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Ubiquitylation and proteasomal degradation of the p21^{Cip1}, p27^{Kip1} and p57^{Kip2} CDK inhibitors

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Abstract

The expression levels of the p21^{Cip1} family CDK inhibitors (CKIs), p21^{Cip1}, p27^{Kip1} and p57^{Kip2}, play a pivotal role in the precise regulation of cyclin-dependent kinase (CDK) activity, which is instrumental to proper cell cycle progression. The stabilities of p21^{Cip1}, p27^{Kip1} and p57^{Kip2} are all tightly and differentially regulated by ubiquitylation and proteasome-mediated degradation during various stages of the cell cycle, either in steady state or in response to extracellular stimuli, which often elicit site-specific phosphorylation of CKIs triggering their degradation.

Keywords

phosphorylation; ubiquitylation; proteasome; p21^{Cip1}; p27^{Kip1}; p57^{Kip2}

Introduction

Ubiquitin (Ub)/proteasome-regulated proteolysis is a key mechanism for regulating many cellular and organismal processes. Conjugation of Ub to substrate proteins requires three enzymes: the Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3). E1 activates Ub through the formation of a thiol-ester bond between the C-terminus of Ub and the active site cysteine (Cys). The activated Ub is then trans-esterified to a conserved Cys of an E2. The E3 ligase interacts with both E2 and the substrate and facilitates polyubiquitylation of the substrate.[1] There are two distinct types of E3 ligases: the enzymatic HECT (homologous to E6-AP C-terminus) domain E3s and the adaptor E3s. HECT domain E3s form a thioester with Ub, which can then be transferred directly to the substrate. The adaptor E3s, containing a RING, SP-RING finger, variant RING/PHD (plant homeodomain) (also named LAP [leukemia-associated protein] domain), or a U box do not form a thioester with Ub but function as adaptors to facilitate the transfer of ubiquitin between a charged E2 and a substrates.[2–5] E3s have a key role in defining the substrate specificity of ubiquitylation.

Cell cycle progression is governed by cyclin-dependent kinases (CDKs), which are activated by cyclin binding and inhibited by CDK inhibitors (CKIs). Although CDK protein levels do not change significantly, the dynamic activities of CDKs during the cell cycle are regulated

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through expression, ubiquitylation, and degradation of cyclins and CKIs, both temporally and spatially, in addition to phosphorylation and dephosphorylation.[1] CKIs can be divided into two families. The Inhibitor of CDKs (INK4) family includes $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$; these CKIs specifically bind CDK4 and CDK6 and inhibit cyclin D association. The other family, the kinase inhibitor protein (KIP) or CDK-interacting protein (CIP) family, includes $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$; these CKIs bind and inhibit all cyclin-bound CDKs.[6,7] The ubiquitylation and proteasome-mediated degradation of $p16^{INK4A}$,[8] and $p19^{INK4D}$ (which depends on binding to CDK4)[9] has been reported. In this review, we discuss the ubiqutylation and degradation of mammalian $p21^{Cip1}$, $p27^{Kip1}$, including its analogues Sic1 and Far1 [in *Saccharomyces (S.) cerevisiae*] and Rum1 (in fission yeast) and $p57^{Kip2}$.

The Skp1/cullin/F-box (SCF) complexes, functioning as E3 ligases, play a pivotal role in ubiquitylation of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. The SCF complexes consist of three invariant subunits—Skp1, CUL1 [cell division cycle (Cdc) 53p in yeast], and the RING finger protein RBX1 (RING box protein-1) (also known as regulator of cullins-1, Roc1)—as well as a variable component known as an F-box protein. F-box proteins bind to Skp1 through their F box and recognize substrates through other domains in the F-box protein.[10] The human genome encodes 69 F-box proteins.[11] Three classes of F-box proteins have been defined on the basis of their substrate recognition motifs: Fbw, F-box proteins containing WD40-repeat domains; Fbl, containing leucine-rich repeats (LRR) domains; and Fbx, containing other domains. The Fbw and Fbl classes of F-box proteins can recognize target phosphodegrons, specific sequences of amino acids in proteins that direct their degradation in a phosphorylation-dependent manner, through their WD40 or LRR domains.[1] The CUL1 scaffold binds RBX1, a RING finger protein that recruits the charged E2, and Skp1. The human genome encodes eight Cullin proteins [CUL1-4A, 4B, 5, 7 and 9 (formerly PARC)] that form similar Cullin-RING Ligase (CRL) complexes.[11] Neddylation of CUL1 is necessary for SCF activity as a Ub ligase, because unneddylated CUL1 holds RBX1 in a closed form bound by an inhibitory protein CAND1, thus preventing Skp1-F-box protein binding. Neddylation of CUL1 loosens the RBX1 structure and dissociates the CAND1 inhibitor, allowing binding of the Skp1-F-box protein complex. The reoriented RBX1 bridges the gap between the associated E2 and substrate bound to the F-box protein, facilitating both initial ubiquitylation and subsequent polyubiquitylation.[11] SCF complexes, working in concert with the UBC3/ Cdc34 E2 (and other E2s, such as Ubc4), which binds to Rbx1, control G_1-S progression and target CKIs ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ in mammals and Sic1 and Far1 in budding yeast) for degradation.[1]

$p21^{Cip1}$

Expression of the $p21^{Cip1}$ mammalian CKI is tightly regulated by signals that control cell division, and also in response to DNA damage, which activates cell cycle checkpoints. Shortly after its discovery, $p21^{Cip1}$ was shown to be a short-lived protein that is degraded by the proteasome.[12] Subsequent work has shown that $p21^{Cip1}$ can be degraded through both ubiqiutin-dependent and independent mechanisms.

Ubiquitylation-dependent proteasomal degradation of $p21^{Cip1}$

Proteolysis of $p21^{Cip1}$ has been shown to require a functional Ub-activating enzyme (E1) and conjugation of the Ub-like protein, Nedd8, whose linkage to CULs is necessary for their Ub ligase activity.[13,14] Consistent with a role for SCF complexes, $p21^{Cip1}$ was shown to be ubiquitylated at four distinct Lys residues located in the C-terminal region by $SCF^{Skp2.15}$ $p21^{Cip1}$ interacts with and is phosphorylated on serine (S)130 by cyclin E/ CDK2, which promotes Skp2-dependent $p21^{Cip1}$ degradation in S phase[16,17] (Fig. 1). Mutation of all six Lys into Arg, disruption of the two nuclear export signal sequences in $p21^{Cip1}$, or treatment

with the nuclear-export inhibitor leptomycin B blocks H_2O_2-induced $p21^{Cip1}$ degradation, implying that cytoplasmic localization is required for $p21^{Cip1}$ degradation.[18] Cytoplasmic $p21^{Cip1}$ degradation is also induced by activation of extracellular signal-regulated kinase 2 (ERK2). ERK2 directly interacts with and phosphorylates $p21^{Cip1}$ at threonine (T)57 and S130, promoting translocation of $p21^{Cip1}$ into the cytoplasm and Ub-dependent degradation by unidentified E3 ligases, thereby resulting in cell cycle progression.[19] In contrast, in transforming growth factor-β1 (TGFβ1) treated cells, p38 and JNK1 are activated and phosphorylate $p21^{Cip1}$ at S130 leading to its stabilization. Whether $p21^{Cip1}$ phosphorylation at S130 by different kinases somehow results in $p21^{Cip1}$ being able to associate with distinct regulators that affect $p21^{Cip1}$ stability differentially warrants further investigation. Similarly, T57 within the CDK binding domain of $p21^{Cip1}$ is also a substrate of glycogen synthase kinase (GSK)3,[20] or JNK,[21] which result in degradation or stabilization of $p21^{Cip1}$, respectively.

HER-2/Neu-activated AKT associates with $p21^{Cip1}$ and phosphorylates it at T145, resulting in cytoplasmic localization of $p21^{Cip1}$ and promotion of cell growth, whereas a nuclear $p21^{Cip1}$ T145A mutant preferentially suppresses growth of transformed cells.[22] AKT-dependent phosphorylation of $p21^{Cip1}$ at T145 prevents complex formation of $p21^{Cip1}$ with proliferating cell nuclear antigen (PCNA) and decreases the binding of CDK2 and CDK4 to $p21^{Cip1}$, thereby attenuating the inhibitory activity of $p21^{Cip1}$ on CDK and DNA replication.[23,24] In addition to T145, S146 of $p21^{Cip1}$ is also phosphorylated by AKT, and this phosphorylation is suggested to stabilize $p21^{Cip1}$.[24] How phosphorylation of two adjacent residues by AKT leads to opposite effects on $p21^{Cip1}$ stability is not yet clear.

Ubiquitylation of $p21^{Cip1}$ by the SCF^{Skp2} complex requires functional interaction between $p21^{Cip1}$ and the cyclin E/CDK2 complex. Mutation of both the cyclin E recruitment motif (RXL) and the CDK2-binding motif (FNF) at the N-terminus of $p21^{Cip1}$ abolishes its ubiquitylation by Skp2 (Fig. 1).[15] $p21^{Cip1}$ accumulates in $Skp2^{-/-}$ mouse embryonic fibroblasts during S phase, suggesting that SCF^{Skp2} plays a role in $p21^{Cip1}$ degradation during the G_1/S transition.[25] In addition to Skp2, overexpression of p53-inducible RING-finger protein (p53RFP) causes degradation of $p21^{Cip1}$,[26] suggesting that multiple E3 ligases are involved in $p21^{Cip1}$ ubiquitylation. Additional evidence shows that, in spite of regulation of $p21^{Cip1}$ by SCF^{Skp2} during the G_1/S transition, $p21^{Cip1}$ reaccumulates during G_2 and is degraded again in prometaphase through the interaction of the anaphase-promoting complex/cyclosome $(APC/C)^{Cdc20}$ E3 ligase with the destruction box (D box) of $p21^{Cip1}$ (Fig. 1). A D-box mutant of $p21^{Cip1}$ is resistant to degradation by APC/C^{Cdc20} E3 ligase, but not to degradation by SCF^{Skp2}. Silencing of APC/C^{Cdc20} induced accumulation and binding of $p21^{Cip1}$ to CDK1, thereby inhibiting CDK1 activity during prometaphase, implying that $p21^{Cip1}$ degradation by APC/C^{Cdc20} is required for the full activation of CDK1 necessary for mitotic events.[25] In contrast to the normal turnover of $p21^{Cip1}$ mediated by the SCF^{Skp2} and APC/C^{Cdc20} complexes at different phases of the cell cycle, ionizing radiation-induced Ub-dependent degradation of $p21^{Cip1}$ requires the $CRL4^{Cdt2}$ E3 ligase (composed of the Cul4A/B, DDB1, and the DCAF subunit Cdt2) and $p21^{Cip1}$ binding to PCNA,[27–30] suggesting that $p21^{Cip1}$ stability is regulated by distinct mechanisms in response to different extracellular stimuli.

Since $p21^{Cip1}$ accumulates in cells harboring a temperature-sensitive mutation in Ub E1 at the restrictive temperature,[13,14] degradation of $p21^{Cip1}$ requires active ubiquitylation, but this does not have to be due to ubiquitylation of $p21^{Cip1}$ itself. Although $p21^{Cip1}$ ubiquitylation can be detected, there has been a lively debate about which residues in $p21^{Cip1}$, if any, need to be ubiquitylated for its degradation, and this issue is complicated by the fact that $p21^{Cip1}$ can be degraded by the proteasome in a ubiquitin-independent fashion (see below). Exogenously expressed $p21^{Cip1}$ K6R, harboring mutations of all six internal

Lys to Arg to prevent ubiquitylation, is still ubiquitylated.[13] Moreover, $p21^{Cip1}$ K6R is unstable, and its abundance also increases in response to proteasome inhibition indistinguishably from WT $p21^{Cip1}$.[31] These findings suggested that Lys-independent ubiquitylation and proteasomal degradation is involved in regulation of $p21^{Cip1}$ stability, and this idea is supported by the evidence that N-terminally-tagged $p21^{Cip1}$ can be ubiquitylated on its free α-NH_2 group.[13,14] However, this occurs because the N-terminal Tyr residue of the tag is not acetylated. In contrast, the majority of endogenous $p21^{Cip1}$ is acetylated at its N-terminal Ser residue,[32] meaning that N-terminal ubiquitylation of $p21^{Cip1}$ is unlikely to be physiologically important. Nevertheless, with the recent discovery that N-terminally-acetylated residues, including N-acetyl Ser, can act as a degradation signal (degron) and specify a short half life for yeast proteins, the role of the acetylated N-terminus in $p21^{Cip1}$ degradation needs further investigation.[33] Such an ^{Ac}N-degron might dictate the basal half life of $p21^{Cip1}$, and involve ubiquitylation of internal Lys residues in $p21^{Cip1}$ leading to its degradation by the proteasome.

$p21^{Cip1}$ degradation can be inhibited by nucleophosmin (NPM)/B23, a multifunctional protein that binds $p21^{Cip1}$. Actinomycin D stimulation increases the interaction and nucleoplasmic translocation of NPM and $p21^{Cip1}$ and stabilizes $p21^{Cip1}$.[34] However, whether NPM-induced $p21^{CIP1}$ stabilization is due to retention of $p21^{Cip1}$ in nucleus thereby preventing $p21^{Cip1}$ degradation in the cytosol remains unclear. $p21^{Cip1}$ can also be stabilized by interaction with WISp39, a tetratricopeptide repeat (TPR) protein. The C-terminal TPR domain of WISp39 recruits Hsp90 and forms a trimeric complex to prevent proteasomal degradation of $p21^{Cip1}$. Point mutations within the C-terminal TPR domain of WISp39, which abolish the binding of WISp39 to Hsp90 but not to $p21^{Cip1}$, fail to stabilize p21, suggesting an essential role of Hsp90 in stabilization of $p21^{Cip1}$.[35]

Ubiquitylation-independent proteasomal degradation of $p21^{Cip1}$

Although the degradation of $p21^{Cip1}$ is generally believed to depend on proteasomes, other observations suggest that proteasomal degradation of $p21^{Cip1}$ does not obligatorily require its ubiquitylation. For instance, MDM2 promotes $p21^{Cip1}$ degradation independently of ubiquitylation.[36] Ubiquitylation- and Skp2-independent proteasomal degradation of $p21^{Cip1}$ also occurs in response to ultraviolet irradiation, which is mediated by phosphorylation of $p21^{Cip1}$ at S114 via GSK3β, a downstream effector of the ATR DNA damage signaling pathway.[37,38] Further, the C8 α-subunit of the 20S proteasome has been shown to interact directly with the non-ubiquitylated C-terminus of $p21^{Cip1}$ and thereby mediate the degradation of $p21^{Cip1}$.[39] The role of the C8 α-subunit of the 20S proteasome in $p21^{Cip1}$ is also evidenced by Ras signaling-induced $p21^{Cip1}$ stabilization. Ras activation promotes the formation of $p21^{Cip1}$-cyclin D1 complexes and prevents $p21^{Cip1}$ from associating with the C8 α-subunit of the 20S proteasome.[40] This observation is further supported by the finding that degradation of unbound $p21^{Cip1}$ by the 20S proteasome occurs in an ATP- and Ub-independent manner. This process is directly mediated by the proteasome activator REGγ, which binds and activates the 20S proteasome.[41] Depletion of REGγ results in increased $p21^{Cip1}$ protein levels and changes in cell cycling and proliferation.

Obviously, further detailed studies on the mechanisms by which $p21^{Cip1}$ is regulated in quiescent and cycling cells will be required for a full understanding of the relative importance of the Ub-dependent and Ub-independent processes for degradation of $p21^{Cip1}$.

p27^{Kip1}, Sic1, Rum1 and Far1 p27^{Kip1}

Regulation of p27^{Kip1} stability by phosphorylation of p27^{Kip1} at T187 by cyclin/CDK complexes

The most well-studied mammalian CKI is p27^{Kip1}, which is abundant in quiescent and G_1 cells and is downregulated in proliferating cells and in S- and G_2-phase cells (Fig. 2). p27^{Kip1} acts in G_0 and early G_1 to inhibit G_1 cyclin/CDK2 complexes, with the primary target being E-type cyclin/ CDK2.[42–44] Before the onset of S-phase, cyclin E/CDK2 binds to and phosphorylates p27^{Kip1} at T187,[45,46] with p27^{Kip1} complexed to cyclin E/CDK2 being a target for phosphorylation by a second, active cyclin E/CDK2 complex. Phosphorylation of T187 creates a phosphodegron that recruits Skp2 through LRR domain binding, resulting in p27^{Kip1} ubiquitylation by SCF^{Skp2} E3 ligase containing either the Rbx1/Roc1 RING finger protein[47–49] or the Ro52 RING finger protein.[50] In addition to using the CUL1-containing SCF complex, Skp2 interacts with CUL4A-DDB1-associated COP9 signalosomes to induce proteolysis of p27^{Kip1}.[51] Depletion of COP9 signalosome subunits 4 and 5 decreases the levels of Skp2 protein, stabilizes p27^{Kip1}, and impairs cell proliferation, and this can be partially reversed by suppression of p27^{Kip1}.[52]

The SCF^{Skp2}-mediated ubiquitylation of p27^{Kip1} requires an additional factor, CDK subunit 1 (Cks1), which is a member of the highly conserved Suc1/Cks family of proteins that bind to some cyclin/CDK complexes and phosphorylated proteins. Cks1 binds to the LRR domain and C-terminal tail of Skp2,[53] in which a negatively charged residue Asp331 is essential for the interaction.[54] The formation of complexes between Cks1 and Skp2 causes conformational changes in both proteins and significantly stabilizes the interaction between Skp2 and Skp1 and enhances the binding of Skp2 to p27^{Kip1} phosphorylated at T187.[53,55] p27^{Kip1} binds to both Cks1 and Skp2 by inserting the side chain of an invariant Glu185 into the interface between Skp2 and Cks1 and interacts with a Cks1 phosphate-binding site via the phosphorylated T187 side chain of p27^{Kip1}.[53] Phosphorylated p27^{Kip1}, complexed to cyclin E/CDK2, binds to the SCF^{Skp2}/Cks1 complex in a cooperative manner. Cyclin E/CDK2 contributes to p27^{Kip1} binding to the SCF^{Skp2}/Cks1 by T187 phosphorylation as well as by a potential direct interaction between cyclin E/CDK2 and the SCF^{Skp2}/Cks1 complex.[56] In contrast, the interaction between phospho-T187 p27^{Kip1} and Cks1 is dramatically reduced by the cis-trans peptidylprolyl isomerase Pin1. Pin1 binds to the phosphorylated Thr187-Pro motif in p27^{Kip1} and can cause cis-trans isomerization of this bond altering the conformation of p27^{Kip1}. p27^{Kip1} exhibits increased polyubiquitylation and has a shorter half life in *Pin1*$^{-/-}$ mouse embryonic fibroblasts than in wild-type cells.[57]

In addition to cyclin E, cyclin D and cyclin A are also involved in p27^{Kip1} degradation by distinct mechanisms. Depletion of cyclin D1 causes p27^{Kip1} accumulation, with a simultaneous decrease in CUL1 neddylation and increased binding to CAND1, which blocks the accessibility of CUL1 to Skp1 and Skp2, thus preventing the formation of the SCF^{Skp2} complex.[58] How cyclin D1 is able to promote SCF^{Skp2} complex formation, however, remains unclear. Cyclin A/CDK2, but not cyclin B/CDK1, forms a stable complex with T187-phosphorylated p27^{Kip1} to stimulate p27^{Kip1} ubiquitylation.[46,59] Distinct interaction regions in cyclin A separately bind to Skp2 and p27^{Kip1}, and Skp2-cyclin A interaction directly protects cyclin A-CDK2 from inhibition by p27^{Kip1} through competitive binding. Disruption of cyclin A-Skp2 binding, which does not affect p27^{Kip1}-cyclin A interaction, compromises Skp2's proliferation stimulatory activity without affecting its ability to degrade p27^{Kip1} and p21^{Cip1}.[60]

Although SCF^{Skp2} mediates the nuclear ubiquitylation of CDK2-bound p27^{Kip1} during S/G_2 and M phases, when p27^{Kip1} is translocated into the cytoplasm its stability is controlled in an SCF^{Skp2}-independent manner (see below).[61–63] In addition, other E3 ligases, such as a

HECT domain family ubiquitin ligase E6-AP,[64] p53-inducible protein with RING-H2 domain (Pirh2),[65] and CUL4A/CUL4B-based E3 ligases in response to active Wnt signaling[66–68] are also reportedly involved in the ubiquitylation and proteasomal degradation of $p27^{Kip1}$,[64] in an extracellular stimuli- or cell type-specific manner.

Regulation of $p27^{Kip1}$ stability by phosphorylation of $p27^{Kip1}$ at S10

Stimulation by hepatocyte growth factor (HGF) increases $p27^{Kip1}$ phosphorylation at S10 and induces nuclear export of $p27^{Kip1}$,[69] (Fig. 2). In keeping with this, ERK activation induces the cytoplasmic localization of $p27^{Kip1}$,[70] and $p27^{Kip1}$ S10A is refractory to Ras-induced cytoplasmic translocation.[71] $p27^{Kip1}$ phosphorylated on S10 at the G_0-G_1 transition binds to the nuclear export protein CRM1, leading to its subsequent cytoplasmic translocation.[62,63] The stability of $p27^{Kip1}$ exported into the cytoplasm can be regulated by interaction with and ubiquitylation by the E3 complex KPC (Kip1 ubiquitylation-promoting complex), which consists of KPC1 and KPC2. KPC2 contains a Ub-like domain and two ubiquitin-associated (UBA) domains. KPC1 possesses a C-terminal RING-finger domain, and its N-terminal region is involved in the interaction with free $p27^{Kip1}$. Thus, KPC may control the degradation of $p27^{Kip1}$ exported from the nucleus in G_1 phase.[72–74] However, it remains unknown whether a direct modification of $p27^{Kip1}$, such as phosphorylation at S10, is required for KPC-mediated $p27^{Kip1}$ degradation. KPC1 itself can be regulated by the USP19 deubiquitinating enzyme, which interacts with and stabilizes KPC1, thereby modulating $p27^{Kip1}$ levels and cell proliferation.[75]

$p27^{Kip1}$ S10 phosphorylation can depend on both tissue and cell type as well as cell cycle stage. CDK5, an unconventional neuronal CDK that is activated in postmitotic neurons but not in proliferative cells, directly phosphorylates $p27^{Kip1}$ at S10, which contributes to neuronal migration in the developing cerebral cortex.[76] CDK5 is also able to phosphorylate T187 in vitro, but the physiological significance remains unclear.[76] Other protein kinases can phosphorylate $p27^{Kip1}$ at S10 at different stages of the cell cycle: Mirk/DYRK1B, which is maximally active in G_0, phosphorylates and stabilizes $p27^{Kip1}$ in quiescent cells;[77] human kinase-interacting stathmin (KIS), which is activated by mitogens, binds the C-terminal domain of $p27^{Kip1}$, phosphorylates S10 at the G_0-G_1 transition, and promotes its nuclear export to the cytoplasm.[78] In addition, corneal endothelial cells treated with fibroblast growth factor (FGF)-2 possess distinct polyubiquitylation pathways for phospho-T187 and phospho-S10 $p27^{Kip1}$; phospho-T187 $p27^{Kip1}$ is ubiquitylated through nuclear SCF^{Skp2} during late G_1 phase, whereas phospho-S10 $p27^{Kip1}$ is ubiquitylated by cytosolic KPC during early G_1 phase.[79] These results further support the conclusion that $p27^{Kip1}$ stability is regulated by phosphorylation at different residues at various stages of the cell cycle. While S10 phosphorylation stabilizes $p27^{Kip1}$ in resting cells (G_0 phase), this phosphorylation promotes $p27^{Kip1}$ nuclear export and cytoplasmic degradation during early and late G_1 phases.[80] Consistently, the S10A $p27^{Kip1}$ mutant has reduced stability compared with WT $p27^{Kip1}$ in G_0 phase whereas mutation of S10 into Asp or Glu to mimic phosphorylation stabilizes $p27^{Kip1}$.[80] A higher proportion of S10A $p27^{Kip1}$ is found in association with cyclin/CDK complexes than WT $p27^{Kip1}$, thus promoting $p27^{Kip1}$ S10A assembly into cyclin-CDK complexes, which is, in turn, necessary for $p27^{Kip1}$ turnover.[71,81] $p27^{Kip1}$ S10A knock-in mice have normal body size but exhibit organ-specific reductions in $p27^{Kip1}$ expression in brain, thymus, spleen and testis.[81] The reason for this organ specificity in the downregulation of $p27^{Kip1}$ S10A remains an enigma.

Regulation of $p27^{Kip1}$ stability by phosphorylation of $p27^{Kip1}$ at tyrosines 74 and 88 and threonines 157 and 198

Distribution of $p27^{Kip1}$ into cyclin/CDK complexes seems to be affected by phosphorylation of $p27^{Kip1}$ at T198, an event that can be mediated by AKT or p90 ribosomal protein S6

kinases (RSK)[82–84] (Fig. 2). Phosphorylation at this residue stabilizes free $p27^{Kip1}$, whereas loss of this phosphorylation site promotes its binding to CDK2-containing complexes.[84]

Tyrosine (Y) 74 and Y88 of $p27^{Kip1}$ can be phosphorylated by the Src-family kinases c-Src and Yes, whereas Lyn and the oncoprotein BCR-ABL appear to phosphorylate predominantly Y88. $p27^{Kip1}$ phosphorylation at Y74/88 reduces its steady-state binding to cyclin E-CDK2, thus restoring partial CDK activity. The activated CDK2 phosphorylates $p27^{Kip1}$ on T187, which in turn promotes SCF^{Skp2}-dependent degradation of $p27^{Kip1}$.[85,86] Consistently, reduced $p27^{Kip1}$ expression levels are observed in Src-activated breast cancer lines, correlating with Src activation in primary human breast cancers.[85] In contrast, $p27^{Kip1}$ phosphorylation at Y88/89 was also proposed to regulate CDK4 activity via a distinct mechanism. $p27^{Kip1}$ associates with cyclin D-CDK4 constitutively. However, Y88 and Y89 phosphorylated preferentially in proliferating cells converts $p27^{Kip1}$ to a non-inhibitor of cyclin D-CDK4 by dislodging $p27^{Kip1}$ from the catalytic cleft of CDK4 to allow ATP binding.[87,88]

$p27^{Kip1}$ T157 can be a substrate of multiple protein kinases. The Pim kinase family members (Pim1, Pim2 and Pim3) bind to and phosphorylate $p27^{Kip1}$ at both T157 and T198. Pim-mediated phosphorylation induces $p27^{Kip1}$ binding to 14-3-3 proteins, resulting in its nuclear export and proteasome-dependent degradation.[89] Phosphorylation of $p27^{Kip1}$ at T157 is also observed upon AKT and SGK1 activation, which interrupts association of $p27^{Kip1}$ with importin-α, thus preventing re-entry of $p27^{Kip1}$ into the nucleus.[90,91] $p27^{Kip1}$ phosphorylation at S83 by the protein kinase CK2,[92] and at S178 downstream of the phosphoinositide 3-kinase pathway[93] are also reported, although the biological significance of these phosphorylations remains unclear.

Significance of the regulation of $p27^{Kip1}$ stability

The degradation of $p27^{Kip1}$ is necessary for entry into S phase, as overexpression of a nondegradable $p27^{Kip1}$ mutant (T187A), microjection of Skp2 antibody, or antisense oligonucleotides targeting Skp2 arrests cells in G_1 phase.[94,95] Cells derived from $p27^{Kip1}$ T187A knock-in mice exhibit rising levels of $p27^{Kip1}$ T187A in late $G_1/S/G_2$ cells and a defect in cell cycle progression and cell proliferation.[96] Mice expressing this protein develop normally, and for unknown reasons, grow to be larger than WT mice. *RBX1*, which encodes an SCF subunit, is an essential gene for mouse embryogenesis, and disruption of *RBX1* causes embryonic lethality due to reduced proliferation as a result of $p27^{Kip1}$ accumulation. Simultaneous loss of $p27^{Kip1}$ extends the life span of *RBX1*-deficient embryos from E6.5 to E9.5,[97] indicating that a failure to downregulate $p27^{Kip1}$ as well as other RBX1 substrates, is detrimental to embryonic development. Both Cks1- and Skp2-deficient mice are smaller than their WT littermates, and their cells tend to proliferate more slowly, which correlates with elevated levels of $p27^{Kip1}$.[98–100] Most of the cellular abnormalities apparent in $Skp2^{-/-}$ mice are not evident in $Skp2^{-/-}$-$p27^{Kip1-/-}$ double-mutant mice, suggesting that $p27^{Kip1}$ is a major physiological target of SCF^{Skp2}.[101] This is further supported by the fact that eliminating $p27^{Kip1}$ phosphorylation on T187 in $p27^{Kip1-/-}$ T187A knock-in mice reproduces the effects of Skp2 knockout in preventing spontaneous tumorigenesis in $Rb1^{+/-}$ mice.[102] In addition, the $p27^{Kip1}$ T187A mutation inhibits progression of intestinal adenomas to carcinomas in a mouse model,[103] and the absence of Skp2, which correlates with increased expression of $p27^{Kip1}$, decreases the leukemogenicity of BCR-ABL in a murine model of chronic myelogenous leukemia (CML).[104] Furthermore, *Skp2* deficiency restricts tumorigenesis induced by inactivation of *Pten* or *Arf*, which is mediated by induction of cellular senescence resulting from concomitant upregulation of $p27^{Kip1}$, $p21^{Cip1}$ and Atf4.[105] These results provide additional in vivo evidence that $p27^{Kip1}$ regulation by Skp2 contributes to the development of some tumor types. However, in certain tumor types $p27^{Kip1}$ T187 phosphorylation-induced protein degradation might not be the

primary mechanism for regulation of $p27^{Kip1}$ stability. For instance, expression of $p27^{Kip1}$ T187A is not higher than that of WT $p27^{Kip1}$, and the expression of both WT and the T187A mutant of $p27^{Kip1}$ is downregulated at the transcriptional level during development of activated K-Ras-induced non-small cell lung cancers in mice.[103] Moreover, regulation of the $p27^{Kip1}$ transcript levels in human breast cancers has been reported.[103]

In agreement with the requirement for phosphorylation by and association with cyclins/CDKs for Skp2-mediated $p27^{Kip1}$ degradation, the expression levels of $p27^{CK-}$ in $p27^{CK-}$ knock-in cells, which carry point mutations in protein interaction domains that abolish $p27^{Kip1}$ binding to cyclins and CDKs and consequently cannot inhibit cyclins/CDKs, are increased in various tissues and mouse embryonic fibroblasts.[71] As expected, an increased growth rate and body size and general organomegaly are observed in $p27^{CK-}$ knock-in mice as well as in $p27^{Kip1-/-}$ mice. $p27^{Kip1-/-}$ mice also exhibit sterility (in females) and disrupted retinal architecture and develop multiorgan hyperplasia and pituitary tumors.[106–108] Furthermore, a decline or loss of $p27^{Kip1}$ protein, with an associated increase in Skp2 protein levels, has been detected in many human cancers and is of prognostic significance.[7] In addition, indirect regulation of $p27^{Kip1}$ may also contribute to human cancer development. For instance, inactivating mutations in the Fbw7 F-box protein (also known as hCdc4 and archipelago) in a number of different human cancers, which result in loss of SCF^{Fbw7}-mediated cyclin E ubiquitylation and accumulation of cyclin E,[109,110] may enhance $p27^{Kip1}$ degradation indirectly by contributing to $p27^{Kip1}$ binding to SCF^{Skp2}.[56]

The Sic1p budding yeast CKI—In *S. cerevisiae*, the CDK inhibitor Sic1p, which is functionally and structurally analogous to the mammalian $p27^{Kip1}$,[111] must be degraded for the onset of B-type cyclin/CDK (Clb/Cdc28) activity and consequent DNA replication.[111] Sic1p is phosphorylated in late G_1 phase by G_1 cyclin/CDK (Cln/Cdc28) activity, and the phosphorylated Sic1p binds to the WD40 repeat domain of the Cdc4 F-box protein.[112–114] Cdc4 dimerization via its D domain, which facilitates Ub conjugation but not substrate recognition, is required for SCF^{Cdc4} function and Sic1p ubiquitylation.[115,116] Six N-terminal lysines of Sic1p serve as the major ubiquitylation sites.[117] Sic1p has nine suboptimal Cdc4 phosphodegrons (CPDs);[118,119] Sic1p mutants that lack multiple CDK phosphorylation sites are stabilized, and hence arrest cells in G_1 phase.[112] The nine Sic1p phosphorylation sites form three separate Cdc4-recognition phosphodegrons (T2/5/9, T33/45/48 and S69/76/80), and each degron contains two essential phosphorylated residues and binds to Cdc4 with similar affinities. Double phosphorylation of a single degron is necessary and sufficient for binding to Skp1-Cdc4.[116] The nine phosphorylation sites can be replaced by a single high affinity CPD, leading to the proposal that multiple suboptimal CPDs in Sic1p serve as a mechanism for setting a threshold of cyclin/CDK phosphorylation before Sic1p degradation can be triggered.[119] Kinetic analysis reveals that Sic1p ubiquitylation has an initiation step, which is a rate-limiting attachment of the first Ub followed by the acidic tail loop of Cdc34 (an E2)-dependent rapid synthesis of K48-linked Ub chains.[120] Subsequently, polyubiquitin chains are built on SCF substrates by sequential transfers of single ubiquitins.[121] Protein kinase CK2-mediated phosphorylation of Cdc34 on the acidic tail domain (S207/216 in yeast and S203/222/231 in humans) stimulates Cdc34-SCF^{Cdc4} ubiquitylation activity toward Sic1p and cell cycle progression.[122] The multiubiquitin chain binding proteins (MCBPs) Rad23 and Rpn10 contribute to recruitment of ubiquitylated Sic1p to the 26S proteasome, where Rpn11 metalloprotease is essential for deubiquitylation and degradation of Sic1p.[123,124] Intriguingly, analysis using an in vitro biochemical reconstitution system shows that Sic1p degradation is essential for triggering the ATP hydrolysis-dependent dissociation and disassembly of the 19S regulatory particles from the 26S proteasome, implying that this is a general process for degradation of other proteins.[125]

During sporulation, Sic1p degradation is independent of Cdc28 but requires Ime2 protein kinase activity.[126,127] The meiosis-specific kinase Ime2 phosphorylates only a subset of the Sic1p sites corresponding to CDK sites, which, by itself, is insufficient to promote Sic1p binding to Cdc4 and Sic1 degradation[128] The identity of the other kinase(s) that phosphorylate Sic1 in meiosis to promote Sic1p degradation in combination with Ime2 remains obscure. Intriguingly, Ime2p kinase is degraded by SCF^{Grr1p} upon glucose stimulation, which increases Sic1p levels, resulting in blockage of meiotic DNA replication.[129]

The Rum1 fission yeast CKI—Rum1, the sole CKI in fission yeast, is essential for proper regulation of the G_1/S transition, inhibiting Cdc13/Cdc2 complex activity. Rum1, the fission yeast analogue of $p27^{Kip1}$ and Sic1p,[130] is a substrate of SCF complexes containing the fission yeast orthologues of Cdc4, Pop1 and Pop2.[131–134] Rum1 degradation requires phosphorylation of Rum1 at T58 and T62 by Cig1/Cdc2 (cyclin/CDK) and covalent attachment of NEDD8 to the Cul-family proteins Pcu1 and Pcu4 (Cul-1 and Cul-4A/Cul-4B orthologues, respectively).[135,136] Pop1 interacts with the N-terminal domain of Pop2 and forms heterooligomeric complexes, which bind and direct polyubiquitylation of Rum1.[137,138]

The Far1 budding yeast CKI—In addition to Sic1p, a second CKI is present in budding yeast, Far1. While Sic1 specifically inhibits Clb/Cdc28 kinases,[111] Far1 inhibits Cln1,2/Cdc28 and Cln3/Cdc28 complexes during the pheromone response.[139,140] Despite the functional similarities between Far1 and $p27^{Kip1}$, these proteins share only a very small amount of amino acid identity.[43,69,141] Far1 is required to arrest the cell cycle of *S. cerevisiae* in response to mating factor. Far1 is phosphorylated at S87 by Cln2/Cdc28 and degraded by SCF^{Cdc4} in the nucleus, through recognition of the pS87 phosphodegron by the Cdc4 WD40 repeat domain.[142] In response to mating pheromone, a fraction of Far1 is stabilized after it is exported into the cytoplasm by Ste21/ Msn5, whereas blockage of nuclear export destabilizes Far1.[143]

$p57^{Kip2}$

$p57^{Kip2}$, the third member of the $p21^{Cip1}$ family of CKIs, is most closely related to $p27^{Kip.1}$ $p57^{Kip2}$ is primarily expressed in terminally differentiated cells and associated with G_1 CDKs, and this can cause cell cycle arrest in the G_1 phase.[144] $p57^{Kip2}$, which accumulates following serum starvation, causing cell cycle arrest of osteoblastic cells, is rapidly degraded upon transforming growth factor (TGF)β1 stimulation.[145] TGFβ1-stimulated ubiquitylation and proteasomal degradation of $p57^{K1p2}$ does not influence the levels of $p21^{Cip1}$ and $p27^{Kip1}$ proteins, indicating that $p57^{Kip2}$ degradation in response to TGFβ1 is mediated by a distinct mechanism. One specific mechanism of $p57^{Kip2}$ degradation is mediated through TGFβ1-activated, Smad-dependent transcription of the gene for the F-box protein FBL12,[146,147] (Fig. 3). FBL12 forms an SCF^{FBL12} complex that binds to and ubiquitylates mouse $p57^{K1p2}$ phosphorylated at T329 (equivalent to human $p57^{Kip2}$ T310), which is conserved between the COOH-terminal QT domains of $p57^{Kip2}$ and $p27^{Kip1}$. Inhibition of FBL12 suppresses TGFβ-induced degradation of $p57^{Kip2}$, increases the steady-state level of $p57^{Kip2}$, and promotes the differentiation of primary osteoblasts.[147]

SCF^{Skp2} is another E3 ligase responsible for regulating the cellular level of $p57^{Kip2}$ by targeting it for ubiquitylation and proteolysis.[148] Overexpression of WT Skp2 promotes degradation of $p57^{Kip2}$, whereas expression of a dominant-negative mutant of Skp2 prolongs the half-life of $p57^{Kip2}$. $p57^{Kip2}$ interacts with Skp2, and mutation of T310 in human $p57^{Kip2}$ abrogates Skp2-induced $p57^{Kip2}$ degradation, suggesting that phosphorylation at this site is required for SCF^{Skp2}-mediated ubiquitylation. Similar to the role of cyclin/CDK

in $p27^{KIP1}$ ubiquitylation, purified recombinant SCF^{Skp2} complex ubiquitinates $p57^{Kip2}$ and this is dependent on the presence of the cyclin E/CDK2 complex. $Skp2^{-/-}$ cells have abnormal accumulation of $p57^{Kip2}$,[148] suggesting that SCF^{FBL12} cannot compensate for the deficiency of Skp2 in the ubiquitylation and degradation of $p57^{Kip2}$.

Whereas the lack of $p21^{Cip1}$ or $p27^{Kip1}$ does not show gross defects in embryonic development,[149] most $p57^{Kip2}$-null mice die after birth and display severe developmental defects resulting from increased apoptosis and delayed differentiation.[144,150] Most of the developmental defects apparent in tissues of the $p57^{Kip2}$ knockout mouse are corrected by replacing the $p57^{Kip2}$ gene with the $p27^{Kip1}$ gene, although the fact that a few developmental defects remain suggests that $p57^{Kip2}$ also has specific functions.[151]

Conclusion

The precise regulation of CDK activity is instrumental to cell cycle progression. Unlike the activity of many other protein kinases, which are often themselves regulated by direct ubiquitylation and degradation of the protein kinase itself,[1] CDK activity is controlled by regulation of cyclins and CKIs. The stability of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ are tightly and differentially regulated by the Ub/proteasome system, in a manner that depends on many factors including the nature of extracellular stimuli, cell cycle stage, differences in subcellular context in different tissues and cells, interaction of CKIs with other regulatory proteins, such as Cks1 for $p27^{Kip1}$ and NPM for $p21^{Cip1}$, involvement of distinct E3 ligases, phosphorylation by distinct protein kinases, and a distinct subcellular compartment for degradation.

$p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ are all targeted by more than one E3 ligase for ubiquitylation. The function of E3 ligases can be overlapping, as illustrated by $p21^{Cip1}$ degradation, where the Cul4-DDB1 and the SCF^{Skp2} E3 ligases are redundant with each other in promoting the degradation of $p21^{Cip1}$ during an unperturbed S phase of the cell cycle.[29] However, these CKIs exhibit specifically regulated ubiquitylation by distinct E3 ligases in response to different extracellular stimuli, such as mitotic stimulation, ionizing radiation, or stress signaling. Under many conditions, phosphorylation of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ is important for their degradation. Many of the regulatory phosphorylation sites lie outside the CDI region in the unique domains of these related proteins, allowing specific regulation of stability and subcellular localization by phosphorylation. Phosphorylation of $p27^{Kip1}$ at S10 or T187 will determine the localization and stages of the cell cycle where degradation of $p27^{Kip1}$ is mediated by distinct E3 ligases, whereas phosphorylation of $p21^{Cip1}$ at T57, S130 or S114 by CDK2, ERK2 or GSK3β will determine its degradation by a Ub-dependent or Ub-independent proteasomal system in cells with or without various stimuli. Given that CKI inactivation by accelerated degradation and mislocalization occurs in cancers,[152] interrupting degradation of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ by targeting the different regulatory steps could open new avenues for cancer therapy.

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Abbreviations

CDKs	cyclin-dependent kinases
CKIs	CDK inhibitors
KIP	kinase inhibitor protein
CIP	CDK interacting protein
Ub	ubiquitin
E1	the Ub-activating enzyme
E2	Ub-conjugating enzyme
E3	Ub ligase
HECT	homologous to E6-AP C-terminus
PHD	plant homeodomain
LAP	leukemia-associated protein
SCF	Skp1/cullin/F-box
LRR	leucine-rich repeats
CRL	cullin-RING ligase
Cks1	CDK subunit 1
Pirh2	protein with RING-H2 domain
HGF	hepatocyte growth factor
UBA domain	ubiquitin-associated domain
FGF	fibroblast growth factor
MCBP	multiubiquitin chain binding protein
ERK	extracellular signal-regulated kinase
PCNA	proliferating cell nuclear antigen
APC/C	anaphase-promoting complex/cyclosome
GSK	glycogen synthase kinase
TGF	transforming growth factor
D box	destruction box
KPC	Kip1 ubiquitylation-promoting complex
RSK	p90 ribosomal protein S6 kinase

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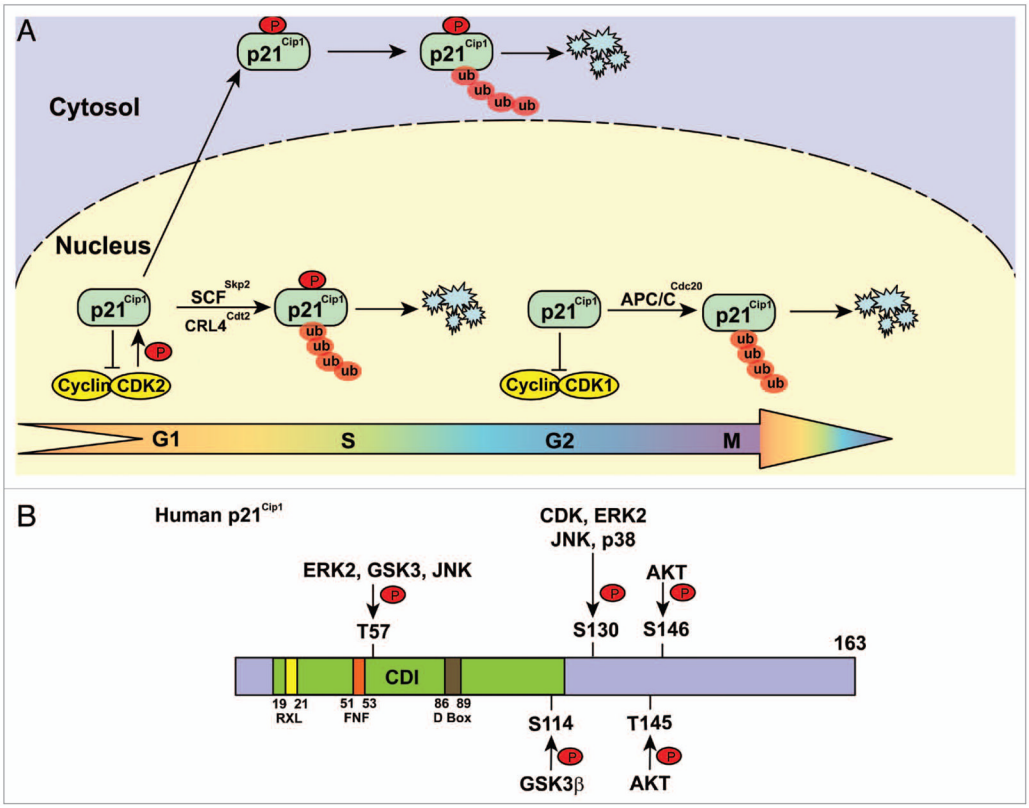

Figure 1.

Phosphorylated $p21^{Cip1}$ is degraded by distinct E3 ligases. (A) E3 ligases involved in $p21^{Cip1}$ degradation. $p21^{Cip1}$ is ubiquitylated and degraded in late G_1 and S phases by SCF^{Skp2} and $CRL4^{Cdt2}$, and in G_2 phase by APC/C^{Cdc20} in the nucleus. A portion $p21^{Cip1}$ is phosphorylated and translocated into the cytosol where it is ubiquitylated and degraded by proteasomes. (B) Schematic structure of $p21^{Cip1}$ showing the regulatory phosphorylation sites and the cognate protein kinases. CDI: CDK inhibitor domain.

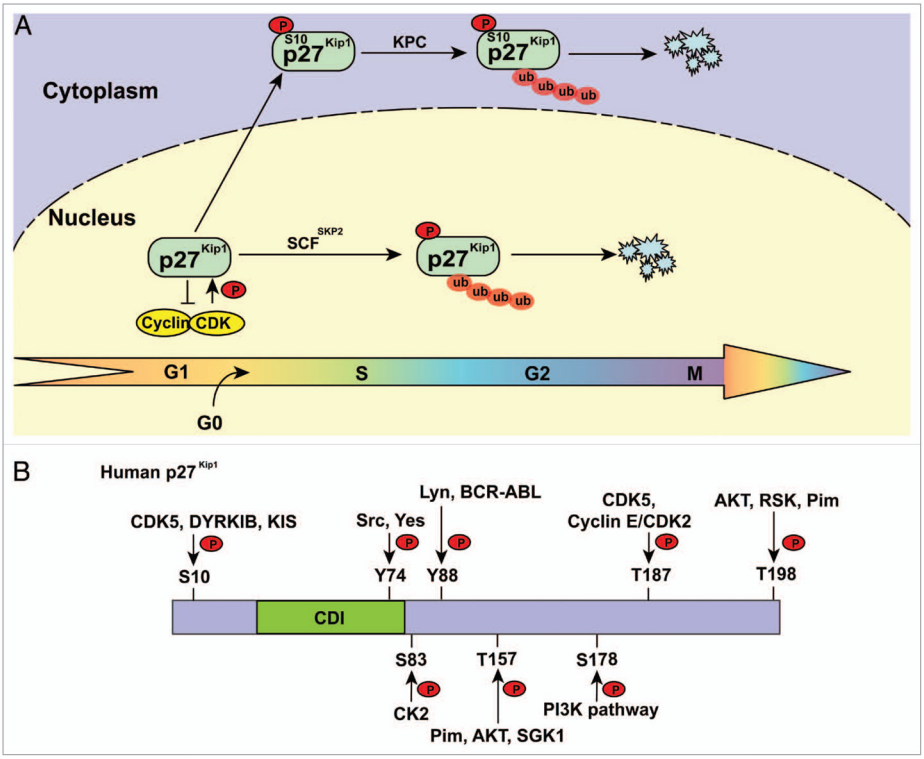

Figure 2.

Phosphorylated 27^{Kip1} is degraded by distinct E3 ligases. (A) E3 ligases involved in $p27^{Kip1}$ degradation. $p27^{Kip1}$ is ubiquitylated and degraded in late G_1, S and G_2 phases by SCF^{Skp2} in the nucleus. $p27^{Kip1}$ phosphorylated at S10 is ubiquitylated by the KPC complex when exported to the cytoplasm. (B) Schematic structure of $p27^{Kip1}$ showing the regulatory phosphorylation sites and the cognate protein kinases. CDI, CDK inhibitor domain.

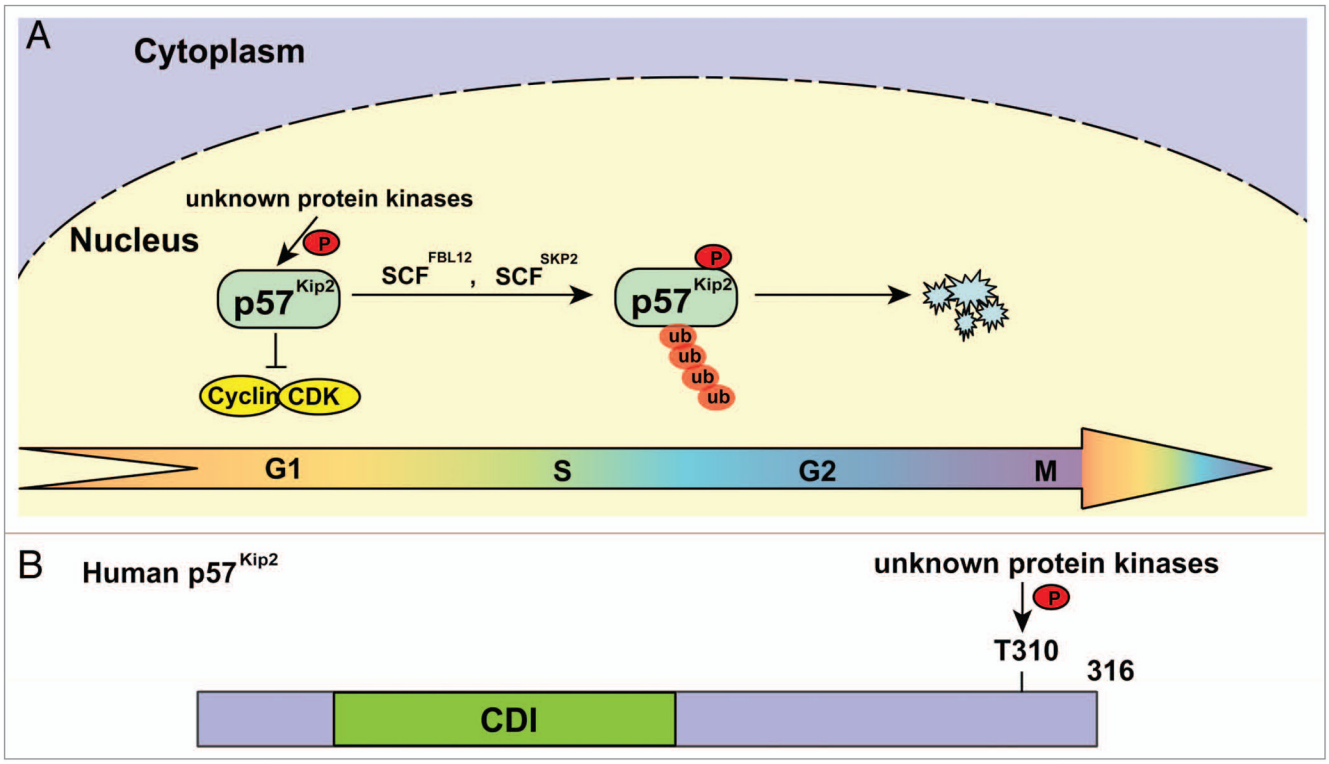

Figure 3.
Phosphorylated $p57^{Kip2}$ is degraded by distinct E3 ligases. (A) E3 ligases involved in $p57^{Kip2}$ degradation. $p57^{KIP2}$ phosphorylated at T329 is ubiquitylated and degraded in late G_1 and S phases by SCF^{FBL12} and SCF^{Skp2}. (B) Schematic structure of $p57^{Kip2}$ showing the single regulatory phosphorylation site. CDI, CDK inhibitor domain.