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Review

CDK Inhibitors: Cell Cycle Regulators and Beyond

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First identified as cell cycle inhibitors mediating the growth inhibitory cues of upstream signaling pathways, the cyclin-CDK inhibitors of the Cip/Kip family p21Cip1, p27Kip1, and p57Kip2 have emerged as multifaceted proteins with functions beyond cell cycle regulation. In addition to regulating the cell cycle, Cip/Kip proteins play important roles in apoptosis, transcriptional regulation, cell fate determination, cell migration and cytoskeletal dynamics. A complex phosphorylation network modulates Cip/Kip protein functions by altering their subcellular localization, protein-protein interactions, and stability. These functions are essential for the maintenance of normal cell and tissue homeostasis, in processes ranging from embryonic development to tumor suppression.

General Features of Cip/Kip Proteins

Progression through the cell-division cycle is regulated by the coordinated activities of cyclin/cyclin-dependent kinases (CDK) complexes. One level of regulation of these cyclin-CDK complexes is provided by their binding to CDK inhibitors (CKIs). In metazoans, two CKI gene families have been defined based on their evolutionary origins, structure, and CDK specificities. The INK4 gene family encodes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, all of which bind to CDK4 and CDK6 and inhibit their kinase activities by interfering with their association with D-type cyclins (Sherr and Roberts, 1999). In contrast, CKIs of the Cip/Kip family bind to both cyclin and CDK subunits and can modulate the activities of cyclin D-, E-, A-, and B-CDK complexes (Sherr and Roberts, 1999). The Cip/Kip family members $p21^{Cip1/Waf1/Sdi1}$ (p21, encoded by *cdkn1a*) (el-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993), $p27^{Kip1}$ (p27, *cdkn1b*) (Polyak et al., 1994a; Polyak et al., 1994b; Toyoshima and Hunter, 1994), and $p57^{Kip2}$ (p57, *cdkn1c*) (Lee et al., 1995; Matsuoka et al., 1995) share a conserved N-terminal domain that mediates binding to cyclins and CDKs but diverge in the remainder of their sequence, suggesting that each of these proteins could have distinct functions and regulation.

A vast body of literature has described the importance of p21, p27, and p57 in restraining proliferation during development, differentiation, and response to cellular stresses (Sherr and Roberts, 1999), although each has specific biological functions that distinguish it from the other family members. Thus, different antiproliferative signals tend to cause elevated expression of only a subset of the Cip/Kip proteins. For example, p21 is an important transcriptional target of p53 and mediates DNA-damage-induced cell-cycle arrest in G1 and G2 (el-Deiry et al., 1993; Gartel and Tyner, 1999). In contrast to p21, p27 expression is usually elevated in mitogen-starved cells and other quiescent states, and the protein is rapidly downregulated as cells enter the cell cycle (Besson et al., 2006; Coats et al., 1996). Several lines of evidence point toward an important role for p57 in the regulation of the cell cycle during embryonic development. Unlike its ubiquitous siblings, p57 has a tissue-restricted expression pattern during embryogenesis and in the adult (Lee et al., 1995; Matsuoka et al., 1995). The transcriptional regulation of p57 is mediated by factors that play critical roles during embryogenesis such as Notch/Hes1, MyoD, BMP-2 and -6, and p73 (Blint et al., 2002; Georgia et al., 2006; Gosselet et al., 2007; Vaccarello et al., 2006). The *cdkn1c* gene is also an imprinted gene with preferred expression of the maternal allele (Matsuoka et al., 1996), which is recognized as a general mechanism to regulate embryonic growth (Andrews et al., 2007). Importantly, p57 is the only CKI to be required for embryonic development, as most mice lacking the *cdkn1c* gene have multiple developmental abnormalities and die at birth (Yan et al., 1997; Zhang et al., 1997).

The importance of the Cip/Kip proteins in cell-cycle regulation is underscored by the phenotypes of the knockout mice for each of these proteins. p27 null mice display an overall increased body size and multiple organ hyperplasia, revealing the importance of p27 in limiting growth (Fero et al., 1996). Although mice lacking p21 do not display an overt hyperproliferative disorder, $p21^{-/-}$ cells fail to undergo DNA-damage-induced cell-cycle arrest and can reach higher saturation density (Deng et al., 1995). Embryos lacking p57 exhibit hyperplasia in several organs and delayed differentiation, probably due to failure to exit the cell cycle in a timely fashion (Zhang et al., 1997).

Although p21, p27, and p57 were initially considered as tumor suppressors based on their ability to block cell proliferation, it rapidly became clear that the situation was not so simple. p21, p27, and p57 are also involved in the regulation of cellular processes beyond cell-cycle regulation, including transcription, apoptosis, and migration, which may be oncogenic under certain circumstances. Moreover, it appears that the loss or subversion of the regulatory mechanisms governing Cip/Kip proteins may lead to the specific loss of the tumor suppressor function of the CKI while maintaining the oncogenic ones. Herein, we will

examine these various functions, how they are regulated, and their significance in vivo, especially in the context of tumorigenesis.

Cip/Kip Proteins in Cell-Cycle Regulation

Cip/Kip proteins were initially characterized as strict inhibitors of all cyclin-CDK complexes, albeit displaying lower affinity toward cyclin B-CDK1 (Sherr and Roberts, 1999). The crystal structure of the N-terminal cyclin and CDK-binding domains of p27 (aa 22–106) bound to cyclin A-CDK2 revealed that the CKI occludes a substrate interaction domain on the cyclin subunit and inserts itself in the catalytic cleft of the CDK, thereby preventing ATP binding and catalytic activity (Russo et al., 1996). However, subsequent studies reported that p21, p27, and p57 participated in the assembly of catalytically active cyclin D-CDK4/6 complexes (LaBaer et al., 1997). Therefore, not only could Cip/Kip proteins promote cyclin D-dependent events, but the sequestration of CKIs into cyclin D-CDK4/6 complexes could allow the downstream activation of cyclin E-CDK2 (Cheng et al., 1998; Perez-Roger et al., 1999; Polyak et al., 1994a; Reynisdottir et al., 1995). The latter is confirmed in cyclin D1 and D2 knockout mice, which display reduced CDK2-associated kinase activity, likely due to the increased availability of Cip/Kip proteins to bind to CDK2 (Geng et al., 2001; Perez-Roger et al., 1999). Nevertheless, there are other reports demonstrating inhibition of cyclin D-CDK4/6 complexes by Cip/Kip proteins, and therefore it was anticipated that the effect of these CKIs on CDK activity would be modulated by other factors.

The Cip/Kip proteins are intrinsically unstructured, adopting specific tertiary conformations only after binding to other proteins (Adkins and Lumb, 2002; Esteve et al., 2003; Lacy et al., 2004). This conformational flexibility suggests that phosphorylation events and protein-protein interactions may modify the folding of the CKIs, thereby modulating their ability to inhibit cyclin-CDK complexes. Likewise, it may explain why CKIs are capable of interacting with a wide diversity of proteins to regulate various cellular functions.

Indeed, it appears that the binding specificity of Cip/Kip proteins is modulated by their phosphorylation on distinct residues, and their potency to inhibit cyclin-CDK complexes can be modified by binding to other proteins. For example, phosphorylation of p21 on Thr-57 (by CDK2 or glycogen synthase kinase 3β [GSK3β]) increases the ability of p21 to bind to cyclin B1-CDK1 complexes at the G2/M transition, without inhibiting the complexes, thus promoting cell-cycle progression (Dash and El-Deiry, 2005). Likewise, the phosphorylation of p27 on Tyr-74, -88 and/or -89 by Src, Lyn, or Abl, greatly decreased the ability of p27 to inhibit CDK2 containing complexes, as Tyr-88 is part of the 3_{10}-helix that normally inserts into the ATP-binding site of the CDK (Chu et al., 2007; Grimmler et al., 2007). In addition, p27 phosphorylation on Tyr-88 and -89 was reported to decrease its affinity for CDK2 while increasing that for CDK4 complexes (Kardinal et al., 2006). A recent report suggested that Tyr-88 phosphorylation was cell-cycle regulated and modulated the ability of p27 to inhibit cyclin D-CDK4 complexes (James et al., 2008). p27 was a potent cyclin D-CDK4 inhibitor in quiescent cells, but not in cycling cells, in which it was tyrosine phosphorylated. Moreover, the weak inhibitory form of p27, phosphorylated on Tyr-88, could be converted to a potent inhibitor by treatment with protein tyrosine phosphatase (PTP) (James et al., 2008). However, the physiological significance of this regulatory pathway of p27 in vivo remains to be investigated. Several other phospho-sites on p21 and p27 also indirectly affect the ability of these proteins to bind to and inhibit cyclin-CDK complexes by controlling their subcellular localization (see below) (Borriello et al., 2007; Child and Mann, 2006).

A number of proteins can either enhance or diminish the inhibitory effect of CKIs on cyclin-CDK complexes by forming quaternary complexes and potentially altering the conformation of the CKI bound to these complexes. Human papillomavirus-16 E7 protein can bind to p21 and p27 and abrogate their inhibitory activity toward CDK2-containing complexes (Funk et al., 1997; Jones et al., 1997). The nuclear protein Set/TAF1 (Template-activating factor-1) was found to associate with the C-terminal part of p21, which reversed the inhibition of cyclin E-CDK2 and enhanced the inhibition of cyclin B-CDK1 complexes (Canela et al., 2003; Estanyol et al., 1999). Two other proteins, TOK1α (p21 and CDK-associated protein-1) (Ono et al., 2000) and the multifunctional domain protein TSG101 (Tumor susceptibility gene-101) (Oh et al., 2002), can also associate with p21 to enhance cyclin-CDK inhibition. Further studies are warranted to elucidate how the binding of these proteins may alter the conformation of the CKI bound to cyclin-CDK complexes and to determine how significant these interactions are in the control of p21's function. The general conclusion is that Cip/Kip proteins are potent inhibitors of cyclin-CDK complexes, although most of the findings in recent years have shown that their inhibitory potential is dependent on cellular context and regulated via phosphorylations and protein-protein interactions.

Cip/Kip proteins also modulate cell-cycle progression independently of cyclins and CDKs via the inhibition of components of the replication machinery. p21 was first reported to bind to proliferating cell nuclear antigen (PCNA), a DNA polymerase δ processivity factor, via its C terminus (aa 143–160), thereby blocking processive DNA synthesis (Luo et al., 1995). This function of p21 is modulated by phosphorylations on Ser-145 (by PKB/Akt [protein kinase B] and possibly PKA), Ser-146 (by PKC ζ), or Ser-160 (by PKC), which prevent p21 from binding to PCNA (Child and Mann, 2006). p57 was subsequently found to interact with PCNA (via aa 271–275), preventing its activity and blocking DNA replication (Watanabe et al., 1998). Although no interaction between p27 and PCNA has been reported, p27 may also inhibit DNA synthesis via the interaction and inhibition of minichromosome maintenance-7 (MCM7), a subunit of the MCM2-7 replication fork helicase, an activity that lies within the C-terminal part of p27 (aa 144–198) (Nallamshetty et al., 2005).

Regulation of p21 and p27 Localization by Phosphorylations

Phosphorylation on various amino acids controls many aspects of Cip/Kip protein biology, not only by altering the Cip/Kip proteins' affinity for specific cyclin-CDK complexes and other proteins, but also their stability (reviewed in Borriello et al., 2007; Child and Mann, 2006), and their subcellular localization. Phosphorylation of p21 on two sites, Thr-145 and Ser-153, by PKB/Akt and PKC, respectively, promote the cytoplasmic retention of p21 (Child and Mann, 2006; Rodriguez-Vilarrupla et al., 2005; Zhou et al., 2001). The first site is proximal to p21's nuclear

localization sequence (NLS) and prevents the interaction with importin when phosphorylated, and the second one occludes a binding site for calmodulin, thus blocking calmodulin-mediated nuclear import (Child and Mann, 2006; Rodriguez-Vilarrupla et al., 2005; Zhou et al., 2001). Three phosphorylation sites on p27 were found to cause cytoplasmic localization. Ser-10 phosphorylation, which stabilizes the protein in quiescent cells, causes its export from the nucleus in G1 phase by providing a binding site for CRM1/exportin1 (Besson et al., 2006; Connor et al., 2003; Rodier et al., 2001). In quiescent cells, Mirk/Dirk kinase has been proposed to phosphorylate this site, whereas in proliferating cells, there are reports implicating kinase interacting with stathmin (KIS), PKB/Akt, and extracellular signal-regulated kinase-2 (ERK2) as the physiologically relevant in vivo kinases (Besson et al., 2004a). On the other hand, phosphorylation of Thr-157 (a site not conserved in the mouse protein) by PKB/Akt or Thr-198 by PKB/Akt or p90 ribosomal S6-kinase ($p90^{RSK}$) causes retention of p27 in the cytoplasm by promoting the association of p27 with 14-3-3, which prevents p27 from interacting with importin α and transport in the nucleus (Fujita et al., 2003; Liang et al., 2002; Sekimoto et al., 2004). The extent to which altered subcellular localization modulates the many biological effects of Kip/Cip proteins is an area of considerable interest and active investigation.

Cip/Kip Proteins, Tumor Suppressors,...and Oncogenes?

There is a great deal of evidence, both from clinical studies and animal models, to support the tumor-suppressor function of p21, p27, and p57. However, the full extent to which p27, and probably also p21, may contribute to tumorigenesis has been underestimated by the study of mouse models that treated these CKIs solely as CDK inhibitors. Only very recently has the development of a new mouse model allowed us to ask whether CDK-independent functions of p27 need to be considered in order to understand the biological effects of p27. Those studies revealed that the CDK-independent functions of p27 play significant roles, not only in normal development, but also during tumorigenesis.

The $p27^{-/-}$ mice, which spontaneously develop adenomas of the intermediate lobe of the pituitary gland and are more susceptible to tumorigenesis induced by chemical carcinogens or irradiation, gave firm evidence for a tumor suppressor role of p27 (Fero et al., 1996, 1998; Kiyokawa et al., 1996; Nakayama et al., 1996). In fact, the loss of one allele of *cdkn1b* is sufficient to predispose to induced tumorigenesis (Fero et al., 1998). Moreover, the tumor-prone phenotypes caused by the loss of other tumor suppressor genes, such as Rb (Retinoblastoma), PTEN (phosphatase and tensin homolog deleted on chromosome ten), $p16^{INK4a}$, $p18^{INK4c}$, or APC (adenomatous poliposis coli), are enhanced by the simultaneous loss of p27 (Di Cristofano et al., 2001; Franklin et al., 2000; Malumbres et al., 2000; Martin-Caballero et al., 2004; Park et al., 1999). In rats, an inherited syndrome of multiple endocrine neoplasia (MEN) was linked to a germline nonsense mutation in the *cdkn1b* gene, and germline mutations in the human *cdkn1b* gene are also associated with MEN (Georgitsi et al., 2007; Pellegata et al., 2006). p27 expression is a prognostic marker for clinical outcome in human cancers. Low amounts of nuclear p27 protein are frequently observed in a broad array of human malignancies, including carcinomas of the breast, colon, prostate, ovary, lung, brain, stomach, and others, and this is associated with increased tumor aggressiveness (Besson et al., 2004a; Blain et al., 2003; Slingerland and Pagano, 2000). However, unlike the classic tumor suppressor genes *Rb* or *p53*, inactivating mutations of the *cdkn1b* gene in tumors are extremely rare (Blain et al., 2003; Fero et al., 1998; Ponce-Castaneda et al., 1995; Slingerland and Pagano, 2000). Instead, p27 is downregulated by other mechanisms, including proteolytic degradation, decreased transcription, or by cytoplasmic mislocalization. In addition, the inhibition of p27 expression by miRNAs in glioblastomas and prostate carcinoma cell lines was recently described, and this might constitute yet another way to decrease p27 levels in tumors (Galardi et al., 2007; le Sage et al., 2007).

Increasing evidence points to the importance of subcellular localization in the control of p27's function and raises the possibility that cytoplasmic p27 may actively contribute to tumorigenesis (Besson et al., 2004a). This is supported by the fact that elevated cytoplasmic localization of p27 is a negative prognostic factor in subsets of certain tumor types, including carcinomas of the breast, cervix, esophagus, ovary, uterus, some leukemias and lymphomas, and in melanomas (Besson et al., 2004a; Blain et al., 2003; Denicourt et al., 2007; Qi et al., 2006; Rosen et al., 2005; Slingerland and Pagano, 2000). In contrast, mice expressing $p27^{S10A}$, which is mostly nuclear, were partially resistant to urethane-induced tumorigenesis despite reduced p27 protein levels (Besson et al., 2006).

An active role for p27 in promoting tumor formation is also supported by several studies in mice. Indeed, in mammary and prostate cancer models, $p27^{+/-}$ mice were more prone to tumorigenesis than $p27^{-/-}$ animals, suggesting an active contribution of the remaining p27 allele during tumor development (Gao et al., 2004; Muraoka et al., 2002). Moreover, a knockin mouse model in which point mutations in p27 prevent its interaction with cyclins and CDKs ($p27^{CK-}$) revealed that in contrast to $p27^{-/-}$ mice, which spontaneously develop only pituitary tumors, $p27^{CK-}$ mice developed hyperplastic lesions and tumors in multiple organs, including the lung, retina, pituitary, ovary, adrenals, spleen, and lymphomas (Besson et al., 2007). Although the mouse models provide a strong evidence for an oncogenic effect of p27, they did not address whether that effect was exerted in the nucleus or cytoplasm, which should be the focus of future studies. Overall, there are clear indications, both from clinical studies and animal models, that p27 has a tumor suppressor function which is exerted in the nucleus via its inhibitory interactions with cyclin-CDK enzymes. Collectively, the data also suggest that p27 can promote oncogenesis and this occurs independently of its interaction with cyclins and CDKs, most likely in the cell cytoplasm.

In the lung and retina of $p27^{CK-}$ mice, the development of tumors was associated with the expansion of stem/progenitor cell populations (Besson et al., 2007). Thus, this mouse model indicates that p27, independently of its role as a CDK inhibitor, could function as an oncogene in vivo, possibly by deregulating the proliferation and/or differentiation of stem/progenitor cells. Therefore, a reasonable hypothesis to account for the lack of mutations in the *cdkn1b* gene in human cancers is that there is a selection both for the loss of the CDK-inhibitory, tumor-suppressive function of p27 in the nucleus and for the maintenance

of the cyclin-CDK-independent, oncogenic functions of p27. The misregulation of stem/progenitor cells by p27 could constitute an important pathway by which it promotes tumorigenesis. The CDK-independent functions of p27 are just beginning to be studied, and the mechanism by which they can promote tumorigenesis and stem-cell amplification remains to be evaluated. In particular, whether the participation of p27 in tumor initiation and progression relies on single or multiple distinct functions of the protein remain unknown.

The evidence supporting a tumor suppressor role for p21 is not as strong, and the many reports offer a contrasting view on p21's role during tumorigenesis. Although $p21^{-/-}$ mice were initially described to remain tumor-free until 7 months of age, a subsequent study found that they were susceptible to spontaneous tumorigenesis at an average age of 16 months and developed mostly histiocytic sarcomas, hemangiomas, and lymphomas (Deng et al., 1995; Martin-Caballero et al., 2001). Also supporting a tumor suppressor role for p21, the tumor-prone phenotype caused by the loss of $p18^{INK4c}$ or APC was enhanced by concomitant loss of p21 (Malumbres et al., 2000). On the other hand, loss of p21 actually delayed tumor development in $ATM^{-/-}$, $p53^{-/-}$, or irradiated wild-type mice; this was attributed to increased apoptosis of tumor cells in the absence of p21 (De la Cueva et al., 2006; Martin-Caballero et al., 2001; Wang et al., 1997). Likewise, PDGF-induced gliomagenesis was dramatically reduced in mice lacking p21 (Liu et al., 2007). The tumor-promoting effect in this model required the cyclin-binding domain of p21 to increase the amount of nuclear cyclin D1 (Liu et al., 2007), providing a clear example of tumorigenic role for p21 dependent on its cyclin-CDK regulatory function. Indeed, binding of p21 to cyclin D1 prevents its export from the nucleus and its subsequent degradation in the cytoplasm (Alt et al., 2002). Therefore, as with p27, the study of the p21-null mouse may have led to a misunderstanding of the complex role that p21 has in tumorigenesis, and, by analogy with p27, the construction of p21 separation-of-function mutations is likely to offer significant new insights into the roles of p21 in tumors.

Indeed, clinical studies analyzing human tumors have also come to conflicting conclusions. Like p27, inactivating mutations in the *cdkn1a* gene are exceedingly rare (Roninson, 2002). While loss of p21 is a negative prognostic marker in some cancer types, it appears that overexpression or cytoplasmic localization of p21 is a marker of poor prognosis and aggressive tumors in carcinomas of the pancreas, breast, prostate, ovary, cervix, and in glioblastomas (Biankin et al., 2001; Roninson, 2002). Thus, it appears likely that similar to p27, exclusion of p21 from the nucleus results in the loss of its tumor-suppressor function while selectively maintaining other tumor-promoting functions.

There is ample evidence indicating a tumor-suppressor role for p57, and, unlike its siblings, no oncogenic activity has been reported for p57 so far. In human tumors, p57 inactivation occurs through several mechanisms, including maternal-specific loss of heterozygosity (LOH); loss of imprinting; promoter methylation in carcinomas of the lung, gastrointestinal tract, liver, pancreas, breast, head, and neck; acute myeloid leukemia; and others (Higashimoto et al., 2006; Kikuchi et al., 2002; Kobatake et al., 2004; Kondo et al., 1996; Lai et al., 2000). The perinatal lethality of mice lacking p57 has limited further investigations to determine the significance of p57 as a tumor suppressor in the adult in vivo, and conditional p57 mutant animals have not yet been constructed. However, in one study in which approximately 10% of p57-null animals survived to adulthood, no spontaneous tumors were observed at the age of 5 months (Yan et al., 1997).

It is not known whether the tumor-suppressor function of p57 relies solely on its cyclin-CDK inhibitory activity or if the additional domains unique to p57 (PAPA repeats and QT domain in human p57), whose functions remain unclear, also participate. C*dkn1c* (encoding p57), a maternally expressed gene, resides within an imprinted gene cluster on chromosome 11p15 that has been implicated in the development of Beckwith-Wiedemann syndrome (BWS). BWS is a heterogeneous overgrowth syndrome associated with various developmental defects and increased risk of embryonal tumor development (Weksberg et al., 2005). *Cdkn1c* mutations are found in 5%–10% of sporadic BWS cases and approximately 40% of inherited BWS cases (Weksberg et al., 2005). In addition, maternal-specific methylation of a chromatin insulator, KvDMR1, is lost in 30%–50% of BWS cases, causing aberrant silencing of *cdkn1c* (Fitzpatrick et al., 2002; Higashimoto et al., 2006). Interestingly, a fraction of BWS patients exhibit point mutations rather than complete deletion or silencing of the p57 gene, and the majority of these mutations are located outside of the cyclin-CDK regulatory region in the C-terminal part of the protein (Bhuiyan et al., 1999; Hatada et al., 1997; Lee et al., 1997; O'Keefe et al., 1997). These support the hypothesis that the role of p57 in tumorigenesis may extend beyond cyclin-CDK regulation.

Cip/Kip Proteins and Apoptosis

CKIs can modulate apoptosis in various ways, depending on the cellular context and the pathway they target (Figure 1). Numerous reports have suggested proapoptotic roles for p21 or p27 by overexpression in cancer cell lines or in response to anticancer agents; however, in these studies, p21 was overexpressed using adenoviral vectors, and their relevance to physiological conditions is uncertain. One way by which Cip/Kip proteins protect against apoptosis is via their CDK inhibitory activity. This was first shown in endothelial cells, where caspase-mediated cleavage of p21 and p27 upregulates CDK2 activity, thereby enhancing the apoptotic program (Levkau et al., 1998). Indeed, both dominant-negative CDK2 and a caspase-resistant mutant of p21 suppressed apoptosis in this model. However, most of the studies investigating a role for Cip/Kip proteins in apoptosis did not test whether this was linked to CDK regulation, and future experiments should aim at exploring how exactly the CKIs are working in this process. There are also numerous in vivo models of apoptosis being increased in CKI-deficient mice, but again whether these phenotypes were CDK mediated was not determined. For example, several of the phenotypes of the p57-null mice were shown to be a consequence of increased apoptosis attributed to a failure to exit the cell cycle and differentiate (Yan et al., 1997; Zhang et al., 1997). Likewise, p27 has been reported to play a protective role in safeguarding normal tissues from excessive apoptosis during inflammatory injury (Ophascharoensuk et al., 1998). This ability of CKIs to limit or prevent apoptosis is also particularly relevant in cancer therapies, and induction of p21 (by p53) and p27 have been associated with resistance to apoptosis induced by cytotoxic drugs or irradiation

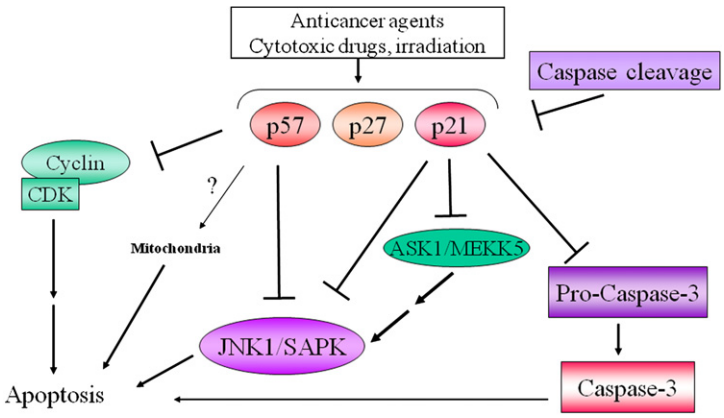

Figure 1. Cip/Kip Proteins and Apoptosis
Cip/Kip proteins can inhibit apoptosis indirectly via the inhibition of cyclin-CDK complexes, and p21 and p27 are targeted for cleavage by caspases to promote cyclin-CDK activation during the apoptotic process. Upregulation of the CKIs by cytotoxic agents can participate in the resistance of tumor cells to anticancer treatments. p21 and p57 may also directly prevent the induction of apoptosis by interfering with activation of the stress-signaling pathways; for instance, both bind to and inhibit the activity of JNK1/SAPK, and p21 can also inhibit ASK1/MEKK5. p21 may also block the processing of pro-caspase-3 into its active form.

(De la Cueva et al., 2006; Eymin and Brambilla, 2004; Martin-Caballero et al., 2001; St Croix et al., 1996; Wang et al., 1997).

The impact of CKIs on apoptosis may not be explained entirely by CDK regulation. For instance, p57 could either promote or inhibit apoptosis. In both cases, this was independent of its role in cyclin-CDK regulation: p57 and a mutant lacking the ability to interact with cyclins and CDKs were equally able to promote staurosporine-induced apoptosis, which involved the translocation of p57 to mitochondria (Vlachos et al., 2007). On the other hand, p57 was found to bind via its QT domain (aa 238–316) to the stress-activated kinase JNK1/SAPK, inhibiting its kinase activity, and expression of the QT domain was sufficient to block UV- or MEKK1-induced apoptosis, mediated by JNK1 (Chang et al., 2003). Substantial evidence points to a role for p21 in protecting against apoptosis, via cell-cycle inhibition, transcriptional regulation, and protein-protein interactions; this constitutes the best-characterized oncogenic function of the protein (Roninson, 2002). p21 could block Fas-mediated cell death by directly binding via its N terminus to pro-caspase-3, which prevented its conversion to the active caspase form (Suzuki et al., 1998). p21 can prevent stress-induced apoptosis mediated by the JNK and p38 signaling pathways by acting at two distinct levels. First, p21 binds to and inhibits the activity of the MAPKKK ASK1/MEKK5 (Huang et al., 2003). Second, like p57, p21 can bind to JNK kinases through its cyclin-CDK binding domain, which both inhibits JNK activity and prevents JNK activation by upstream kinases (Shim et al., 1996). So far, there have not been any reports to suggest that p27 can directly interfere with pro- or antiapoptotic pathways.

Cip/Kip Proteins in Transcriptional Regulation

Similar to the regulation of apoptosis, Cip/Kip proteins can repress transcription indirectly by inhibiting cyclin-CDK complexes, in turn preventing the phosphorylation of Rb-family proteins (p107, p110, and p130). In their hypophosphorylated state, Rb-related proteins sequester E2F family members, thereby repressing their transcriptional targets (Sherr and Roberts, 1999). In addition, Cip/Kip proteins can also modulate the activity of transcription factors through direct binding to transcription factors (Figure 2). The N-terminal cyclin-CDK binding region of p57 can interact with MyoD, protecting MyoD from degradation and thus promoting transactivation of muscle-specific genes (Reynaud et al., 2000). Interestingly, a similar mechanism is observed between p27 and Neurogenin-2, and p27-mediated stabilization of Neurogenin-2 promotes the differentiation of neuronal progenitors in the cortex (Nguyen et al., 2006). This interaction is evolutionarily conserved, since in *Xenopus* primary neurogenesis also relies on the stabilization of X-NGNR1, a Neurogenin-2 homolog, by $p27^{Xic}$ (Vernon et al., 2003).

p21 is also a potent regulator of several transcription factors. Through direct binding, p21 inhibits the activities of E2F1, c-Myc, and STAT3 (Coqueret and Gascan, 2000; Delavaine and La Thangue, 1999; Kitaura et al., 2000). On the other hand, p21 can stimulate p300/Creb-binding protein (CBP) histone acetyl transferase complex-mediated transcriptional activation (Snowden et al., 2000). Through direct binding to p300, p21 can disrupt the activity of the CRD1-transcriptional-repression domain of p300 and derepress transcription of target genes (Snowden et al., 2000). The biological significance of the role of p21 in transcriptional regulation is still poorly understood. For example,

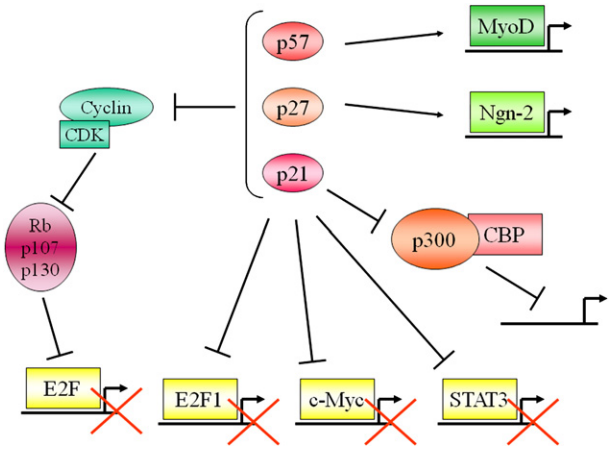

Figure 2. Transcriptional Regulation by Cip/Kip Proteins
The CKIs p21, p27, and p57 can repress E2F-mediated transcription indirectly via the inhibition of cyclin-CDK complexes, thereby maintaining Rb-family proteins (Rb/p110, p107, and p130) in a hypophosphorylated state in which they sequester E2F transcription factors. Cip/Kip proteins also regulate transcriptions factors directly; for instance, p57 and p27 can interact via their N termini with MyoD and Neurogenin-2 (Ngn-2), respectively, stabilizing them and promoting transcription of their target genes. On the other hand, p21 binds to E2F1, c-Myc, and STAT3 to inhibit their activities. p21 may also derepress p300/CBP targets by inhibiting the transcriptional repression domain of p300.

p21, by associating with E2F1, mediates the transcriptional repression of Wnt genes downstream of Notch to regulates keratinocyte fate and growth (Devgan et al., 2005). One could envision that in tumors in which p21 is highly expressed, this function could play a significant role in regulating tumor progression. Some indications suggest that p21 participates in the transcriptional changes taking place in cells that undergo DNA-damage-induced senescence following treatment with chemotherapeutic agents, and these cells secrete a number of factors that promote growth and survival of surrounding tumor cells (Roninson, 2003).

Cip/Kip Proteins in the Regulation of Rho Signaling and Cytoskeletal Dynamics

Studies in yeast first suggested a direct functional link between CDK inhibitors and regulators of cytoskeletal organization. During vegetative growth, the CDK inhibitor Far1 binds to and sequesters Cdc24, a guanine-nucleotide exchange factor (GEF) for Cdc42, in the nucleus. Activation of Cln/Cdc28 in the G1 phase leads to phosphorylation and degradation of Far1, thereby releasing Cdc24, which is then exported to the bud site on the plasma membrane where it regulates cytoskeletal organization and cell polarity (Shimada et al., 2000). On the other hand, during mating, Far1 and Cdc24 are exported from the nucleus as a complex, and Far1 is required for the recruitment of Cdc24 to sites of $G_{\beta\gamma}$ activation at the cell cortex for polarized growth in response to pheromones (Butty et al., 1998; Shimada et al., 2000).

In mammals, the Rho family of GTPases, composed of at least 20 members, regulates multiple signaling pathways with pleiotropic cellular responses. Rho and its effector Rho-kinase (ROCK) are best known for their role in the regulation of actin-stress-fiber formation, focal-adhesion assembly, as well as actin-myosin contractility (Etienne-Manneville and Hall, 2002). Stress-fiber assembly is mediated by the activation of LIM-kinase (LIMK) by ROCK, which in turn phosphorylates and inactivates the actin-depolymerization factor cofilin to promote stress-fiber formation. On the other hand, Rac and its effector p21-activated kinase (PAK) induce actin rearrangements that control the formation of lamellipodia and new focal contacts at the cell edges (Ridley et al., 2003). Cell migration requires a tight balance of Rho and Rac activities, and the dynamic activation and inhibition of both GTPases are spatially and temporally coordinated. Indeed, insufficient levels of Rho-GTP will inhibit migration by preventing cells from achieving the level of adhesiveness and traction needed for movement (Nobes and Hall, 1999; Worthylake et al., 2001). Conversely, too much Rho activity increases adhesion and prevents focal adhesion turnover, resulting in the inhibition of cell migration (Ren et al., 2000; Sahai et al., 2001; Vial et al., 2003).

In a remarkable example of convergent evolution, CKI-mediated regulation of cytoskeletal dynamics arose independently in metazoans. Thus, all three Cip/Kip proteins inhibit the Rho/ROCK/LIMK/Cofilin signaling pathway, albeit acting at distinct levels (Figure 3) (Besson et al., 2004a). Interestingly, Rho can also negatively regulate the levels of the p27 and p21 proteins (Hu et al., 1999; Olson et al., 1998; Vidal et al., 2002). Thus, Rho and p21 and p27 could participate in a negative-feedback loop. The network of interactions between CDK inhibitors and the Rho signaling pathway could very well represent a basic mechanism to coordinate cytoskeletal functions with cell division.

p57 was found to interact with LIMK1 without inhibiting its activity, and overexpression of p57 resulted in nuclear localization of LIMK1 (Yokoo et al., 2003). This was accompanied by loss of LIMK1-associated stress fibers, suggesting that the p57-LIMK1 interaction inhibits LIMK1 by sequestering it in the nucleus. In another report, knockdown of p57 delayed the migration of neurons in the cortical plate during mouse development; however, whether this resulted from the sequestration of LIMK1 was not investigated (Itoh et al., 2007).

Cytoplasmic p21 has been shown to bind and inhibit the Rho-kinase ROCK1 (Lee and Helfman, 2003; Tanaka et al., 2002). p21-mediated ROCK1 inhibition promoted neurite extension of neuroblastoma cells and hyppocampal neurons in vitro (Tanaka et al., 2002). Moreover, in rat, the transduction of a TAT-p21 fusion protein at the site of spinal-cord injury promoted axonal regeneration and improved recovery of the animals' motor function (Tanaka et al., 2004).

p21 inhibition of ROCK may also be selected for during tumor progression to enhance cell motility. Ras-transformed cells are highly motile and invasive, despite having high levels of Rho activity. In fact, these cells have found ways to uncouple the effector pathways downstream of Rho. For instance, Ras-induced ERK signaling uncouples Rho from inducing actin stress fibers and focal adhesions by downregulating ROCK expression (Sahai et al., 2001). Alternatively, Ras activation can induce p21 expression via ERK and its cytoplasmic localization via PI-3K activation. Once in the cytoplasm, p21 inhibits ROCK activity, thereby uncoupling Rho from stress-fiber formation (Lee and Helfman, 2003). Further studies of tumor cells with high levels of cytoplasmic p21 are needed to directly confirm that p21 can participate in the acquisition of a motile and invasive phenotype.

The involvement of p27 in the regulation of migration was first reported in hepatocellular carcinoma cells, in which transduction of a TAT-p27 protein induced migration (Nagahara et al., 1998). Moreover, a recent report has shown that expression of even subphysiological levels of cytoplasmic p27 in melanoma cells results in a profound increase in metastatic potential in vivo (Denicourt et al., 2007). In these cells, both the export of p27 to the cytoplasm through its phosphorylation on Ser-10 and a region in the C-terminal half of p27 were required for hepatocyte-growth-factor-induced migration (McAllister et al., 2003). Further analysis showed that the motility of p27-null mouse fibroblasts was impaired due to an increase in RhoA activity and that normal migration could be restored by inhibiting ROCK (Besson et al., 2004b). Indeed, $p27^{-/-}$ cells had increased numbers of stress fibers and focal adhesions and elevated levels of Rho-GTP (Besson et al., 2004b). Overexpression experiments revealed that p27 interacted with RhoA, thereby preventing RhoA activation by interfering with RhoA binding to its GEFs (Besson et al., 2004b). The regulation of RhoA by p27 was critical for proper migration of neuronal progenitor cells in the subventricular cortex of developing mice (Kawauchi et al., 2006; Nguyen et al., 2006). Thus, CKIs may be crucial for proper neurogenesis, given that neuronal migration in the cerebral cortex is regulated by p27 in the subventricular zone and by p57 in the cortical plate.

These results indicate that p27 regulates cell migration by preventing Rho activation. Different migratory responses, either

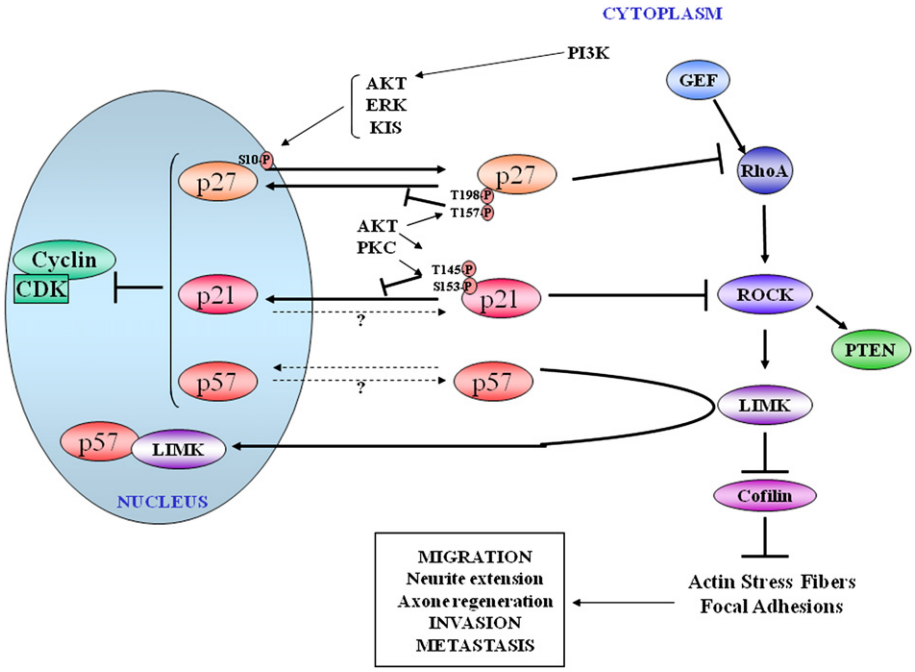

Figure 3. Regulation of Rho Signaling and Cytoskeletal Dynamics by Cip/Kip Proteins

In the nucleus, Cip/Kip proteins primarily function to restrict the activities of cyclin-CDK complexes. Phosphorylation of p27 on Ser-10 promotes its binding to the exportin CRM1 and nuclear export. On the other hand, phosphorylations on T157 (by Akt) or T198 (by Akt or $p90^{Rsk}$) promote binding to 14-3-3 proteins and prevent the reentry of p27 in the nucleus. In the cytosol, p27 can bind to RhoA, preventing its activation by its GEFs (guanine-nucleotide exchange factors), leading to decreased actin stress fiber and focal-adhesion formation and resulting in several cell types in increased migration, invasion, and metastasis. PI3K-AKT induction of cytoplasmic localization of p27 is also involved in the inhibition of PTEN activation via p27-mediated inhibition of the RhoA-ROCK pathway. p21 cytoplasmic localization is induced by phosphorylation on T145 and S153 by Akt and PKC, respectively; however, how p21 is exported from the nucleus is unclear. Cytoplasmic p21 can bind to ROCK, inhibiting its kinase activity, resulting in decreased actin stress fibers formation. The mechanism of nucleo-cytoplasmic shuttling of p57 is unknown at this point. Cytoplasmic p57 can bind to LIMK and induce its translocation into the nucleus, resulting in loss of actin stress fibers.

inhibition or promotion, have been observed in various cell types by manipulating p27 levels (Besson et al., 2004a). It is therefore possible that the effect of RhoA inhibition by p27 could vary, depending on the cellular context, and reflect distinct requirements for Rho and Rac for migration. For instance, cells that migrate with an elongated morphology require Rac activity, whereas cells migrating with a rounded morphology depend on Rho/ROCK signals (Sahai and Marshall, 2003). In breast cancer and glioblastoma cell lines, cytoplasmic p27-mediated inhibition of RhoA activity promoted motility and invasion, and knockdown of p27 impaired tumorigenicity and invasiveness of tumor xenografts in mice (Wu et al., 2006). Recently, cyclin D1 was found to promote cell motility in a p27-dependent manner (Li et al., 2006). Cyclin D1 is overexpressed is several types of cancers in human, and this correlates with tumor metastasis in some tumors. Thus, it is possible that sequestration of p27 in cyclin D1-CDK4/6 complexes could both inactivate the cell-cycle inhibitory function of p27 while promoting its role in cell migration.

While most studies have focused on the effect of p27 inhibition of RhoA on migration, a new study suggests that p27 could be more widely involved in the modulation of the pleiotropic effects of RhoA signals. Indeed, RhoA was recently found to positively regulate PTEN, the lipid phosphatase best known to counteract phosphatidyl inositol 3-kinase (PI 3-K) activation. It turns out that PI 3-K can negatively regulate PTEN in part via a pathway involving PKB/Akt-induced cytoplasmic localization of p27, which inhibits RhoA (Papakonstanti et al., 2007). Thus, cytoplasmic p27 could participate in the inactivation of the tumor suppressor PTEN. p27 inhibits RhoA activation by preventing the RhoA/GEF interaction. However, p27 did not interfere equally with the activity of all GEFs tested, suggesting that p27 may only inhibit RhoA activation by select upstream pathways (Besson et al., 2004b; A.B. and J.M.R., unpublished data).

Although the fact that Cip/Kip proteins can affect both the cell-division cycle or cytoskeletal structure may represent functions that are executed independently of one another, a more interesting hypothesis is that this organization underlies a pathway that serves to coordinately regulate both processes. Indeed, it is common to observe an alternation between episodes of cell movement and periods of cell proliferation. Some examples include neural-crest cell migration, which occurs without concomitant cell division (Perris, 1997); wound healing, during which keratinocytes first migrate into the wound bed before beginning to proliferate (Martin, 1997); and gastrulation, during which time cell division is suppressed by a prolongation of the cell cycle (Nance and Priess, 2002). Cell migration and cell division are often activated by the same upstream signaling pathways (Besson et al., 2004a; Collins et al., 1999; Frey et al., 2004), but the mechanisms that determine whether a cell chooses to migrate or divide are not well understood. That CKIs directly modulate both processes and their regulation by mitogenic stimuli suggests that they may also regulate the choice between cell movement and cell proliferation. For instance, high levels of p27 would inhibit cell proliferation and promote cell migration and,

conversely, low levels of p27 would stimulate proliferation and inhibit cell movement. Misregulation of p27 in tumors may result in the simultaneous promotion of both cell proliferation and cell migration and therefore contributes to the invasive phenotype of aggressive cancers.

Perspectives

Over the years, it has become evident that CKIs of the Cip/Kip family are not merely involved in limiting cell division but are multifunctional proteins playing key roles in the regulation of the cell cycle, cell survival, transcription, differentiation, cytoskeletal dynamics, cell migration, and probably other, as yet undiscovered, functions. The importance of the cyclin-CDK-independent functions of these CKIs is just beginning to be grasped, and much work remains to be done before we have a clear understanding of the regulation and interplay between the various roles of these proteins. For example, why some terminally differentiated cells, like neurons, express cyclins, CDKs, and their inhibitors remains a mystery. An attractive hypothesis is that CKIs may act as molecular switches to coordinate cell proliferation with the other cellular processes they govern.

From the perspective of cancer biology, CKIs play a dual role during tumorigenesis, acting as both tumor suppressors and oncogenes. Indeed, in tumors the dysregulation of various signaling pathways, such as PKB/Akt, inactivates the tumor-suppressor functions of these proteins but maintains or even exacerbates the oncogenic ones. Thus, for therapeutic purposes, simply increasing the expression of these CKIs may not be beneficial and could have consequences opposite to those intended. A major challenge is now to gain the knowledge that will permit the specific targeting the oncogenic functions of CKIs while maintaining or restoring the tumor suppressive functions.

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