesses the additional ability to mediate Skp2-p27 binding. [119] Cks2 functions in germ cells. They play a role of safeguard in DNA replication and cell differentiation. [129] Cks proteins are essential components of mitotic cyclin/Cdk complexes, mice with double knockout of Cks1 and Cks2 are non-viable.

1.6.1. Structure and Function

Cks1 is a small protein formed from only one polypeptide chain of 79 amino acids long (~9.6 kDa) and has a half-life of approximately 4 to 6.6 hours. [130-132] It is folded into four antiparallel β-strands, involved in Cdk binding, and two short α-helices, involved in Skp2 binding. [133] (see Fig.5B) Cks1 is folded in a way that there is a β-hairpin followed by an exposed α-helical hairpin on the amino-terminal and a β-hairpin on the carboxyl-terminal, resulting in a four-stranded β-sheet. [128] Cks1 contains 3 protein-protein

interaction domains for Cdk-binding, anion-binding and Skp2-binding. The anion-binding site is responsible for recognizing and binding phosphate, sulfate, or acidic residues of proteins, including phosphorylated p27. [133] (see Fig.5) A function of Cks1 is to promote binding of Cdks to partially phosphorylated proteins and mediate their poly-phosphorylation. The targets of multi-phosphorylation are often substrates of APC/C and G_2- and M-phase regulators, including Cdc25 and Wee1. [127]

Moreover, Cks1 greatly increases the affinity binding of Skp2 to p27. This interaction is Skp2-specific, since it was found that ubiquitination of cyclin E by SCF^{Skp2} is also mediated by Cks1. [133] Skp2 associates with Skp1 of the SCF complex by its F-box motif while its LRR domain and C-terminal binds to Skp2-binding domain of Cks1. [119] Cks1 anion-binding site recognize the phosphorylated Thr187 side chain of $p27^{Kip1}$ and along with Skp2, promotes mutual binding to C- and N-terminal of p27 respectively. An invariant glutamic acid 185 of p27's central chain inserts into the interface between Skp2 and Cks1, interacting with both. [119] Sitry et al. proposed that interaction of Skp2 with the substrate is further strengthened by the association of the Cdk-binding site of Cks1 with Cdk2/cyclin E, to which phosphorylated p27 is bound. [133] (see Fig.4) $p21^{Cip1}$, $p57^{Kip2}$ and p130/Rb all have been shown to be ubiquitinated and degraded via the SCF^{Skp2}-Cks1 pathway. p57 shares a homologous C-terminal with p27 that mediate its interaction with Skp2-Cks1, while the mechanism of p21 ubiquitination still remain unclear. [119] p130/Rb is an inhibitor of Cdk2 and the transcription factor E2F, degradation of this protein is thus essential for mitotic entry. [134]

Cks1 also exert multiple p27-independent functions in the G_1 phase. In yeast, Cks1 is essential for Cdk activities. [135, 136] Along with Cdc28, the yeast homolog of Cdk1, Cks1 is recruited to gene promoters to modulate a subset of genes. The ubiquitin-binding domain of Cks1 allows it to selectively recruit ubiquitylated subunits of the proteasome as a mechanism of regulation of the transcriptional process. In mammalian cells, various studies also confirmed that Cks1 partake in cdks pathways, stimulate the Cdc27 component of the APC/C complex, and increase Cdc20 expression. This latter protein is known to be involved in two microtubule-dependent processes, therefore suggesting that cytoplasmic Cks1 might play a role in cell migration. Recently, Hoellein et al. has shown genetic evidence that Cks1 association with Cdk2 instead of SCF^{Skp2} is regulated for the G_1/S transition. [137]

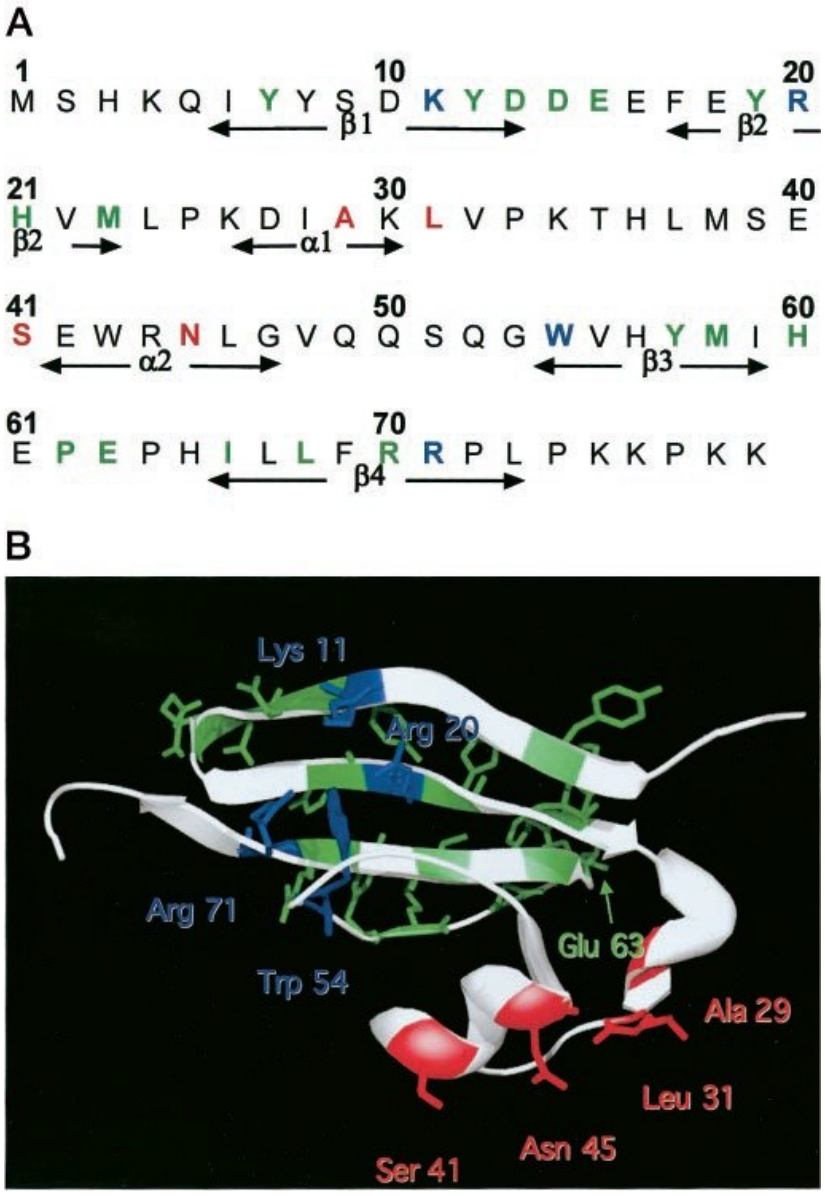

Figure 5 The amino acid sequence (A) as well as the crystal structure (B) of the human form of Cks1. (adapted from *{Sitry et al., J Biol Chem, 2002, 277(44)}*)

Cks1 forms a single stranded polypeptide of 79 amino acids long. It has 3 distinct binding sites for interaction with anion (shown in blue), Skp2 (in red), and Cdk (in green).

1.6.2. Relevance to cancer

Cks1 expression level fluctuates during the cell cycle and correlates with expression of Skp2. Both are ubiqitinated by the APC/C^{Cdh1} complex. [130] The main activator of Cks1 transcription is the known nuclear factor Y (NF-Y). [138] However, FoxM1 was also shown to promote expression Cks1, along with Skp2. [139] Up-regulation of Cks1 is mediated by a tandem combination of cell cycle dependent element (CDE) and cell cycle genes homology region (CHR) found on the promoter region of *Cks1B* gene. On the opposite, the tumor suppressor p53 repress Cks1 expression in a NF-Y-independent way. [138]

The gain of an extra chromosome 1q in cancer leads to nuclear Cks1 and Skp2 overexpression in various cancer cells, including lung cancer [140], oral squamous cell carcinoma [141] and multiple myeloma. [142] With Cks1 being the rate-limiting component of SCF^{Skp2}-Cks1, its overexpression often results in increased degradation of cell cycle inhibitor p27 and poor prognosis. Unlike common beliefs, Cks1 overexpression alone was insufficient to cause cancer formation in healthy cells. [143] However, it does correlate with tumor size and metastasis, thus conferring great diagnostic and prognostic value. [144, 145] Chang et al. proposed the use of immunohistochemistry for detecting Cks1 to predict outcomes and survival rates of p27-reduced cancers. [142] Moreover, recent studies also noted that overexpressed Cks1 is also involved in various p27-independent pathways also essential in cancer development, such as increased metastasis [126], radiotherapy and drug resistance [145], as well as promotion of cell survival via increased interleukin-8 (IL-8) expression [146] and activation the MEK-Erk pathway. [126] Considering the importance of

Cks1 overexpression in cancer, inhibition of Cks1 is of great therapeutic potential. The use of small interference RNA to inhibit Cks1 translation was used by various groups and it indeed induced G_2/M arrest in cancerous cells and not in normal human cells. [140, 142] Other methods such as Skp2-Cks1 ligase inhibitors [147] and Cks1-related protein inhibitors are also being considered.

2. Hypothesis & Objectives

Clinical studies have shown that in a variety of cancers, p27 is either over degraded in the nucleus or mislocalized to the cytoplasm. Endogenously, nuclear p27's degradation is mediated by the E3 ubiquitin ligase SCF^{Skp2} complex, which targets it to the 26S proteasome. Binding of Cks1 to both the F-box protein Skp2 and p27 greatly increases their affinities for each other and stabilizes their interactions. Incidentally, overexpression of Skp2 and Cks1 is observed in most of the p27-disregulated cancers.

A previous project in the lab was aimed to identify all interactions of p27 and p27-associated proteins with their substrates. A Tandem Affinity Purification (TAP tag) approach was used to identify specific binding proteins of Cks1. Among the candidate interactors, α1 importin (also known as kpna2) as well as β3 importin (also known as imp5) were identified. Concordantly, the amino acid sequence of Cks1 reveals a C-terminal ending with lysine – lysine – proline – lysine – lysine (K-K-P-K-K) (see Fig.5), which resembles to the classic NLS, formed of a lysine – lysine/arginine – any amino acid – lysine/arginine (K-K/R-X-K/R) sequence.

From those observations, I believe that despite its small molecular size, Cks1 travels through nuclear pores via classic α-β importins pathways rather than passive diffusion. Therefore, control of Cks1 activity can be achieved by interrupting its nuclear translocation. Hence, my objectives were to further investigate the evidence of Cks1 interaction with α-

importins. Then, using Cks1 C-terminal mutants, verify the functional importance of this candidate NLS. Finally, study the consequence of β importin inhibition on Cks1 localization.

3. Materials & Methods

3.1. Plasmids & Mutagenesis

The human Cks1 homolog, Cks1B, sequence was obtained from pcDNA$_3$-HA-Cks1 plasmid, previously created by an ancient member of the lab, Benjamen Turgeon. PCS3-MT-5Myc-Cks1 plasmids were generated, using the digestion enzymes BamH1 and Xba1, for co-immunoprecipitation (Co-Ip), GST pull down, and immunofluorescence purposes. The 5Myc tag rather than the HA tag was selected due to the stability it confers to Cks1 mutants (see Results). Six clones were created, five mutants and one deletion, named respectively Cks1_m11, Cks1_m12, Cks1_m21, Cks1_m22, Cks1_m4, and Cks1_del according to the number and site of mutagenesis. All changes were made to the C-terminal end of the protein on the candidate NLS. The C-terminal amino acid sequence of the wild type Cks1 protein is ILLFRRPLPKKPKK. The point mutation mutants were each mutated at one or more of the lysine (K) residues. The amino acid lysine was changed into alanine (A). Consequently, Cks1_m11 ends with ILLFRRPLPAKPKK, Cks1_m12 ends with ILLFRRPLPAAPKK, Cks1_m21 ends with ILLFRRPLPKKPAK, Cks1_m22 ends with ILLFRRPLPKKPAA and Cks1_m4 ends with ILLFRRPLPAAPAA. The Cks1_del ends at ILLFRRP.

Most importin plasmids were given by Dr. Marc Servant, professor of the Faculty of Pharmacy at Université de Montréal while Flag-Kpna2 and the control HA-Chk2 plasmids were given by Dr. Laura Zannini of the Istituto Nazionale Tumori, Italy.

For infection purposes, the 5Myc-Cks1 wild type (wt) and mutants were subcloned into pBabe-puro vector for retroviral infections using the digestion enzymes BamH1 and SnaB1.

3.2. Cell culture

Immortalized transformed human embryonic kidney cell line HEK 293, rat fibroblast cell line Rat1, cervical cancer-derived cell line HeLa, the human osteosarcoma cell line U2OS, the mouse embryonic fibroblast cell line NIH3T3, and the human glioblastoma-derived cell line T98G were used. All cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine and antibiotics, penicillin (50 μg/ml) and streptomycin (28.9 μg/ml). Only HEK293 cells were plated in both supplemented MEM and DMEM depending of the desired growth rate. All plated cells are kept at 37^{o}C under 5% CO_2 conditions. For storage purposes, the cells were frozen and kept at -80^{o}C in complete DMEM + 10% dimethyl solfoxide (DMSO).

3.3. Transfection & Infection

Calcium phosphate ($CaPO_4$) transfection

A total of 14 µL plasmids were added to 500 µL $CaCl_2$ and vortexed. 500 µL phosphate buffer with a pH of 7.1 was added drop by drop 5 min later and vortexed. The mixture was incubated at room temperature (RT) for 20 min. The plasmid-$CaPO_4$ mixtures were then added drop by drop to the HEK 293 cells pre-plated at 30-50 % confluence in 10 cm plates. Incubation at 37 ^{o}C ensued for 12-14 h. The transfection medium was then replaced with fresh complete DMEM. Cells were lysed for experiments after 24-30 h incubation at 37 ^{o}C.

Lipofectamine transfection

Rat1, NIH3T3, T98G, U2OS or HeLa cells were pre-plated onto glass coverslips for 24 h at 20 % confluence in 6 cm wells. A total of 5 µg plasmids were added to 250 µL Opti-MEM and vortexed. 15 µL Lipofectamine were added to 250 µL Opti-MEM and vortexed. After 5 min incubation at RT, the lipofectamine mixture was added drop by drop to the plasmids and vortexed. After 20 min of incubation at RT, the plasmid-lipofectamine mixture was added to pre-plated cells, which is also in Opti-MEM. Incubation ensued for 4-6 h. The transfection medium was then replaced with fresh complete DMEM. Cells were lysed for experiments after 40 h incubation at 37 ^{o}C.

Lentiviral infection

HEK 293 cells were pre-plated at 20 % confluence for 24 h. Plasmids containing the shRNA were mixed with pMD_2-VSVG, pMDLg-pREE and pRSV-Rev in a ratio of 4:2:1:1 respectively. The DNA mixture was transfected to HEK 293 cells using $CaPO_4$ transfection. After 12-14 h incubation, the transfection medium was replaced with fresh complete DMEM. Rat1, T98G or NIH3T3 cells were pre-plated at ~30 % confluence for 24 h. Medium of the infected HEK 293 cells was then filtered with 0.45 μm filters and then added to the latter pre-plated cells along with polybrene at 4 μg/ml of filtrate. After 24 h incubation, Rat1 cells were selected for successful infections with complete DMEM supplemented with 2 μg/ml puromycin. Successfully infected cells were all kept in DMEM 2μM puromycin.

Retroviral infection

HEK 293 cells were pre-plated at 20 % confluence for 24 h. pBabe-puro 5Myc-Cks1-wt and mutants' plasmids were mixed separately with pCL-Ampho at a ratio of 1:1. The DNA mixture was transfected to HEK293 cells using $CaPO_4$. 12-14 h later, the transfection medium was replaced with fresh complete DMEM. Rat1, T98G or NIH3T3 cells were plated at ~30 % confluence for 24 h. Medium of the infected HEK293 cells was filtered with 0.45μm filters and then added to Rat1, T98G or NIH3T3 cells along with polybrene at 4 μg/ml of filtrate. After 24 h incubation, Rat1 cells were selected for successful infections with DMEM supplemented with 2 μg/ml puromycin. Successfully infected cells were all kept in DMEM 2μg/ml puromycin.

3.4. Antibodies

For Western blots, mouse monoclonal anti-flag M2 was used to detect Flag-tagged Kpna2, while HA-tagged β importin and Chk2 was detected using house made mouse monoclonal anti-HA 12ca5 and Covance's anti-HA. House made anti-Myc 9e10 and Santa Cruz anti-Myc Sc40 were used for detection of 5Myc-Cks1. In immunofluorescence experiments, Sigma's anti-Myc rabbit was used along with the Alexa 488 rabbit as secondary antibodies. Santa Cruz's anti-Imp 5 and anti-Kpna2 were used to determine efficacies of the shRNAs.

3.5. Co-immunoprecipitation

Cell lysis

Plasmids were previously co-transfected using $CaPO^4$ transfection method in HEK 293 cells. The transfected cells are then lysed using either classic Co-IP lysis buffer (5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.1 % NP-40) or ELB buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 0.1 % NP-40) with 1/1000 protease inhibitor Leupeptin (Leu), Phenylmethylsulfonyl fluoride (PMSF) and Pepstatin A (PA), and 1/100 phosphatase inhibitor sodium orthovanadate (Van). Cell lysates, used for Co-IP, were obtained after 5 min centrifugation, at 12000 rpm and RT, to spin out cell debris of the lysed cells.

Immunoprecipitation

For anti-Flag Co-IP, commercial anti-Flag beads were washed with Co-IP buffer. For anti-Myc and anti-HA Co-IP, antibodies were incubated with immunoglobulin binding protein A and protein G beads respectively at 4^{o}C for 3 h in TNET buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA, 0.5 % Triton X-100). Beads were then washed and resuspended in Co-IP buffer at 8-15 µg beads per sample. Cell lysates were added to beads and incubated at 4^{o}C overnight on turning wheel. After 3 washes, the beads were resuspended in 150 µL 2X sample buffer (SB) and heated at 90^{o}C for 5 min for protein unfolding. Samples were then loaded onto gels for Western blot.

3.6. GST-pull down

GST-beads

Laboratory grown *E.coli* BL21 bacteria were transformed with PGEX-KG-importin plasmids, given to us by Dr. Marc Servant. The bacteria were grown overnight on LB-agar plate, selected, and grown in liquid LB. Bacteria culture was further diluted 1/50 and incubated at 37^{o}C until exponential growth phase. IPTG was added to induce production of proteins encoded by plasmids. Incubation of bacteria culture at RT overnight ensued. Lysis of bacteria in TB buffer (1 % PBS, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 % Triton X-100, 1 % sarcosyl) with 1/1000 lysozyme. Clarified lysate was obtained after freeze-thaw

and sonication. The lysates were continuously mixed on turning wheel with previously TB buffer-washed glutathione-sepharose beads for 2 h at 4^{o}C. Beads were washed and kept in TB buffer.

Pull down

HEK 293 cells were previously transfected with 5Myc-Cks1 plasmids using $CaPO_4$ and lysed with Co-IP buffer. The centrifuged lysate was then incubated with Co-IP buffer-washed GST beads at 4^{o}C overnight, on a turning wheel. Beads were then washed with Co-IP buffer, eluted in 2X SB and heated for 5 min at 90^{o}C. Samples were analysed by Western blot.

3.7. Radioactive *in vitro* translation

Following Invitrogen's *in vitro* translation kit, 0.5 µg Cks1 and 0.5 µg Kpna2 plasmids were mixed with 25 µL rabbit reticulocyte lysate, 2 µL reaction buffer, 1 µL RNA polymerase, 1 µL methionine-free amino acid mixture, 1 µL ribonuclease inhibitor and 1 µL of radioactive [^{35}S] methionine (10 µCi/µl) in 18 µL RNase-free water. After incubation of 90 min at 30^{o}C with shaking, the mixture was eluted in SB and heated at 90^{o}C for 5 min. Western blot was then performed to determine the presence of Cks1-Kpna2 complexes.

3.8. Western Blot

Cell lysis

For test of plasmids expression, HEK 293, Rat1, NIH3T3, T98G, U2OS or HeLa cells were plated and allowed to grow overnight. They were incubated with or without 2 μM of proteasome inhibitor MG132 for 6 hours. Then, they were lysed in classic lysis buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 % Triton X-100) with protease inhibitors 0.01 mg/mL PMSF, PA and Leu, and 1 mM Van. Centrifugation of lysed cells was performed to spin out cell debris. Lysates were dosed and aliquots containing 150 μg proteins were prepared. These samples were diluted in SB and heated at 95^{o}C for 5 min for proteins unfolding. Samples were loaded onto gel for Western blot with appropriate weight ladder.

Gels and loading

Resolving gel's (375 mM Tris pH 8.8, 1 % SDS, 1% APS, 0.1 % TEMED and acrylamide) concentration varied from 8 % to 18 % depending of the protein's size. All samples containing HA-Cks1 (~9 kDa) were loaded into 18 % acrylamide gel while 5Myc-Cks1 (~19 kDa) samples were loaded in 12 % acrylamide gel. Overnight migration in running buffer (25 mM Tris, 192 mM Glycine, 0.1 % SDS, pH 8.3) was set to 7-8 milliamps per gel. Transfer of proteins from gel to cellulose membrane was done at 45 volts for 2h in transfer buffer (25 mM Tris, 192 mM Glycine, 0 % or 10 % Methanol, pH 8.3). Membranes were then stained with Ponceau red to mark out the molecular weight ladder.

Blotting

Membrane was blocked with 5 % dry milk/TBS/0.1 % Tween for an hour and then washed 5 times with TBS/0.1 % Tween (TBS/T) for at least 5min each time. Incubation of membrane with primary antibody diluted in 5 % dry milk/TBS/T for an hour at RT or overnight at 4^{o}C ensued. After 5 washes of membrane with TBS/T of 5 min each time, the membrane was further incubated with horseradish peroxidase-conjugated anti-primary antibody secondary antibodies diluted in 5 % dry milk/TBS/T for an hour at RT. Proteins were revealed with ECL or GE Healthcare's ECL+ after a third thorough washes with TBS/T.

3.9. Immunofluorescence

Infected or transfected cells were plated onto glass coverslips at 20-40 % confluence. Cells were incubated with 3.7 % paraformaldehyde (PFA) at 37^{o}C for 20 min to be fixed onto the slides, 0.1 M Glycine/PBS at RT for 10 min to quench any remaining PFA, and 0.1 % Triton X-100/PBS at RT for 5 min to permeabilize cell membranes for antibody entry. Cells were further washed and incubated in a stepwise fashion for an hour each time at 37^{o}C, 5 % CO_2, with PBS/0.1 % BSA to inhibit non-specific antibody recognition, primary antibody diluted in PBS/0.1 % BSA, and Alexa 488-labelled secondary FITC or TRITC anti-mouse/rabbit fluorochrome. DAPI was added to mark cell

nucleus. Slides are finally conserved with Mowiol and kept in the dark at RT. Observation were done using inverted microscopy while pictures were taken using an LSM510 confocal microscope.

3.10. β Importin Inhibitor

Rat1 cells infected with the retroviral pBabe puro 5Myc-Cks1-wt were incubated in DMEM supplemented with 2 μM puromycin at 37°C. Cells were grown at 40 % confluence on glass cover slides overnight. Proteins expression was inhibited by incubating plated cells with 50 μM of protein biosynthesis inhibitor, cycloheximide, during 8 and 10 hours. After washing, cells were incubated with supplemented DMEM in presence of 25 μM of either karyostatin 1A or DMSO for 0.5 to 6 hours to allow proteins production. Karyostatin 1A is a small molecule found to specifically inhibit β importin [148] and synthesized by the chemical platform of IRIC (see Fig. 6). Cells were fixed for immunofluorescence.

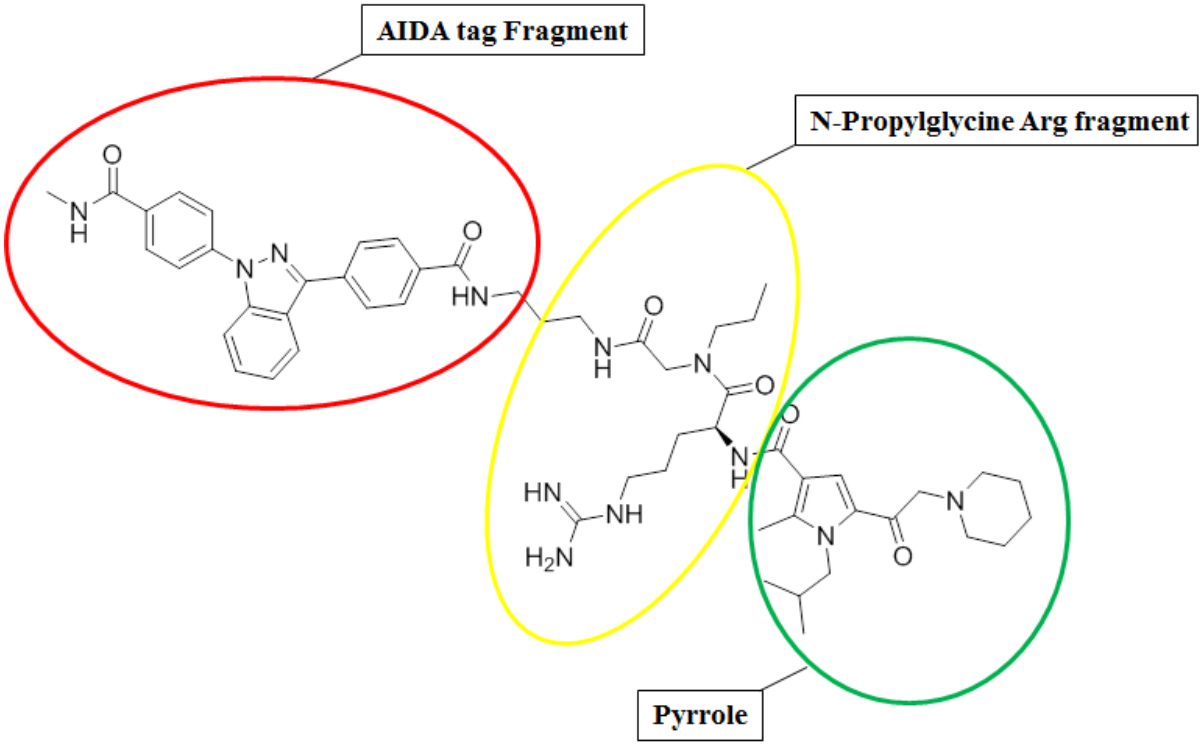

Figure 6 Inhibitor synthesis (by IRIC chemical platform)

Hintersteiner et al. first discovered Karyostatin 1A in 2010 using confocal on-bead screening of tagged one-bead one-compound library. This small molecule could bind to importin β with high nanomolar affinity even at low concentration. It was also found to specifically inhibit importin α/β mediated nuclear import by disrupting the interaction between importin β and the GTPase Ran. No transportin-mediated nuclear import impediments were observed.

4. Results

4.1. Tandem Affinity Purification (TAP)

A previous project of previous member of the lab, Christel Boutonnet, was aimed to identify all interactions of p27 and p27-associated proteins with their substrates. TAP was considered as an approach since it allows gentle protein complex purification under native conditions. However, considering the nature and size of the protein of interest (especially Cks1), the classic purification assays was found to be limited in various ways. Its requirement for a large starting concentration of the bait protein not only increases non-specific interaction, but also renders impossible assays of natively low expressed proteins. Then, its bulky double tag can block certain interactions especially for small proteins. Furthermore, its multiple washes and gel extraction step can disrupt weak interactions as well as protein recovery.

In order to maximize bindings, rule out non-specific interactions, decrease the steric hindrance by the double tag, and recovery after protein dissociation, a modified version of TAP tag was generated. (see Fig. 7) Expression of the retroviral bait protein (BP) tagged with C-terminal Protein C-$Flag_3$ (Cks1-PC-$Flag_3$) was induced in HEK 293 cells. Cell lysates were passed through affinity purification column to isolate Cks1 protein complexes, which were then fractioned and sent to liquid chromatography mass spectrometry (LS/MS). The analysis results were then compared to the negative control in which GFP-PC-$Flag_3$ is used to eliminate background and non-specific interactions.

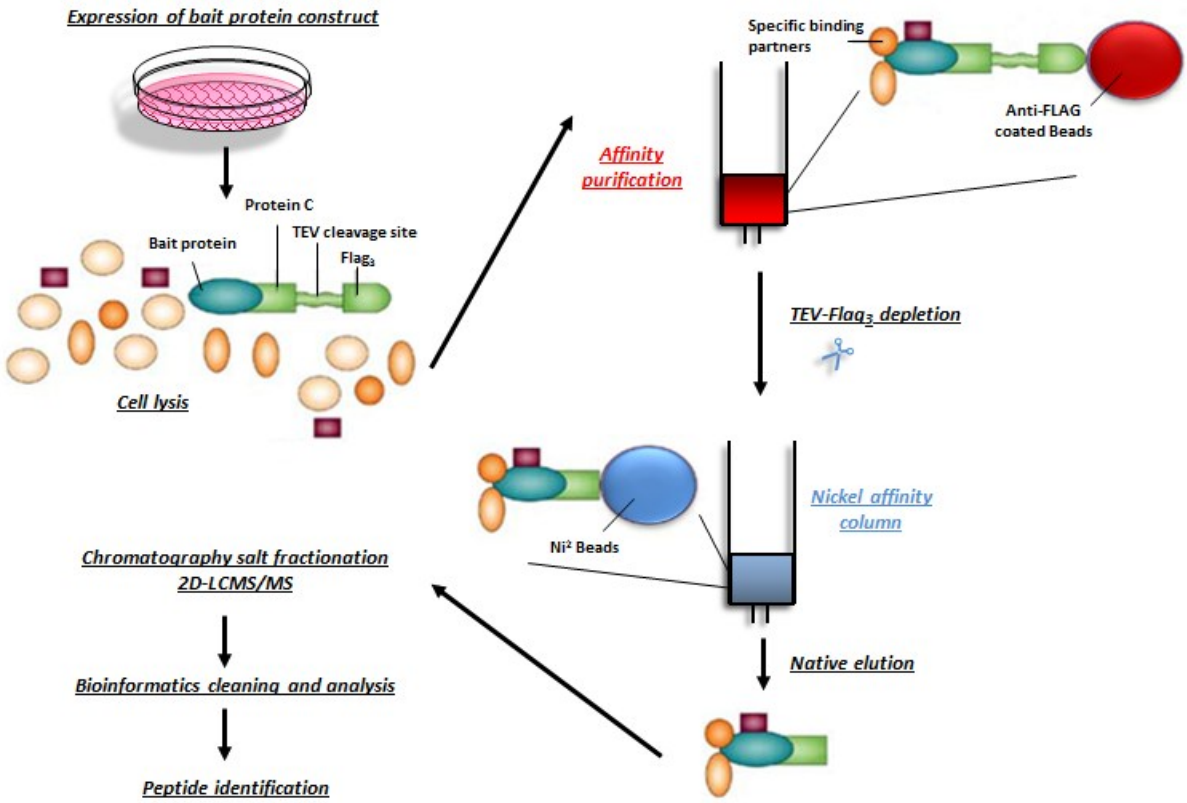

Figure 7 Process of TAP purification (adapted from *{Huber LA, Nat Rev Mol Cell Biol, 2003. 4(1)}*) [149]

HEK293 cells infected with retroviral plasmid containing BP-PC-TEV-Flag$_3$ were induced to express the bait protein for a few hours and then lysed with a low stringency buffer. Cell lysate was then passed through the affinity purification column containing anti-Flag coated beads to select for Flag-tagged protein complexes. The elution was TEV depleted using TEV protease to rule out background protein interactions and passed through a second nickel affinity column to eliminate residual non-specific bindings. The purified protein complex was fractioned and sent to liquid chromatography mass spectrometry for analysis.

Systematic background cleaning was done and the result was compared to the negative control in which the bait protein was replaced by GFP. The remaining peptides were blasted then identified.

4.2. Cks1 expression

Plasmids containing different mutants of Cks1 were first transfected into HEK 293 cells to determine whether or not the clones can be properly translated. (see Fig. 8) However, the HA-Cks1 mutants were found to be very unstable. Even in presence of the MG132 proteasome inhibitor, only HA-Cks1 m4 was weakly expressed. The 5Myc tag was then used and all mutants were highly expressed.

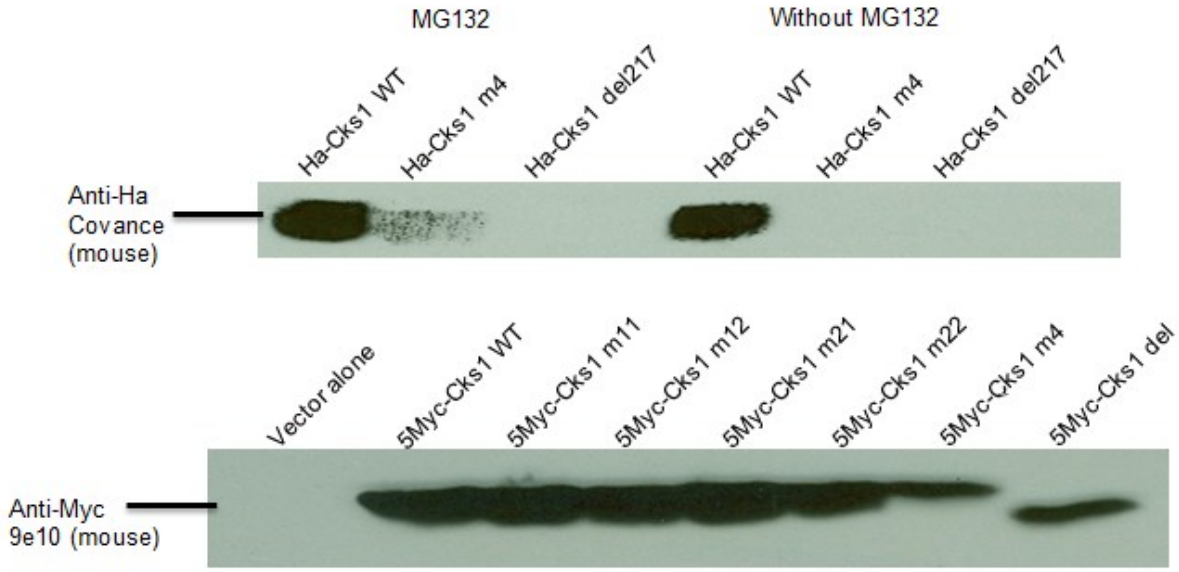

Figure 8 Cks1 expressions in HEK 293 cells. 150 µg proteins were blotted for each sample. Despite the stability conferred by the 5Myc tag, Cks1-m4 and Cks1-del mutants in infected cells were still very unstable and degraded rapidly. Therefore, all transformed cells were incubated with 2 µM MG132, 4 hours prior to experiment, such as IF, to prevent degradation of the protein of interest. (see Fig. 9) Even then, the expression level of m4 and del were much lower than the other mutants.

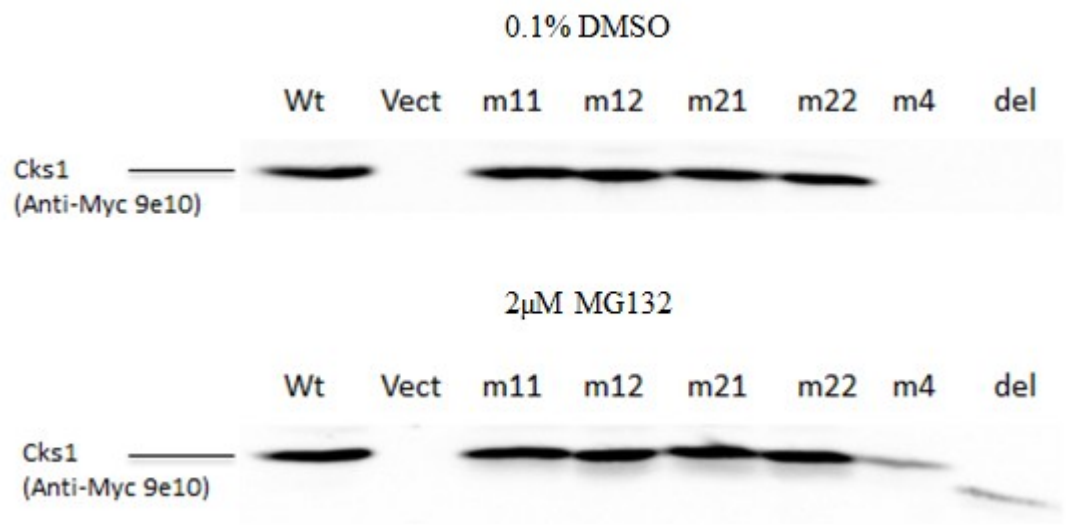

Figure 9 Cks1 expression in infected Rat1 cells. 150 µg proteins were blotted for each sample. Cks1 m4 and del were weakly expressed even in presence of 2 µM MG132 during 4 hours. As negative control, Rat1 cells were incubated with 0.1% DMSO for the same amount of time. This blot was representative of 3 separate infections of Rat1 cells.

4.3. Interaction of Cks1 with importin

Cks1-wt protein was first co-immunoprecipitated with Flag-Kpna2 with either classic Co-Ip buffer or ELB buffer at different NaCl and NP-40 concentration. Furthermore, Co-Ips were done in both ways, using anti-Flag and anti-Myc beads. Co-Ip with Flag-tagged beads revealed a positive binding of Cks1. However, Cks1 alone was also able to bind the beads. (see Fig. 10A). Thus, this interaction had to be deemed non-specific. On the other hand, Co-Ip with Myc-tagged beads was characterized by the absence of interaction of Kpna2 with the beads even with the gentlest Co-Ip buffer at 0.1% NP-40. The

Cks1 bound as predicted (see Fig. 10B). At the same time, a control Co-Ip of Flag-Kpna2 with HA-Chk2 was done. No interaction was observed either.

Since the Flag Co-Ip was not conclusive due to the interaction of Cks1 with the Flag-tagged beads, we wanted to rule out any possible non-specific interactions. After consideration, *in vitro* translation was chosen since only Cks1 and Kpna2 are produced. However, no Cks1-Kpna2 complex was detected after staining. Hence, an intermediate protein was suspected to facilitate the Cks1-kpna2 interaction. Consequently, HA-Skp2 and HA-impβ were separately added to induce protein complex formation. Still, no positive interaction was observed for neither of the Myc or Flag Co-Ip.

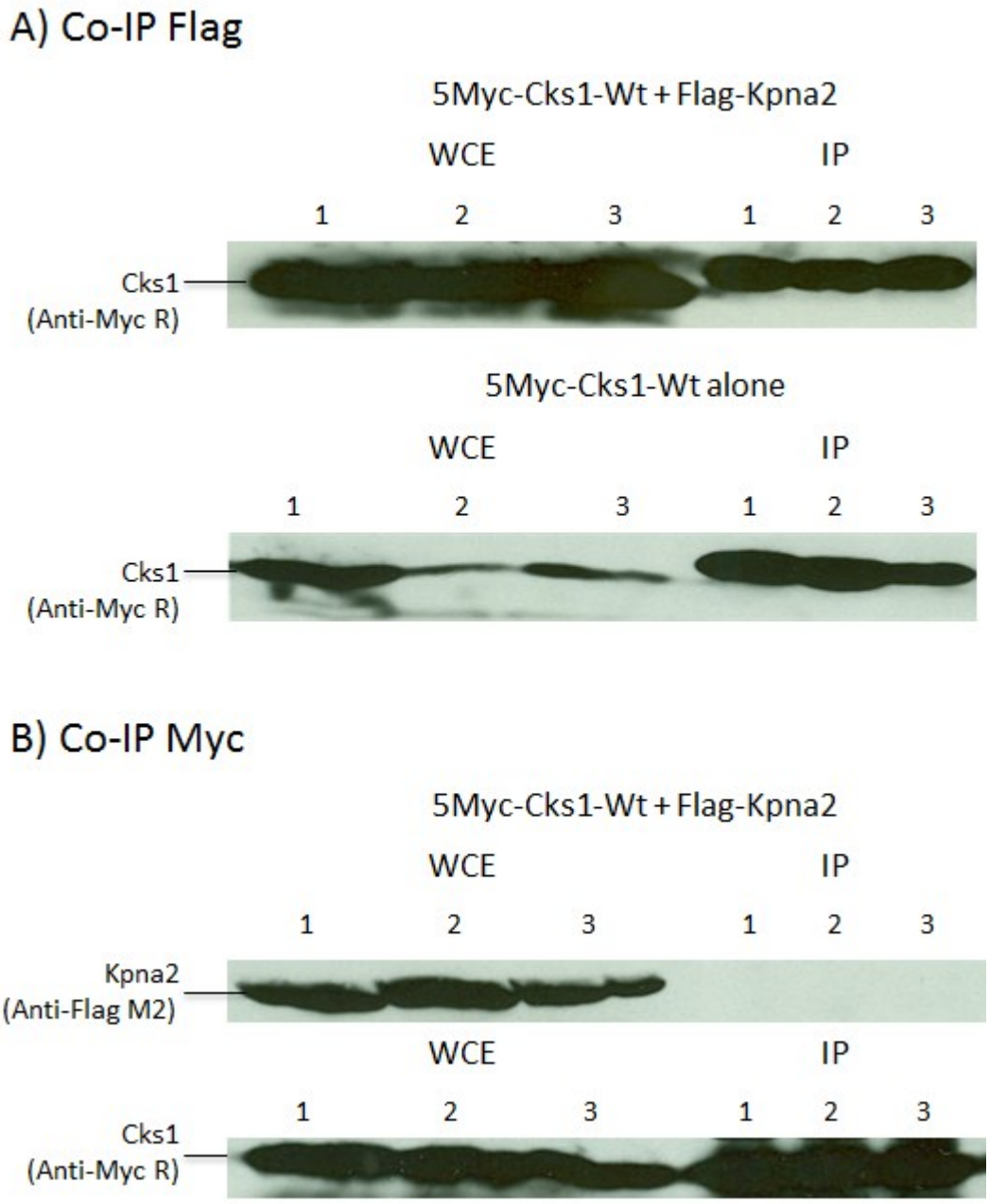

Figure 10 Co-IP results of Cks1 with Kpna2. Co-Ip were done in three different buffer conditions. A) Non-specific binding of Cks1 to the Flag-beads even in the absence of flag-Kpna2. B) No binding of Kpna2 despite presence of Cks1 on Myc-beads.

The GST-pull down of Cks1 by α importin-tagged beads gave the same results as the Myc Co-Ip. No interaction of Cks1 with any of the importin was detected. Additionally, β importin expression was also induced to mediate formation of the Cks1 nuclear import complex. Nonetheless, no binding was observed between 5Myc-Cks1-wt with any of α importins, including Flag-Kpna2 (see Fig. 11).

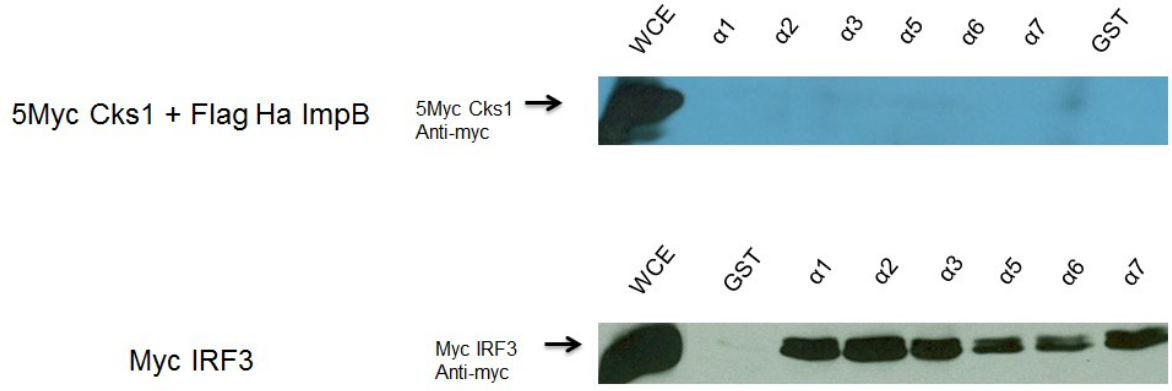

Figure 11 Results of the pull down of Cks1 by α importin-conjugated GST beads. Myc-IRF3 was used as positive control for α importin bindings.

4.4. Subcellular localization of Cks1

Transfections were first performed in U2OS, NIH3T3, MCF10A, T98G, Rat1, and HeLa cell lines for IF purposes. However, these cells were unable to express Cks1 evenly and most cells expressing Cks1 demonstrated rather odd morphologies, which rendered the observation of Cks1 localization difficult (see Fig. 12A). Various assays using different methods of transfection in a variety of cell lines were done in order to generate cells with a regular morphology expressing Cks1 clearly and evenly. Finally, retroviral infection in Rat1 cells was found to be the best way to maximize Cks1 expression uniformly without disrupting regular cellular activities (see Fig. 12B).

Figure 12 Cells expressing 5Myc-Cks1. A) Cells transfected with Cks1-wt showed bizarre morphologies. B) Rat1 cells infected with Cks1-wt had no visible sign of cellular alteration.

The cells were classified into three types according to Cks1 localizations: nuclear more than cytoplasm (N>C), even distribution (N=C) and nuclear less than cytoplasm (N<C). For N>C, a clear defined nuclear staining can be seen (see Fig. 13A). For N=C, an even staining was observed and no nucleus was defined (see Fig. 13B). Finally, for N<C, a darker, less illuminated nucleus can be seen (see Fig. 13C). Sometimes, an aggregation of Cks1 on the surface of the nucleus envelop were observed in the form of a peri-nuclear ring, suspecting their accumulation at nuclear pores (see Fig. 13D).

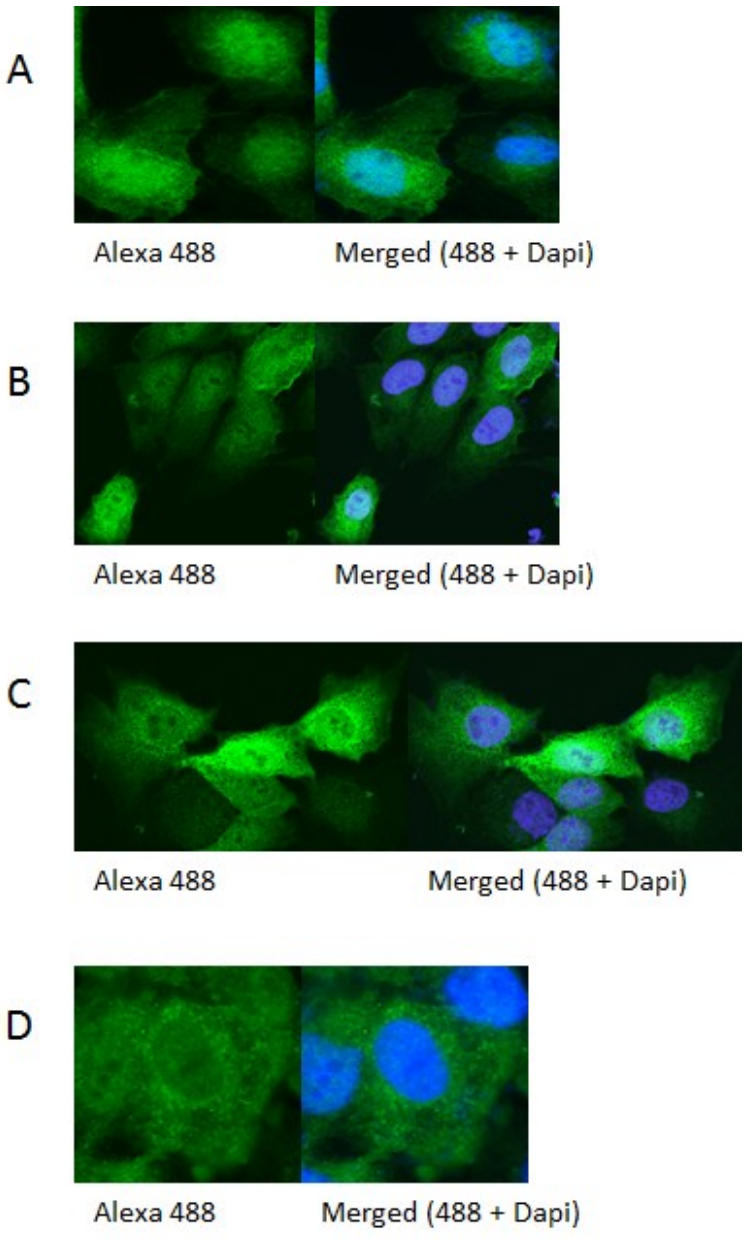

Figure 13 Immunofluorescence analysis of Cks1 localization. Rat1 cells infected with either 5Myc-Cks1-wt or mutant were fixed and stained with primary anti-Myc and secondary alexa 488. The nucleus was counter-stained with Dapi. A) N>C, the nucleus is much brighter than the cytoplasm. B) N=C, the cell has a uniform stain, nucleus is undefined. C) N<C, the cytoplasm is much brighter than the nucleus. D) The nucleus and cytoplasm has the same brightness, a clear ring is formed around the nucleus.

The localization experiment was repeated three times. The Rat1 cells were infected separately with the retroviral Cks1-wt and mutants, and examined under the same IF conditions. The analysis of all Cks1 slides revealed a decrease in nuclear Cks1 in cells infected with Cks1 mutants (see Fig. 14). When compared to the wild type Cks1, the mutants also demonstrated an increase in cytoplasmic Cks1, especially the ones in which the last two lysines were mutated, namely m21, m4 and del.

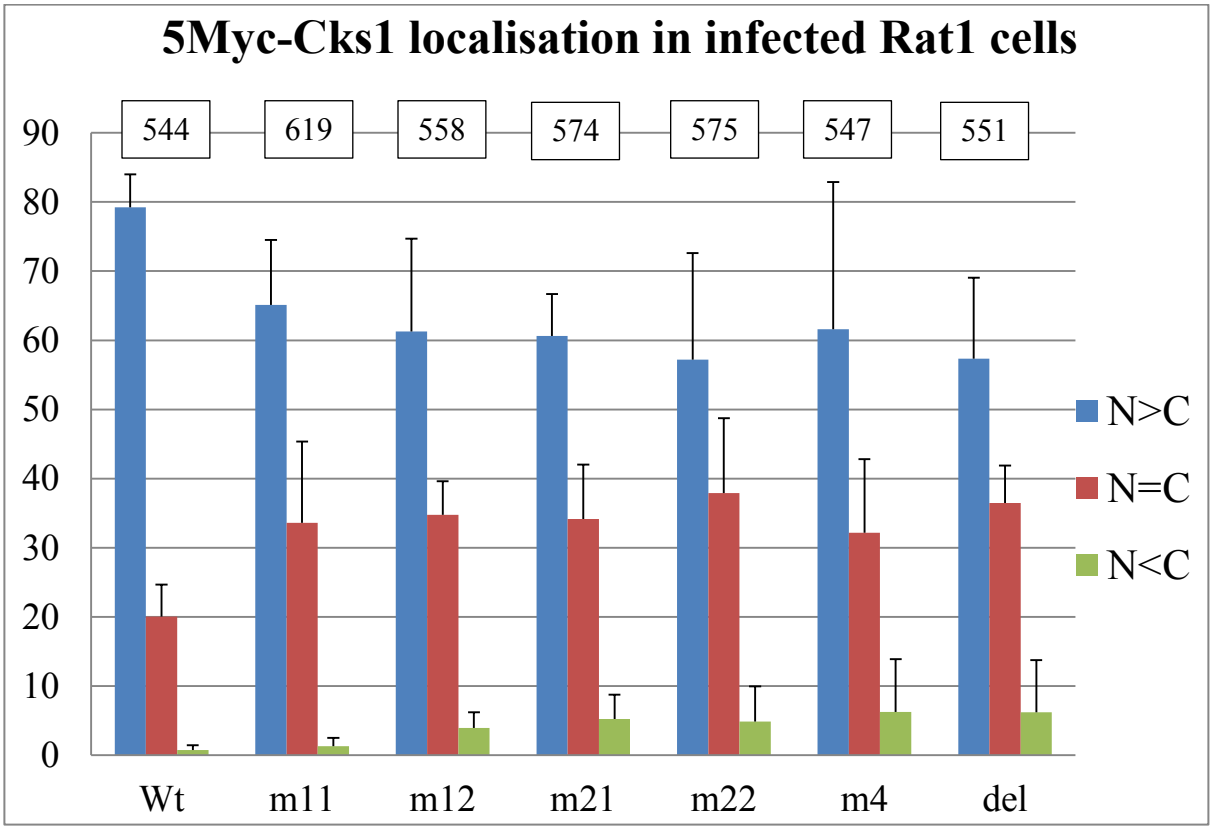

Figure 14 Cks1-wt and mutants localization in infected Rat1 cells. The ratio of each Cks1 localization (N=nuclear, C=cytoplasm) is represented in percentage. The graph represents the mean and the standard deviation of three separate experiments. Each time, at least 155 cells were scored for any of the Cks1 slides. The numbers of cells in total counted for each construct are written on top of the graph.

4.5. β Importin inhibition

Short hairpin RNAs (shRNA) were used to deplete α1 importin or β3 importin of the cells and thus blocking the NLS-mediated nuclear import of Cks1. However, the lentiviral infected NIH3T3, T98G and Rat1 cells were unable to express Cks1 evenly. As

an alternative approach, karyostatin 1A was synthesised and used instead since it was found to be able to inhibit β importin and achieve the same effect as the shRNA. [131]

Observations of 5Myc-Cks1-Wt infected Rat1 cells revealed the presence of overexpressed Cks1 throughout the cell, in the cytoplasm and especially in the nucleus. To determine whether or not the inhibition of β importin has an impact on the nuclear import of Cks1, the infected Rat1 cells were first depleted of existing nuclear 5Myc-Cks1 using cycloheximide for 8 hours (see Fig. 15). Quantification and classification of the cells displayed a drastic decrease in nuclear Cks1 in comparison to the no-cycloheximide negative control (see Fig. 16).

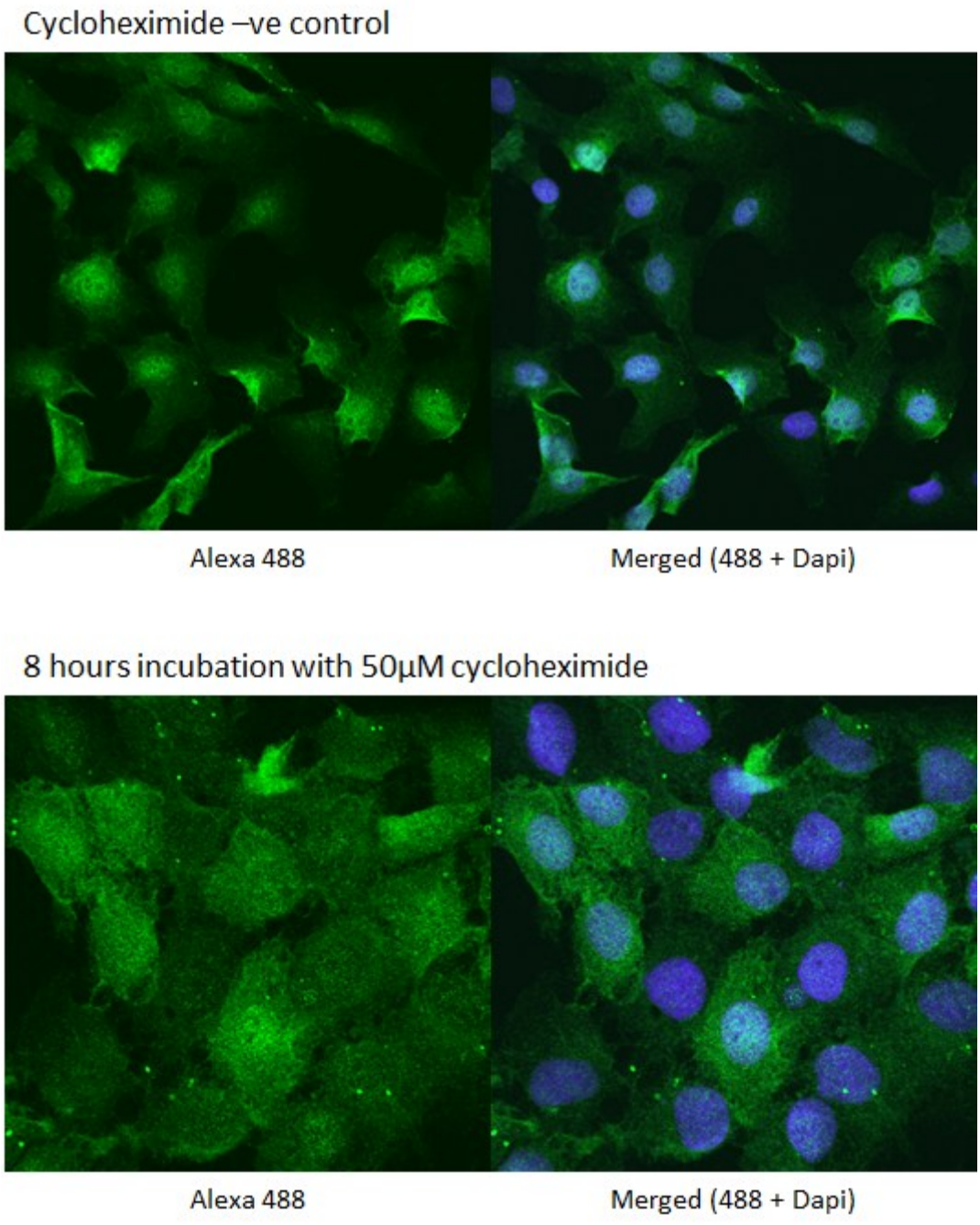

Figure 15 Immunofluorescence of Cks1-wt infected Rat1 cells after protein depletion by 8 hours of cycloheximide incubation.

After 4 hours of incubation with DMSO, we can see a recovery, although not back to normal, of nuclear Cks1 (see Fig. 16). The cells incubated in presence of karyostatin 1A however, showed a much slower recovery of nuclear Cks1 and a higher accumulation of cytoplasmic Cks1. A separate experiment was done in parallel, in which nuclear Cks1 was first depleted by cycloheximide for 10 hours and allowed to recover in either DMSO or inhibitor for 6 hours. The results were similar in every way.

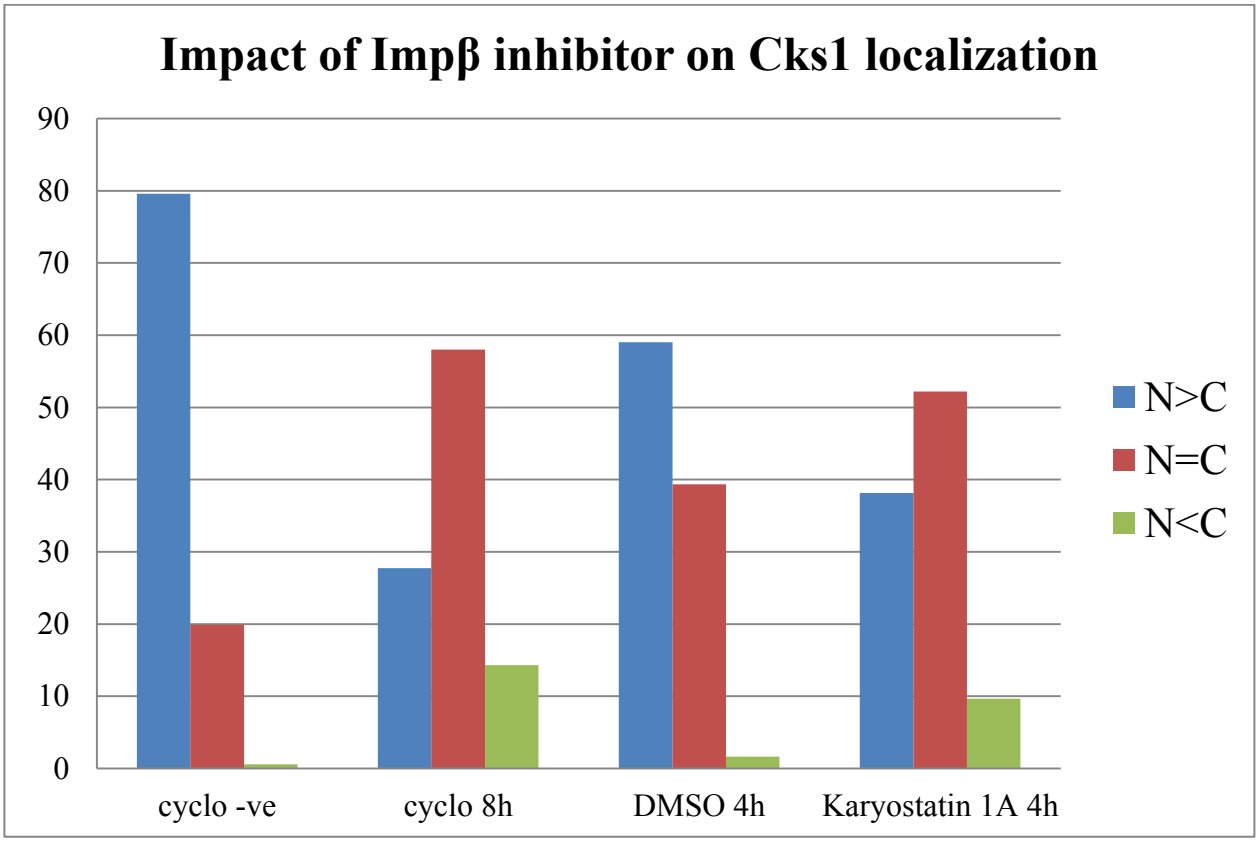

Figure 16 Cks1 localization after 8 h incubation of cycloheximide and 4 h DMSO or inhibitor. The ratio of each Cks1 localization (N=nuclear, C=cytoplasm) is represented in percentage. Each sample counts at least 115 cells. This graph is representative of 2 separate experiment results.

5. Discussion & Perspectives

Cks1 performs a large array of tasks throughout the cell cycle (see Fig.17). Its main and most documented function is to facilitate SCF^{Skp2} in targeting phosphorylated Cdk inhibitors p21, p27 and p57, as well as Rb-related p130 for degradation. This step is crucial for cell cycle progression. Moreover, Cks1 is also required to bind to Cdk2 for G_1/S transition. In yeast, Cks1 was found to modulate genes expressions in a kinase-independent manner. [150] By associating with Cdk1, Cks1 is able to recruit the proteasome via its 19S component and induce transcription of specific genes.

Recent findings also pointed out Cks1's role in cancer cell aggressiveness and treatment resistance. Cks1 have been shown stimulate APC/C to induce production of Cdc20, involved in cell migration. Furthermore, Cks1 activates both STAT3 and MEK/ERK pathways for increased cell survival and drug resistance. In addition, Cks1 also seems to be able to confer radiation resistance to cancer cells via an unknown mechanism.

It is clear that Cks1 plays a critical role in cell division and survival in both normal and cancer cells. Yet, its value as potential target for cancer therapy has not been recognized. Our objectives were to have a better understanding of its post-translational regulation as well as to determine how its localization can affect its function as a tumor suppressor inhibitor and a cell survival promoter.

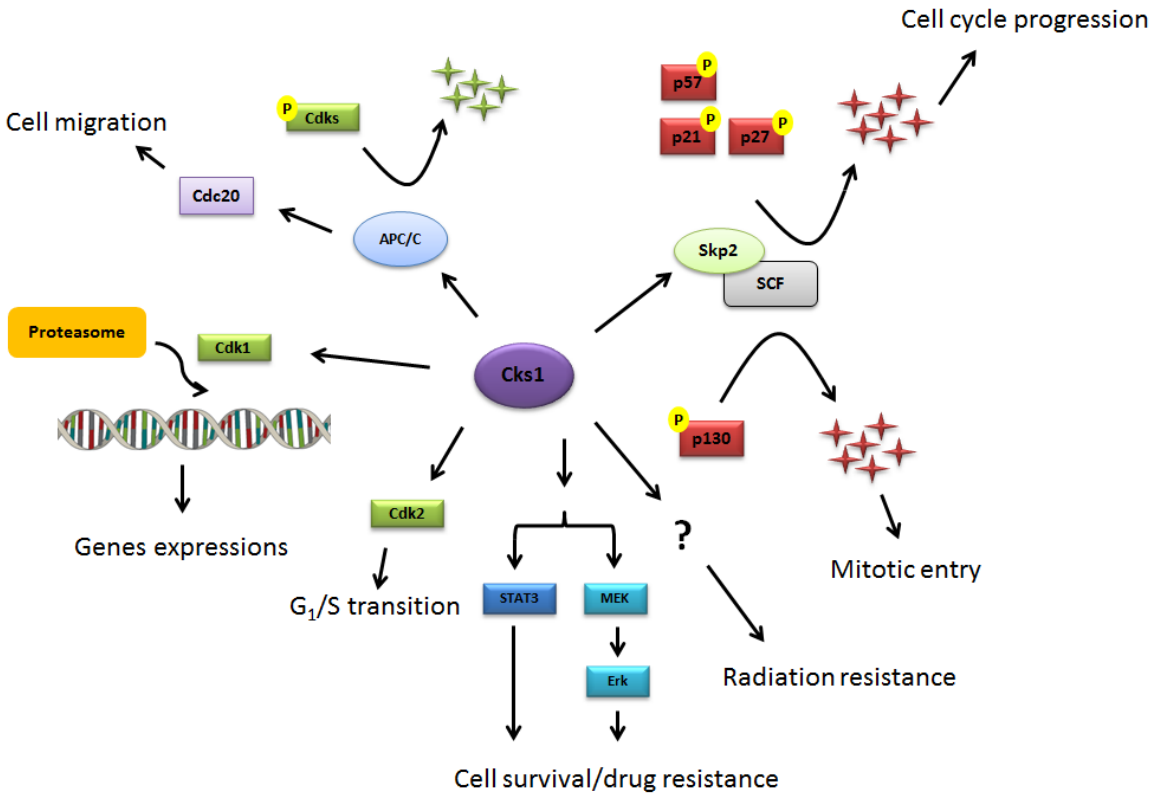

Figure 17 Cks1 functions

Cks1's roles are mainly focused on cell cycle progression and cell survival. Cks1 associates with SCF^{Skp2} to target Cdk inhibitors p21, p27 and p57 for degradation. While, SCF^{Skp2}-Cks1 complex also inhibit Rb-related p130 protein for mitotic entry; Cks1/Cdk2 is required for G_1/S transition. In yeasts, Cks1 associates with the human Cdk1 homolog Cdc28 to modulate expressions of various genes. Cks1 also promotes cell migration via Cdc20 production. Recent studies pointed out Cks1's role in multi-drug and radiation resistance of cancer cells.

5.1. Cks1 expression

Most studies involving Cks1 have been performed in cancer cell lines or by knockdown and rescue in mouse models. These experiments seldom explore Cks1 interactions and mainly focus on the effects of overexpressing or drastically reducing this protein. Little information is known about the regulation and properties of Cks1; Cks1 is a relatively small protein, composed of 79 amino acids with a mass of 9.66 kDa, according to ExPASy-Protparam, the online bioinformatics tool of the Swiss Institute of Bioinformatics. Protein secondary and tertiary structures are formed based on the properties of the amino acids in a protein. A deletion of only seven amino acids at Cks1's C-terminus represents a 9% reduction of its total weight (see Fig. 5). Hence, the amino acid substitutions of Cks1-m4 and the amino acid deletions of Cks1-del constitute modifications large enough to lead to conformational changes and are enough to offset protein stability.

We suspected a problem when the small HA-tagged deletion mutant of Cks1 was not expressed in HEK 293 cells, which are well known for their high production of proteins (Fig. 8). Therefore, the bulky 5myc-tag was chosen because it has been shown to stabilize the structure of Cks1 mutants. The 5myc-tag, comprised of five EQKLISEEDL repeats, has a molecular weight of ~6kDa, representing about two-thirds the mass of Cks1. However, Cks1-m4 and Cks1-del proteins were rapidly degraded and were only present in quantities large enough for assays when infected Rat1 cells were treated with the proteasome inhibitor MG132.

The high expression of Cks1 in transfected as well as infected cells is concordant with the Cks1 levels found in cancer cells; the gain of an extra 1q chromosome in cancer cells often leads to increased expression of Cks1. Statistical analysis of the breast cancer cell line MDA-MB-231 has shown that overexpressed Cks1 is primordial for cell proliferation, migration, and invasion [126], therefore explaining the bizarre morphology of certain cells lines, such as MCF10A and T98G, when expressing a high level of wild type of mutant Cks1 (see Fig.12 A).

5.2. Cks1 interactions

We were not able to document any stable interaction between Cks1 and any of the importins. There may be two reasons for the absence of binding. First, an absence of co-factors could have lowered the affinity and stability of Cks1 binding. When performing Co-IP, Skp2 was added. Endogenously, Skp2 is a Cks1-associated protein and its binding to Cks1 might be involved in co-transport into the nucleus. In addition, classical β importin was added. During nuclear import, NLS-containing cargo proteins are bound to both α and β importin, which may act synergistically. None of these co-factors aided the Cks1-importin interaction. From the results of TAP-tagging, 150 proteins in total were shown to interact with Cks1, of which at least 88 are involved in the Skp2-Cks1 pathway (results not shown). One or several of these binding factors could take part in Cks1 nuclear co-translocation.

Secondly, the large size of the N-terminal tag was a problem. Despite being an N-terminal tag, the cumbersome 5Myc tag might still cause steric interference of Cks1 when

binding with the importins. Nonetheless, the bulky tag was needed because Cks1 mutants were expressed when tagged with 5Myc but not with Ha. An intermediate-sized tag, such as a Flag3, similar to the TAP-tag or 3Myc tag, might have been adequate to allow both protein stability and protein-protein interaction.

In summary, although Cks1's C-terminus is indeed an NLS, no interaction with any of the karyopherins has been confirmed except with the TAP-tagged molecule, which represents a different tag and a different interaction protocol. Additional study is needed to better understand the mechanism of interaction between karyopherins and Cks1.

5.3. Cks1 localization

The analysis of the various Cks1 mutants shows that there are significant differences in Cks1 localization between each mutant. Approximately 80% of wild type Cks1 cells exhibit the N>C profile, which is expected because endogenous Cks1 is required to degrade p27 in the nucleus. In contrast, the mutants show a tendency for the N=C profile. There is an average of 18.69% difference in the ratio of nuclear Cks1 between wild type and the mutants, large enough to be significant. Cks1-m21, m22, m4, and del, in particular, have a remarkably high ratio of cytoplasmic retention.

As mentioned, mutations in both Cks1-m4 and Cks1-del might induce conformational changes that offset their stability relative to wild type. They are most likely passively transported through the nuclear pore complexes rather than actively imported into

the nucleus. Therefore, the difference in their localization may not be caused by alteration to the suspected NLS. However, Cks1-m21 and m22 are both point mutations, with only one or two amino acid changes, and are likely to be folded correctly. Moreover, in each experiment, the cells demonstrating the greatest decrease in nuclear Cks1 are point mutations, namely m12, m21, and m22.

The large standard deviations seen in the results are mainly caused by the second experiment, which exhibited a much higher level of cytoplasmic Cks1. Taking this into consideration; the ratio of cytoplasmic Cks1 against nuclear Cks1 can only be greater. Therefore, one can conclude that the C-terminus of Cks1 contains a bona fide NLS. Modification of only one amino acid is enough to impede nuclear import. However, the difference in ratio of nuclear to cytoplasmic Cks1 was not as great as predicted due to the small size of Cks1 and thus its ability to be transported passively through the nuclear pore complex.

5.4. Effect of pharmacological β importin inhibition on Cks1 localization

Endogenously, Cks1 is produced in the cytoplasm, translocated, and retained in the nucleus. The overexpressed Cks1 of infected Rat1 cells follows the same pattern. To determine the effects of impβ inhibition on Cks1 import, nuclear Cks1 must first be

eliminated. However, studies have shown discrepancies in the half-life of Cks1, varying from 4 hours to 6.6 hours. [130-132] In addition, the 5Myc tag confers stability to Cks1. After cycloheximide chase analysis, the protein depletion times were set at 8 and 10 hours to ensure better results.

The analysis of Rat1 cells after cycloheximide exposure confirmed the efficiency of the treatment. Most of these cells were characterized by the absence Cks1 in the nucleus, while the untreated cells were characterized by their high ratio of nuclear Cks1. After rescue, cells incubated in DMSO as a negative control were compared to those with karyostatin. Among the latter, cells displayed a slower recovery rate and were denoted by a high ratio of cytoplasmic Cks1. In contrast, a large portion of the non-inhibited cells demonstrated a large increase in nuclear Cks1, a recovery toward the wild type phenotype. This indicates that impairment of β-importin also impairs Cks1 import.

Despite the lack of evidence in vitro, this difference in Cks1 localization strongly suggests that Cks1 is transported into the nucleus via the β importin pathway in vivo. The low level of nuclear Cks1 following karyostatin treatment demonstrates that the inhibition of β importin indeed blocked Cks1 import and affected its localization. However, because we could not confirm the interaction of Cks1 with any of the importins, the possibility of an indirect mechanism cannot be ruled out. β importin is required for multiple pathways and its inhibition will affect many cell functions, one of which could be the import of Cks1 into the nucleus.

5.5. Impact on cancer

One of the key cell cycle inhibitors is the protein p27. A drastic or total depletion of this tumor suppressor has been observed in approximately 50% of cancers, including breast, prostate, colon, intestinal, lung, esophagus, and ovarian. [151] p27 dysregulation is usually observed as degradation and/or cytoplasmic retention of this nuclear protein; however, its gene is seldom mutated. Thus, p27 is an interesting target for cancer treatment because it does not require gene therapy, with associated serious side effects, and it affects many cancer types.

Cks1 and Skp2 overexpression have also been observed in the same cancer cells that present with p27 dysregulation. [113] This suggests that the rate of degradation of nuclear p27 is increased due to increased expression of the SCFSkp2-Cks1 complex. Therefore, understanding the regulation of these molecules is crucial in restoring p27 function and cell cycle arrest.

Interestingly, recent studies by Westbrook et al. showed that high Cks1 expression in certain mammary tumors does not always reduce p27. [135] In their experiments, mammary cancers were initiated in transgenic mice using various carcinogens including ErbB2, methylnitrosourea, and polyoma middle-T. In these tumors, despite the expected high level of Cks1, unexpectedly, the level of p27 increased only slightly. This suggested that Cks1 has oncogenic roles on its own, independent of SCFSkp2 or p27. This theory was further supported in a recent experiment in which Myc-induced B cell lymphoma was studied. [143]

It was found that loss of Skp2 also resulted in elevated p27 levels, regardless of the high Cks1 expression. Surprisingly, no impact on the tumor onset was observed.

Various studies and analysis of multiple malignant cancers have also shown that knockdown of Cks1 significantly decreased the angiogenesis, anchorage-independent growth, migration activities, and overall aggressiveness of the tumor cells. [152] Considering the p27-independent functions of Cks1, it is not surprising that Cks1 overexpression is required for tumor malignancy. Additionally, Cks1 is able to positively regulate nuclear factor kappa B (NF-κB) and stimulate the production of the pro-inflammatory chemokine IL-8. This positive feedback further promotes angiogenesis, tissue remodeling, and tumor progression. [146, 153]

In clinical studies, Cks1 has been used as a diagnostic marker for tumor size and stage. It is also an independent prognostic marker for recurrence of and mortality from certain cancers. [144] The level of Cks1 can also predict the efficacy of treatment. A significant negative correlation has been found between Cks1 expression and patient survival; it was suspected that in some cases, Cks1 overexpression might cause treatment resistance. An article was recently published that also reported the ability of Cks1 to cause treatment resistance to radiotherapy. [145] Patients with esophageal squamous cell carcinomas presenting a higher level of Cks1 were less responsive to radiotherapy. The knockdown of Cks1 in these carcinoma tissues was able to sensitize the cells to radiation and a degradation-resistant form of Cks1 was able to rescue this effect.

In 2009, a study suggested that Cks1 levels regulate the responsiveness of ER+ breast cancers to estrogens and anti-estrogens. [135] It was observed that stable

overexpression of Cks1 in human breast carcinoma MCF-7 cells conferred resistance to Faslodex, an estrogen receptor inhibitor used to treat hormone-receptor positive breast cancers. However, the mechanism by which it worked was unknown. In 2010, another group studying multiple myeloma found that forced expression of Cks1 in these cells induced multidrug-resistance. [154] It was discovered that overexpressed Cks1, in a SCFSkp2 independent manner, activated both the STAT3 and the MEK/ERK pathways. The transcription activator STAT3 transcribes a set of genes and, along with BCL2, the downstream target of the MEK/ERK signaling pathway. This mechanism promotes cell survival, thus leading to drug resistance during multiple myeloma therapy.

In general, the overexpression of Cks1 is essential in both cancer progression and survival. In addition to degradation of the tumor suppressor p27, it also promotes cell cycle transitions and cell motility via interaction with Cdks, transcription activators, and microtubule modulators. Cks1 will have different impact on cancers depending on its localization. While nuclear Cks1 mainly mediates carcinogenesis and cell survival, cytoplasmic Cks1 promotes tumor aggressiveness and metastasis. Recent findings also revealed that Cks1 is involved in multidrug and radiation resistance, significantly decreasing patient survival.

5.6. Perspectives

In view of the important role played by Cks1 in cancer, a better understanding of Cks1 is warranted. Various treatments aimed at reducing Cks1 levels have been studied. It is known that the other member of the Cks family, Cks2, is involved in guarding DNA replication fidelity and that in response to DNA damage, Cks2 is required to initiate cell-cycle arrest. [129] Data from a mouse model suggests that Cks2 counteracts Cks1 and stabilizes p27. Cks2 has the same anion pocket capable of recognizing p27 as Cks1, yet has no N-terminal residues that recruit Skp2. Therefore, it is suspected that Cks2 blocks p27 degradation by competing with Cks1 for binding with p27. However, overexpression of Cks2 in cancers with elevated levels of Cks1 does not show benefit. Knockdown studies of Cks2 have revealed its participation in promoting tumorigenicity and inhibiting programmed cell death. [152]

Many studies to date have suggested the use of Cks1 siRNA as the most direct way to reduce Cks1 levels in cancer cells; however, only a few studies have employed this approach. Tsai et al. demonstrated that treatment of lung cancer cells with Cks1 siRNA caused downregulation of Cdc2 activity as well as cell cycle arrest at the G2/M transition. Long-term use of the siRNA induced caspase activation and apoptosis in these cells. [155] Interestingly, normal lung fibroblasts were barely affected and still viable under the same conditions. In addition, an in vivo high-throughput screening technique for inhibitors of Cks1-Skp2 interaction has been developed. [156] Various compounds have shown significant inhibition of APC/C activity and Skp2-Cks1 interaction. [157] These inhibitors are very

effective; their half maximal inhibitory concentrations are all below 20 μM. However, no chemical details have been revealed for commercial reasons.

Target specificity is the main issue facing SCFSkp2-Cks1 ligase inhibitors because this multi-protein complex has many distinct binding domains common to other proteins; this is the reason some sought to target Cks1-related proteins. One of the well-known drugs currently on the market is Vorinostat. Commercialized under the name Zolinza, it is a histone deacetylase inhibitor that blocks cancer cell proliferation through the regulation of cyclin-dependent kinase inhibitors. [158] This drug is also able to prolong the half-life of p21 and p27; however, this effect can be reversed by overexpression of Cks1 and Skp2. An analysis of Cks1's drug resistance properties by Shi et al. determined that CKS1-overexpressing multiple myeloma cells exhibit significant cell death and growth inhibition when their STAT3 or MEK/ERK signaling pathways have been targeted with specific inhibitors. [153] Furthermore, the use of inhibitors of both pathways resulted in synergy.

In light of my research results, controlling intracellular Cks1 localization appears to be a potential new method of inhibiting Cks1 activity in malignant tumor cells in which high expression of Cks1 is crucial for cell survival. There would be many benefits to blocking Cks1 entry into the nucleus. First, it would prevent the degradation of the cell cycle inhibitors p21, p57, p130/Rb, and, especially, p27, thereby inhibiting uncontrolled cell division. A quantification of the tumor suppressor protein p27 could be performed following Cks1's import inhibition in p27-reduced cancer cells to determine if indeed Cks1 is responsible for the major reduction of nuclear p27. On parallel, a cell viability MTT-assay could be performed to determine if the absence of nuclear Cks1 would lead to cell

cycle arrest. Whether p27-dependent or independent, Cks1 partakes in various steps of cell cycle progression. Also, a decrease in nuclear Cks1 would lead to reduced activation of the MEK/ERK and STAT3 signaling pathways; therefore, reducing cell survival and cancelling drug and radiation resistance. The use of NLS-defective Cks1 mutants in cancer cell lines expressing high level of Cks1 would be able to confirm whether or not their drug and radiation resistance could be overridden. However, the invasiveness and metastasis of malignant tumor cells would not be affected and might even increase due to accumulation of cytoplasmic Cks1.

Despite all the potential of a Cks1-specific import inhibitor, the main concern, as with all therapeutic agents, would be target specificity. The α-β karyopherins nuclear import pathway is common for lots of proteins. Further work on the mechanism of Cks1-karyopherin interaction as well as in depth study of Cks1 structure are needed. Furthermore, Cks1 assists in different pathways, such as cell cycle progression and gene expression of regular cells as well. How the inhibition of nuclear import of Cks1 affects healthy cells is yet to be determined. Lots of works still need to be done on this important yet often overlooked protein.

References

1. Viallard, J.F., et al., *[Molecular mechanisms controlling the cell cycle: fundamental aspects and implications for oncology]*. Cancer Radiother, 2001. **5**(2): p. 109-29.
2. Malumbres, M., *Physiological relevance of cell cycle kinases.* Physiol Rev, 2011. **91**(3): p. 973-1007.
3. Cooper, G.M., ed. *The Eukaryotic Cell Cycle*. 2nd ed. The Cell: A Molecular Approach. 2000, Sunderland (MA): Sinauer Associates.
4. Pardee, A.B., *A restriction point for control of normal animal cell proliferation.* Proc Natl Acad Sci U S A, 1974. **71**(4): p. 1286-90.
5. Callegari, A.J. and T.J. Kelly, *UV irradiation induces a postreplication DNA damage checkpoint.* Proc Natl Acad Sci U S A, 2006. **103**(43): p. 15877-82.
6. Pinsky, B.A. and S. Biggins, *The spindle checkpoint: tension versus attachment.* Trends Cell Biol, 2005. **15**(9): p. 486-93.
7. Nigg, E.A., *Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle.* Bioessays, 1995. **17**(6): p. 471-80.
8. Marrocco, K., et al., *Selective proteolysis sets the tempo of the cell cycle.* Curr Opin Plant Biol. **13**(6): p. 631-9.
9. Castro, A., et al., *The anaphase-promoting complex: a key factor in the regulation of cell cycle.* Oncogene, 2005. **24**(3): p. 314-25.
10. Qiao, X., et al., *APC/C-Cdh1: from cell cycle to cellular differentiation and genomic integrity.* Cell Cycle, 2010. **9**(19): p. 3904-12.
11. Cardozo, T. and M. Pagano, *The SCF ubiquitin ligase: insights into a molecular machine.* Nat Rev Mol Cell Biol, 2004. **5**(9): p. 739-51.
12. Hershko, A., *Roles of ubiquitin-mediated proteolysis in cell cycle control.* Curr Opin Cell Biol, 1997. **9**(6): p. 788-99.
13. Chang, E.J., et al., *Prediction of cyclin-dependent kinase phosphorylation substrates.* PLoS One, 2007. **2**(7): p. e656.
14. Evans, T., et al., *Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division.* Cell, 1983. **33**(2): p. 389-96.
15. Nugent, J.H., et al., *Conserved structural motifs in cyclins identified by sequence analysis.* J Cell Sci, 1991. **99 (Pt 3)**: p. 669-74.
16. *Cyclin family of proteins*. [cited 2011 2011/11/11]; Available from: http://www.scienceslides.com/LhP/cyclin-family-of-proteins#.
17. Musgrove, E.A., *Cyclins: roles in mitogenic signaling and oncogenic transformation.* Growth Factors, 2006. **24**(1): p. 13-9.
18. Sánchez I, D.B., *New insights into cyclins, CDKs, and cell cycle control.* Semin Cell Dev Biol., 2005. **16**: p. 311-321.
19. D'Angiolella, V., et al., *SCF(Cyclin F) controls centrosome homeostasis and mitotic fidelity through CP110 degradation.* Nature. **466**(7302): p. 138-42.

20. Ren, S. and B.J. Rollins, *Cyclin C/cdk3 promotes Rb-dependent G0 exit.* Cell, 2004. **117**(2): p. 239-51.
21. Sage, J., *Cyclin C makes an entry into the cell cycle.* Dev Cell, 2004. **6**(5): p. 607-8.
22. Musgrove, E.A., et al., *Cyclin D as a therapeutic target in cancer.* Nat Rev Cancer, 2011. **11**(8): p. 558-72.
23. Bienvenu, F., et al., *Transcriptional role of cyclin D1 in development revealed by a genetic-proteomic screen.* Nature. **463**(7279): p. 374-8.
24. Geng, Y., et al., *Kinase-independent function of cyclin E.* Mol Cell, 2007. **25**(1): p. 127-39.
25. Hwang, H.C. and B.E. Clurman, *Cyclin E in normal and neoplastic cell cycles.* Oncogene, 2005. **24**(17): p. 2776-86.
26. Fung, T.K. and R.Y. Poon, *A roller coaster ride with the mitotic cyclins.* Semin Cell Dev Biol, 2005. **16**(3): p. 335-42.
27. Kalaszczynska I, G.Y., Iino T, Mizuno S, Choi Y, Kondratiuk I, Silver DP, Wolgemuth DJ, Akashi K, Sicinski P., *Cyclin A is redundant in fibroblasts but essential in hematopoietic and embryonic stem cells.* Cell., 2009. **138**(2): p. 352-65.
28. Cardoso, M.C., H. Leonhardt, and B. Nadal-Ginard, *Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication.* Cell, 1993. **74**(6): p. 979-92.
29. Pemberton, L.F. and B.M. Paschal, *Mechanisms of receptor-mediated nuclear import and nuclear export.* Traffic, 2005. **6**(3): p. 187-98.
30. Toyoshima, F., et al., *Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G2 checkpoint.* EMBO J, 1998. **17**(10): p. 2728-35.
31. Fung, T.K., H.T. Ma, and R.Y. Poon, *Specialized roles of the two mitotic cyclins in somatic cells: cyclin A as an activator of M phase-promoting factor.* Mol Biol Cell, 2007. **18**(5): p. 1861-73.
32. Lalioti, V., D. Pulido, and I.V. Sandoval, *Cdk5, the multifunctional surveyor.* Cell Cycle. **9**(2): p. 284-311.
33. Morgan, D.O., *The Cell-Cycle Control System*, in *The Cell Cycle: Principles of Control*. 2007, New Science Press: London. p. 27-56.
34. Lee MG, N.P., *Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2.* Nature, 1987. **327**(6117): p. 31-35.
35. Satyanarayana, A. and P. Kaldis, *Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms.* Oncogene, 2009. **28**(33): p. 2925-39.
36. Malumbres, M., et al., *Cyclin-dependent kinases: a family portrait.* Nat Cell Biol, 2009. **11**(11): p. 1275-6.
37. Li S, M.S., Matsuguchi T, Blethrow JD, Shokat KM, Blackburn EH., *Cdk1-dependent phosphorylation of Cdc13 coordinates telomere elongation during cell-cycle progression.* Cell, 2009. **136**(1): p. 50-61.
38. Sarita Rajender.P, R.D., Bhargavi. K, Vasavi. M, and Uma. V, *Selective inhibition of proteins regulating CDK/cyclin complexes: strategy against cancer - a review.* Journal of Receptors and Signal Transduction, 2010. **30**(4): p. 206-213.

39. Ladner, R.D., et al., *Identification of a consensus cyclin-dependent kinase phosphorylation site unique to the nuclear form of human deoxyuridine triphosphate nucleotidohydrolase.* J Biol Chem, 1996. **271**(13): p. 7752-7.
40. Mendenhall, M.D. and A.E. Hodge, *Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae.* Microbiol Mol Biol Rev, 1998. **62**(4): p. 1191-243.
41. Database, N.G. *CDK1 cyclin-dependent kinase 1 [Homo sapiens]*. 2009 18-Nov-2011; Available from: http://www.ncbi.nlm.nih.gov/gene/983.
42. Timofeev, O., et al., *Cdc25 phosphatases are required for timely assembly of CDK1-cyclin B at the G2/M transition.* J Biol Chem. **285**(22): p. 16978-90.
43. Database, N.G. *CDK2 cyclin-dependent kinase 2 [Homo sapiens]* 2008 20-Nov-2011; Available from: http://www.ncbi.nlm.nih.gov/gene/1017.
44. Matsuura, I., et al., *Cyclin-dependent kinases regulate the antiproliferative function of Smads.* Nature, 2004. **430**(6996): p. 226-31.
45. Hofmann, F. and D.M. Livingston, *Differential effects of cdk2 and cdk3 on the control of pRb and E2F function during G1 exit.* Genes Dev, 1996. **10**(7): p. 851-61.
46. Zheng, D., et al., *Cyclin-dependent kinase 3-mediated activating transcription factor 1 phosphorylation enhances cell transformation.* Cancer Res, 2008. **68**(18): p. 7650-60.
47. Wang, H., et al., *BRCA1 proteins are transported to the nucleus in the absence of serum and splice variants BRCA1a, BRCA1b are tyrosine phosphoproteins that associate with E2F, cyclins and cyclin dependent kinases.* Oncogene, 1997. **15**(2): p. 143-57.
48. Meyerson, M. and E. Harlow, *Identification of G1 kinase activity for cdk6, a novel cyclin D partner.* Mol Cell Biol, 1994. **14**(3): p. 2077-86.
49. Slomiany, P., et al., *Changes in motility, gene expression and actin dynamics: Cdk6-induced cytoskeletal changes associated with differentiation in mouse astrocytes.* J Cell Biochem, 2006. **99**(2): p. 635-46.
50. Nakayama, K.I. and K. Nakayama, *Ubiquitin ligases: cell-cycle control and cancer.* Nat Rev Cancer, 2006. **6**(5): p. 369-81.
51. Thrower, J.S., et al., *Recognition of the polyubiquitin proteolytic signal.* EMBO J, 2000. **19**(1): p. 94-102.
52. Zhu, Q., et al., *Deubiquitination by proteasome is coordinated with substrate translocation for proteolysis in vivo.* Exp Cell Res, 2005. **307**(2): p. 436-51.
53. Voges, D., P. Zwickl, and W. Baumeister, *The 26S proteasome: a molecular machine designed for controlled proteolysis.* Annu Rev Biochem, 1999. **68**: p. 1015-68.
54. Peters, J.M., *The anaphase-promoting complex: proteolysis in mitosis and beyond.* Mol Cell, 2002. **9**(5): p. 931-43.
55. Risseeuw, E.P., et al., *Protein interaction analysis of SCF ubiquitin E3 ligase subunits from Arabidopsis.* Plant J, 2003. **34**(6): p. 753-67.
56. Bai, C., et al., *SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box.* Cell, 1996. **86**(2): p. 263-74.

57. Kipreos, E.T. and M. Pagano, *The F-box protein family.* Genome Biol, 2000. **1**(5): p. REVIEWS3002.
58. Cenciarelli, C., et al., *Identification of a family of human F-box proteins.* Curr Biol, 1999. **9**(20): p. 1177-9.
59. Nakayama, K.I. and K. Nakayama, *Regulation of the cell cycle by SCF-type ubiquitin ligases.* Semin Cell Dev Biol, 2005. **16**(3): p. 323-33.
60. Database, N.G. *SKP2 S-phase kinase-associated protein 2 (p45) [Homo sapiens]* 2011 18-Nov-2011 [cited 2011 22/11]; Available from: http://www.ncbi.nlm.nih.gov/gene?term=%28skp2[gene]%29%20AND%20%28Homo%20sapiens[orgn]%29%20AND%20alive[prop]%20NOT%20newentry[gene].
61. Smith, T.F., et al., *The WD repeat: a common architecture for diverse functions.* Trends Biochem Sci, 1999. **24**(5): p. 181-5.
62. Zhang, Z., et al., *The APC/C subunit Cdc16/Cut9 is a contiguous tetratricopeptide repeat superhelix with a homo-dimer interface similar to Cdc27.* EMBO J, 2010. **29**(21): p. 3733-44.
63. Page, A.M. and P. Hieter, *The anaphase-promoting complex: new subunits and regulators.* Annu Rev Biochem, 1999. **68**: p. 583-609.
64. Herzog, F., et al., *Structure of the anaphase-promoting complex/cyclosome interacting with a mitotic checkpoint complex.* Science, 2009. **323**(5920): p. 1477-81.
65. Ayad NG, R.S., Murakami M, Jebanathirajah J, Gygi S, Kirschner MW., *Tome-1, a trigger of mitotic entry, is degraded during G1 via the APC.* Cell, 2003. **113**(1): p. 101-13.
66. Sgambato, A., et al., *Multiple functions of p27(Kip1) and its alterations in tumor cells: a review.* J Cell Physiol, 2000. **183**(1): p. 18-27.
67. Viglietto, G., M.L. Motti, and A. Fusco, *Understanding p27(kip1) deregulation in cancer: down-regulation or mislocalization.* Cell Cycle, 2002. **1**(6): p. 394-400.
68. Fahraeus, R., et al., *Inhibition of pRb phosphorylation and cell-cycle progression by a 20-residue peptide derived from p16CDKN2/INK4A.* Curr Biol, 1996. **6**(1): p. 84-91.
69. Sherr, C.J. and J.M. Roberts, *CDK inhibitors: positive and negative regulators of G1-phase progression.* Genes Dev, 1999. **13**(12): p. 1501-12.
70. Mosavi, L.K., et al., *The ankyrin repeat as molecular architecture for protein recognition.* Protein Sci, 2004. **13**(6): p. 1435-48.
71. Roussel, M.F., *The INK4 family of cell cycle inhibitors in cancer.* Oncogene, 1999. **18**(38): p. 5311-7.
72. Choi, B.Y., et al., *The tumor suppressor p16(INK4a) prevents cell transformation through inhibition of c-Jun phosphorylation and AP-1 activity.* Nat Struct Mol Biol, 2005. **12**(8): p. 699-707.
73. Jenkins, N.C., et al., *The p16(INK4A) tumor suppressor regulates cellular oxidative stress.* Oncogene, 2011. **30**(3): p. 265-74.
74. Faderl S, K.H., Estey E, Manshouri T, Chan CY, Rahman Elsaied A, Kornblau SM, Cortes J, Thomas DA, Pierce S, Keating MJ, Estrov Z, Albitar M., *The prognostic*

significance of p16(INK4a)/p14(ARF) locus deletion and MDM-2 protein expression in adult acute myelogenous leukemia. Cancer, 2000. **89**(9): p. 1976-82.
75. Rich, J.N., et al., *Transforming growth factor-beta-mediated p15(INK4B) induction and growth inhibition in astrocytes is SMAD3-dependent and a pathway prominently altered in human glioma cell lines.* J Biol Chem, 1999. **274**(49): p. 35053-8.
76. Krimpenfort, P., et al., *p15Ink4b is a critical tumour suppressor in the absence of p16Ink4a.* Nature, 2007. **448**(7156): p. 943-6.
77. Bai, F., et al., *p18 Ink4c and Pten constrain a positive regulatory loop between cell growth and cell cycle control.* Mol Cell Biol, 2006. **26**(12): p. 4564-76.
78. Franklin, D.S., et al., *CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis.* Genes Dev, 1998. **12**(18): p. 2899-911.
79. Uziel, T., et al., *The tumor suppressors Ink4c and p53 collaborate independently with Patched to suppress medulloblastoma formation.* Genes Dev, 2005. **19**(22): p. 2656-67.
80. Carcagno, A.L., et al., *E2F1-mediated upregulation of p19INK4d determines its periodic expression during cell cycle and regulates cellular proliferation.* PLoS One, 2011. **6**(7): p. e21938.
81. Zindy, F., et al., *Control of spermatogenesis in mice by the cyclin D-dependent kinase inhibitors p18(Ink4c) and p19(Ink4d).* Mol Cell Biol, 2001. **21**(9): p. 3244-55.
82. Denicourt, C. and S.F. Dowdy, *Cip/Kip proteins: more than just CDKs inhibitors.* Genes Dev, 2004. **18**(8): p. 851-5.
83. Besson, A., S.F. Dowdy, and J.M. Roberts, *CDK inhibitors: cell cycle regulators and beyond.* Dev Cell, 2008. **14**(2): p. 159-69.
84. Bates, G.J., et al., *The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumour suppressor.* EMBO J, 2005. **24**(3): p. 543-53.
85. Weinberg, R.L., D.B. Veprintsev, and A.R. Fersht, *Cooperative binding of tetrameric p53 to DNA.* J Mol Biol, 2004. **341**(5): p. 1145-59.
86. Warbrick, E., et al., *Homologous regions of Fen1 and p21Cip1 compete for binding to the same site on PCNA: a potential mechanism to co-ordinate DNA replication and repair.* Oncogene, 1997. **14**(19): p. 2313-21.
87. Gervais, J.L., P. Seth, and H. Zhang, *Cleavage of CDK inhibitor p21(Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis.* J Biol Chem, 1998. **273**(30): p. 19207-12.
88. Watanabe, H., et al., *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**(4): p. 1392-7.
89. Tanaka, H., et al., *Cytoplasmic p21(Cip1/WAF1) regulates neurite remodeling by inhibiting Rho-kinase activity.* J Cell Biol, 2002. **158**(2): p. 321-9.
90. Yokoo, T., et al., *p57Kip2 regulates actin dynamics by binding and translocating LIM-kinase 1 to the nucleus.* J Biol Chem, 2003. **278**(52): p. 52919-23.

91. Borriello, A., et al., *Targeting p27Kip1 protein: its relevance in the therapy of human cancer.* Expert Opin Ther Targets, 2011. **15**(6): p. 677-93.
92. Borriello, A., et al., *p27Kip1 metabolism: a fascinating labyrinth.* Cell Cycle, 2007. **6**(9): p. 1053-61.
93. Baldassarre, G., et al., *p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion.* Cancer Cell, 2005. **7**(1): p. 51-63.
94. Jin, L., et al., *Transforming growth factor-beta, transforming growth factor-beta receptor II, and p27Kip1 expression in nontumorous and neoplastic human pituitaries.* Am J Pathol, 1997. **151**(2): p. 509-19.
95. Nguyen, L., et al., *[p27Kip1 independently promotes neuronal differentiation and migration in the cerebral cortex].* Bull Mem Acad R Med Belg, 2007. **162**(5-6): p. 310-4.
96. McAllister, S.S., et al., *Novel p27(kip1) C-terminal scatter domain mediates Rac-dependent cell migration independent of cell cycle arrest functions.* Mol Cell Biol, 2003. **23**(1): p. 216-28.
97. Moeller, S.J., E.D. Head, and R.J. Sheaff, *p27Kip1 inhibition of GRB2-SOS formation can regulate Ras activation.* Mol Cell Biol, 2003. **23**(11): p. 3735-52.
98. Hu, M.C., et al., *IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a.* Cell, 2004. **117**(2): p. 225-37.
99. Jonuleit, T., et al., *Bcr-Abl kinase down-regulates cyclin-dependent kinase inhibitor p27 in human and murine cell lines.* Blood, 2000. **96**(5): p. 1933-9.
100. Coleman, J. and W.K. Miskimins, *Structure and activity of the internal ribosome entry site within the human p27 Kip1 5'-untranslated region.* RNA Biol, 2009. **6**(1): p. 84-9.
101. Lee, J. and S.S. Kim, *The function of p27 KIP1 during tumor development.* Exp Mol Med, 2009. **41**(11): p. 765-71.
102. Shin, I., et al., *PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization.* Nat Med, 2002. **8**(10): p. 1145-52.
103. Tomoda, K., Y. Kubota, and J. Kato, *Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1.* Nature, 1999. **398**(6723): p. 160-5.
104. Sekimoto, T., M. Fukumoto, and Y. Yoneda, *14-3-3 suppresses the nuclear localization of threonine 157-phosphorylated p27(Kip1).* EMBO J, 2004. **23**(9): p. 1934-42.
105. Carrano, A.C., et al., *SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27.* Nat Cell Biol, 1999. **1**(4): p. 193-9.
106. Chu, I., et al., *p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2.* Cell, 2007. **128**(2): p. 281-94.
107. Kamura, T., et al., *Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27(Kip1) at G1 phase.* Nat Cell Biol, 2004. **6**(12): p. 1229-35.
108. Sugiyama, Y., et al., *Direct binding of the signal-transducing adaptor Grb2 facilitates down-regulation of the cyclin-dependent kinase inhibitor p27Kip1.* J Biol Chem, 2001. **276**(15): p. 12084-90.

109. Chen, Q., et al., *Targeting the p27 E3 ligase SCF(Skp2) results in p27- and Skp2-mediated cell-cycle arrest and activation of autophagy.* Blood, 2008. **111**(9): p. 4690-9.
110. Denicourt, C., et al., *Relocalized p27Kip1 tumor suppressor functions as a cytoplasmic metastatic oncogene in melanoma.* Cancer Res, 2007. **67**(19): p. 9238-43.
111. Chu, I.M., L. Hengst, and J.M. Slingerland, *The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy.* Nat Rev Cancer, 2008. **8**(4): p. 253-67.
112. Zhang, Q., et al., *Inducible expression of a degradation-resistant form of p27Kip1 causes growth arrest and apoptosis in breast cancer cells.* FEBS Lett, 2005. **579**(18): p. 3932-40.
113. Frescas, D. and M. Pagano, *Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer.* Nat Rev Cancer, 2008. **8**(6): p. 438-49.
114. Ji, P., et al., *Skp2 contains a novel cyclin A binding domain that directly protects cyclin A from inhibition by p27Kip1.* J Biol Chem, 2006. **281**(33): p. 24058-69.
115. Chan, C.H., et al., *Regulation of Skp2 expression and activity and its role in cancer progression.* ScientificWorldJournal, 2010. **10**: p. 1001-15.
116. Tedesco, D., J. Lukas, and S.I. Reed, *The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF(Skp2).* Genes Dev, 2002. **16**(22): p. 2946-57.
117. Marti, A., et al., *Interaction between ubiquitin-protein ligase SCFSKP2 and E2F-1 underlies the regulation of E2F-1 degradation.* Nat Cell Biol, 1999. **1**(1): p. 14-9.
118. Ho, M.S., et al., *The utility F-box for protein destruction.* Cell Mol Life Sci, 2008. **65**(13): p. 1977-2000.
119. Hao, B., et al., *Structural basis of the Cks1-dependent recognition of p27(Kip1) by the SCF(Skp2) ubiquitin ligase.* Mol Cell, 2005. **20**(1): p. 9-19.
120. Wei, W., et al., *Degradation of the SCF component Skp2 in cell-cycle phase G1 by the anaphase-promoting complex.* Nature, 2004. **428**(6979): p. 194-8.
121. Li, J.Q., et al., *Correlation of Skp2 with carcinogenesis, invasion, metastasis, and prognosis in colorectal tumors.* Int J Oncol, 2004. **25**(1): p. 87-95.
122. Kawakami, K., et al., *Increased SKP2 and CKS1 gene expression contributes to the progression of human urothelial carcinoma.* J Urol, 2007. **178**(1): p. 301-7.
123. Ji, P., et al., *Disrupting Skp2-cyclin A interaction with a blocking peptide induces selective cancer cell killing.* Mol Cancer Ther, 2007. **6**(2): p. 684-91.
124. Wu, L., et al., *Specific small molecule inhibitors of Skp2-mediated p27 degradation.* Chem Biol, 2012. **19**(12): p. 1515-24.
125. Database, N.G. *CKS1B CDC28 protein kinase regulatory subunit 1B [Homo sapiens]* 4/12/2011 [cited 2011 08/12]; Available from: http://www.ncbi.nlm.nih.gov/gene/1163.
126. Wang, X.C., et al., *Role of Cks1 amplification and overexpression in breast cancer.* Biochem Biophys Res Commun, 2009. **379**(4): p. 1107-13.
127. Ganoth, D., et al., *The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27.* Nat Cell Biol, 2001. **3**(3): p. 321-4.

128. Arvai, A.S., et al., *Crystal structure of the human cell cycle protein CksHs1: single domain fold with similarity to kinase N-lobe domain.* J Mol Biol, 1995. **249**(5): p. 835-42.
129. Frontini, M., et al., *The CDK Subunit CKS2 Counteracts CKS1 to Control Cyclin A/CDK2 Activity in Maintaining Replicative Fidelity and Neurodevelopment.* Dev Cell, 2012. **23**(2): p. 356-70.
130. Bashir, T., et al., *Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase.* Nature, 2004. **428**(6979): p. 190-3.
131. Keller, U.B., et al., *Myc targets Cks1 to provoke the suppression of p27Kip1, proliferation and lymphomagenesis.* EMBO J, 2007. **26**(10): p. 2562-74.
132. Holic, R., et al., *Cks1 activates transcription by binding to ubiquitylated proteasome.* Mol Cell Biol, 2010.
133. Sitry, D., et al., *Three different binding sites of Cks1 are required for p27-ubiquitin ligation.* J Biol Chem, 2002. **277**(44): p. 42233-40.
134. Westbrook, L., et al., *Cks1 regulates cdk1 expression: a novel role during mitotic entry in breast cancer cells.* Cancer Res, 2007. **67**(23): p. 11393-401.
135. Westbrook, L., et al., *High Cks1 expression in transgenic and carcinogen-initiated mammary tumors is not always accompanied by reduction in p27Kip1.* Int J Oncol, 2009. **34**(5): p. 1425-31.
136. Holic, R., et al., *Cks1 activates transcription by binding to the ubiquitylated proteasome.* Mol Cell Biol, 2010. **30**(15): p. 3894-901.
137. Hoellein, A., et al., *Cks1 promotion of S phase entry and proliferation is independent of p27Kip1 suppression.* Mol Cell Biol, 2012. **32**(13): p. 2416-27.
138. Rother, K., et al., *Expression of cyclin-dependent kinase subunit 1 (Cks1) is regulated during the cell cycle by a CDE/CHR tandem element and is downregulated by p53 but not by p63 or p73.* Cell Cycle, 2007. **6**(7): p. 853-62.
139. Wang, I.C., et al., *Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase.* Mol Cell Biol, 2005. **25**(24): p. 10875-94.
140. Tsai, Y.S., et al., *RNA silencing of Cks1 induced G2/M arrest and apoptosis in human lung cancer cells.* IUBMB Life, 2005. **57**(8): p. 583-9.
141. Kitajima, S., et al., *Role of Cks1 overexpression in oral squamous cell carcinomas: cooperation with Skp2 in promoting p27 degradation.* Am J Pathol, 2004. **165**(6): p. 2147-55.
142. Chang, H., et al., *CKS1B nuclear expression is inversely correlated with p27Kip1 expression and is predictive of an adverse survival in patients with multiple myeloma.* Haematologica, 2010. **95**(9): p. 1542-7.
143. Kratzat, S., et al., *Cks1 is required for tumor cell proliferation but not sufficient to induce hematopoietic malignancies.* PLoS One, 2012. **7**(5): p. e37433.
144. Liu, Z., et al., *Prognostic implication of p27Kip1, Skp2 and Cks1 expression in renal cell carcinoma: a tissue microarray study.* J Exp Clin Cancer Res, 2008. **27**: p. 51.
145. Wang, X.C., et al., *Overexpression of Cks1 increases the radiotherapy resistance of esophageal squamous cell carcinoma.* J Radiat Res, 2012. **53**(1): p. 72-8.

146. Lee, E.K., et al., *Cell-cycle regulator Cks1 promotes hepatocellular carcinoma by supporting NF-kappaB-dependent expression of interleukin-8.* Cancer Res, 2011. **71**(21): p. 6827-35.
147. Calvisi, D.F., et al., *SKP2 and CKS1 promote degradation of cell cycle regulators and are associated with hepatocellular carcinoma prognosis.* Gastroenterology, 2009. **137**(5): p. 1816-26 e1-10.
148. Hintersteiner, M., et al., *Identification of a small molecule inhibitor of importin beta mediated nuclear import by confocal on-bead screening of tagged one-bead one-compound libraries.* ACS Chem Biol, 2010. **5**(10): p. 967-79.
149. Huber, L.A., *Is proteomics heading in the wrong direction?* Nat Rev Mol Cell Biol, 2003. **4**(1): p. 74-80.
150. Yu, V.P., et al., *A kinase-independent function of Cks1 and Cdk1 in regulation of transcription.* Mol Cell, 2005. **17**(1): p. 145-51.
151. Ciaparrone, M., et al., *Localization and expression of p27KIP1 in multistage colorectal carcinogenesis.* Cancer Res, 1998. **58**(1): p. 114-22.
152. Lan, Y., et al., *Aberrant expression of Cks1 and Cks2 contributes to prostate tumorigenesis by promoting proliferation and inhibiting programmed cell death.* Int J Cancer, 2008. **123**(3): p. 543-51.
153. Suzuki, S., et al., *Up-regulation of Cks1 and Skp2 with TNFalpha/NF-kappaB signaling in chronic progressive nephropathy.* Genes Cells, 2011. **16**(11): p. 1110-20.
154. **Shi, L.**, et al., *Over-expression of CKS1B activates both MEK/ERK and JAK/STAT3 signaling pathways and promotes myeloma cell drug - resistance. Oncotarget*, 2010. **1**(1): p. 22-33.
155. Tsai, Y., et al., *RNA silencing of Cks1 induced G2/M arrest and apoptosis in human lung cancer cells.* IUBMB Life, 2005. **57**(8): p. 583-9.
156. Huang, K.S. and L.T. Vassilev, *High-throughput screening for inhibitors of the Cks1-Skp2 interaction.* Methods Enzymol, 2005. **399**: p. 717-28.
157. cardozo, t. and m. pagano, *Wrenches in the works: drug discovery targeting the SCF ubiquitin ligase and APC/C complexes.* BMC Bioinformatics, 2007. **8**(suppl 1): p. s9.
158. Uehara, N., K. Yoshizawa, and A. Tsubura, *Vorinostat enhances protein stability of p27 and p21 through negative regulation of Skp2 and Cks1 in human breast cancer cells.* Oncol Rep, 2012. **28**(1): p. 105-10.