PERSPECTIVE

Cip/Kip proteins: more than just CDKs inhibitors

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Members of the Cip/Kip family of cyclin-dependent kinases inhibitors (CKIs) are well characterized for their role as negative regulators of G₁-phase cell-cycle progression (Sherr and Roberts 1999). In eukaryotic cells, progression through the cell cycle is governed by a suite of cyclins and cyclin-dependent kinase (CDKs) complexes (Murray 2004). Regulation of cyclin-CDKs complexes occurs at multiple levels, including assembly of cyclin and CDK subunits, inhibitory and activating phosphorylation and dephosphorylation events, and association of cyclin-CDK complexes with CKIs. During these regulatory processes, cyclin-CDK complexes positively drive progression of the cell cycle, whereas by binding to and inactivating cyclin-CDKs, CKIs negatively regulate progression through the cell cycle. Based on their sequence homology and specificity of action, CKIs are divided into two distinct families: INK4 and Cip/Kip (Sherr and Roberts 1999). Members of the INK4 family, namely p15, p16, p18, and p19 specifically inhibit the activity of CDK4 and CDK6, whereas Cip/Kip members, that is, p21, p27, and p57 inhibit a broader spectrum of cyclin-CDK complexes (el-Deiry et al. 1993; Gu et al. 1993; Harper et al. 1993; Polyak et al. 1994; Toyoshima and Hunter 1994; Lee et al. 1995).

Until recently, Cip/Kip members were almost solely viewed as nuclear proteins with a principal function of inhibiting cyclin-CDK activity and hence, cell-cycle progression. However, emerging studies now suggest that Cip/Kip proteins play additional roles outside of the nucleus (Coqueret 2003). Indeed, previous reports have linked p27^{kip1} to the regulation of actin dynamics and cell migration (Nagahara et al. 1998; McAllister et al. 2003). Moreover, several recent papers have shown that p21^{cip1} can act as a Rho-kinase (ROCK) inhibitor and that p57^{kip2} modulates subcellular localization of LIMK, a serine/threonine kinase involved in the regulation of actin filaments (Tanaka et al. 2002; Yokoo et al. 2003; Lee and Helfman 2004). In this issue of *Genes & Development*, a report by Besson et al. (2004) shows that cytoplasmic p27^{kip1} modulates actin dynamics by direct regulation of the small GTPase RhoA pathway.

p27^{kip1} was originally identified as an inhibitor of cyclin-CDK complexes in cells arrested by transforming growth factor-beta (TGF-β; Polyak et al. 1994; Slingerland et al. 1994). Forced expression of p27^{kip1} results in a G₁ phase cell-cycle arrest in most cell types by associating with complexes of cyclins D1-D3:CDK4 or CDK6 and cyclin E or cyclin A:CDK2 (Polyak et al. 1994; Toyoshima and Hunter 1994). Growth arrest by contact inhibition is also thought to be mediated in part by p27^{kip1} (Polyak et al. 1994). In normal cells, p27^{kip1} protein levels are highest during G₀ and early G₁ phases, then rapidly decline in late G₁ and S phases (Nourse et al. 1994; Reynisdottir et al. 1995). p27^{kip1} abundance is primarily controlled by phosphorylation-regulated ubiquitin-mediated proteolysis (Pagano et al. 1995). Phosphorylation of p27^{kip1} on Thr-187 by CDK2 allows for SCF^{Skp2} ubiquitin-protein ligase recognition and subsequent degradation (Carrano et al. 1999; Sutterluty et al. 1999; Tsvetkov et al. 1999). p27^{kip1} harbors its cyclin-CDK inhibition and binding in an N-terminal domain that contacts both the cyclin and the CDK subunits (Russo et al. 1996). Importantly, to exert its CDK inhibitory functions, p27^{kip1} is required to be localized in the nucleus. p27^{kip1} contains a phosphorylation-regulated nuclear localization signal (NLS) harbored in the C terminus (Zeng et al. 2000). Transport of p27^{kip1} between the nucleus and the cytoplasm is also regulated by phosphorylation events. Upon mitogen stimulation, the human kinase interacting stathmin (hKIS) phosphorylates p27^{kip1} on Ser 10 to signal its nuclear export into the cytoplasm (Ishida et al. 2000; Rodier et al. 2001; Boehm et al. 2002). Furthermore, phosphorylation of p27^{kip1} on the NLS Thr-157 by AKT disables its nuclear localization capacity (Liang et al. 2002; Shin et al. 2002; Viglietto et al. 2002). Thus, nuclear binding of cyclin-CDK complexes appears to account for most or all of the previously associated properties of p27^{kip1} in negative regulation of cell-cycle progression.

However, emerging data have suggested a role for cytoplasmic p27^{kip1} in the regulation of cell migration independent of cyclin-CDK inhibition. Nagahara et al. (1998) first demonstrated that delivery of a transducible form of p27^{kip1} (TAT-p27^{kip1}) into HepG2 hepatocellular carcinoma cells induced cell migration or scattering to a similar extent as HGF. Subsequent work found that

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1205304>.

p27^{kip1}-deficient fibroblasts were impaired for cell motility and that reconstitution of p27^{kip1} protein rescued the cell migration defect (McAllister et al. 2003). The ability of p27^{kip1} to induce cell migration resides in a C-terminal "scatter domain" that has a loose sequence similarity with the yeast cell-cycle inhibitor homolog Far1p, which is also involved in actin rearrangement (McAllister et al. 2003). Loss or disruption of the C-terminal p27^{kip1} scatter domain abolished both actin rearrangement and cell scattering independent of cyclin–Cdk binding or inhibition. Importantly, phosphorylation of p27^{kip1} on Ser 10 is necessary for both cytoplasmic localization and induction of cell migration. These observations solidified a genetic role for p27^{kip1} in cell migration that is independent of cyclin–CDK inhibition. However, exactly how and through which pathway p27^{kip1} mediates this process remained unresolved.

Besson et al. (2004) now shed light on how p27^{kip1} regulates actin dynamics to promote cell migration. Building on the observation that p27^{kip1}-deficient fibroblasts have decreased motility, they first observed that p27^{kip1}-deficient cells displayed an increased amount of stress fibers and focal adhesions compared to wild-type cells. The addition of PDGF to MEFs normally induces rearrangements of focal adhesion and actin stress fibers,

accompanied by formation of membrane ruffles and lamellipodia. However, treatment of p27^{kip1}-deficient cells failed to induce these actin-associated rearrangements. Reintroduction of either a wild-type p27^{kip1} or a p27^{kip1} mutant with impaired binding to cyclin–CDKs into p27^{kip1}-deficient cells significantly decreased both the number of stress fibers and focal adhesions. These observations suggested that p27^{kip1} was regulating actin dynamics necessary for cell migration independent of cyclin–CDKs complexes.

Members of the Rho family of small GTPases are important regulators of cell motility involving the actin cytoskeleton (Moon and Zheng 2003; Fig. 1). RhoA and its effector ROCK (also known as Rho kinase) are well characterized for their role in mediating the formation of stress fibers and focal adhesions (Riento and Ridley 2003). GTP-bound, active RhoA stimulates ROCK, which phosphorylates and activates LIMK, a serine/threonine kinase also involved in the regulation of actin filament dynamics. LIMK phosphorylation of cofilin, an actin-depolymerizing protein that regulates actin filament turnover, inhibits its actin-depolymerizing activity. Building on their actin dynamics clue, Besson et al. (2004) then went on to analyze the level of RhoA activity in the p27^{kip1}-deficient cells. Surprisingly, compared to

Figure 1. Inhibition of the Rho pathway by the Cip/Kip family members leads to reorganization of the actin stress fiber filaments and promotion of cell migration. In addition to their role as cyclin–CDK inhibitors, members of the Cip/Kip family (p21, p27, p57) regulate actin dynamics through inhibition of the Rho–ROCK–LIMK pathway. Rho GTPases work as molecular switches that cycle between inactive GDP-bound and active GTP-bound states. GTPase-activating proteins (GAPs) promote the inactive state by enhancing the rate of intrinsic GTPase activity of nucleotide hydrolysis. The active GTP-bound state is promoted by guanine-nucleotide exchange factors (GEFs) that induce the release of bound GDP and facilitate GTP binding. Upon stimulation, RhoA is activated by GEFs, and then GTP-bound RhoA activates ROCKs to phosphorylate one of its substrate LIMKs. By doing so, ROCK1 enhances the ability of LIMKs to phosphorylate cofilin, an actin-depolymerizing protein. This phosphorylation inhibits the activity of cofilin. Phosphorylation of LIMKs by ROCKs inhibits disassembly of actin filaments and thereby leads to an increase in stress fibers. Besson et al. (2004) showed that p27^{kip1} inhibits activation of RhoA by the GEFs. To exert this function, p27^{kip1} needs to relocate to the cytoplasm via Ser 10 phosphorylation. Cytoplasmic p21^{cip1} can inhibit ROCK activity; however, the mechanism underlying p21^{cip1} export to the cytoplasm is currently unknown. p57^{kip2} inhibits the function of LIMK by sequestering it in the cytoplasm, away from its substrate cofilin.

wild-type cells, p27^{Kip1}-deficient cells had a significantly higher level of active GTP-bound RhoA and an increased level of phospho-cofilin. Furthermore, inhibition of ROCK activity by a small molecule (Y27632) rescued the migration defect of p27^{Kip1}-deficient cells, further solidifying a direct role for Rho inhibition of cell migration in p27^{Kip1}-deficient cells.

Thus, the migration defect observed in p27^{Kip1}-deficient cells is due to a higher level of activation of the RhoA pathway. Could p27^{Kip1} be directly involved in the regulation of RhoA activity? As it turns out, Besson et al. (2004) showed that p27^{Kip1} binds to RhoA in vitro, suggesting a direct interaction. Likewise, co-immunoprecipitation experiments in HEK 293 T cells showed that p27^{Kip1} could interact with both the active and inactive forms of Rho. As these experiments relied on overexpression of both p27^{Kip1} and RhoA, we will need confirmation of these direct interactions by physiologic protein levels. Interestingly, only the C-terminal half of p27^{Kip1} is responsible for the interaction with RhoA, again reaffirming that this cell migration function is independent of binding to cyclin-CDKs. Exactly how does p27^{Kip1} inhibit RhoA activation? p27^{Kip1} could inhibit Rho activity by preventing GTP loading by guanine-nucleotide exchange factors (GEFs) or by inhibiting the interaction with its effectors, for example ROCK. To answer this question, Besson et al. (2004) tested the capacity of p27^{Kip1} to inhibit wild-type RhoA or constitutively active RhoA mutant (RhoA-63L; always bound to its effector) and found that p27^{Kip1} could only inhibit the activation of wild-type RhoA, suggesting that p27^{Kip1} does not interfere with RhoA binding to its effectors. Consistent with this, p27^{Kip1} had the ability to inhibit the activation of RhoA by two Rho-specific GEFs, p115-RhoGEF and Lbc. Taken together, these observations indicated that p27^{Kip1} interferes with the interaction of RhoA with its GEFs and thereby interferes with RhoA activation. Thus, Besson et al. (2004) fit the pieces together and show that loss of p27 leads to the observed phenotype of decreased cell motility due to increased Rho activity.

However, there are several unanswered questions. What is the signaling event that induces p27^{Kip1} to interact with and inhibit RhoA in the cytoplasm? Previous work showed that induction of scattering in hepatocellular carcinoma cells through HGF and Met receptor signaling correlated with phosphorylation of p27^{Kip1} on Ser 10 and relocation to the cytoplasm (McAllister et al. 2003). hKIS is the major kinase phosphorylating p27^{Kip1} on Ser 10 and mediating its nuclear export (Boehm et al. 2002). It will be of interest to determine whether hKIS activity is necessary for the interaction of p27^{Kip1} with RhoA. It was recently shown that p21^{Cip1} can also act as an inhibitor of ROCK (Tanaka et al. 2002). Cytoplasmic p21^{Cip1} was associated with neural differentiation, neurite outgrowth, and suppression of actin stress fibers in NIH 3T3 fibroblasts. During osteoblast differentiation, cytoplasmic p57^{Kip2} was found to inhibit LIMK by translocating and sequestering it in the nucleus (Yokoo et al. 2003). The interaction of p57^{Kip2} and LIMK also correlated with reorganization of actin filaments.

Interestingly, cytoplasmic localization of p27^{Kip1} has also been associated with the differentiation process of normal myeloid cells (Yaroslavskiy et al. 1999). However, it remains to be determined whether in the course of myeloid differentiation, cytoplasmic p27^{Kip1} is involved in regulating actin dynamics through the Rho pathway.

The physiologic relevance of the p27^{Kip1}-RhoA link also remains to be determined. p27^{Kip1}-deficient mice do not display any obvious migration defect-related phenotype (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). However, this could be due in part to compensation by p21^{Cip1} and/or p57^{Kip2}. The analogy between p27^{Kip1} and the yeast Far1 is very interesting (McAllister et al. 2003). Far1p is the yeast cell-cycle inhibitor homolog that upon α -factor pheromone signaling mediates both G₁ cell-cycle arrest and bud formation that requires polarization of the actin cytoskeleton toward the opposite mating partner (O'Shea and Herskowitz 2000). Far1p sequesters the Cdc24p GEF that activates the small GTPase Cdc42p in the nucleus. Activation of the pheromone response pathway leads to phosphorylation of Far1p, nuclear export of the Far1p-Cdc24p complex into the cytoplasm, and targeting Cdc24p to the polarity site through its association with G $\beta\gamma$. Given their functionally analogous roles, it is possible that p27^{Kip1} acts in a similar fashion. Besson et al. (2004) did not rule out the possibility that p27^{Kip1} could bind to and sequester a specific Rho-GEF to inhibit activation of RhoA. Although much remains to be learned about this novel link, these new observations highlight the importance of the members of the Cip/Kip family in actin dynamics regulation.

Due to its growth-inhibitory function, p27^{Kip1} is considered a tumor suppressor, and p27^{Kip1}-deficient mice are predisposed to the development of spontaneous tumors (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). In humans, decreased p27^{Kip1} levels have been correlated with tumor aggressiveness and poor patient survival (Alkarain and Slingerland 2004). Interestingly in light of the observations discussed here, a subset of breast, thyroid, esophagus, and colon carcinomas contain normal levels of p27^{Kip1}, but p27^{Kip1} is found in the cytoplasm (Doki et al. 1997; Fredersdorf et al. 1997; Sgambato et al. 1997; Fiorentino et al. 2000). Indeed, cytoplasmic sequestration of p27^{Kip1} is considered a poor prognostic indicator (Slingerland and Pagano 2000). Oddly, although p27^{Kip1} is characterized as a tumor suppressor, inactivating point mutations with loss of heterozygosity are almost never observed in human cancer (Kawamata et al. 1995; Ponce-Castaneda et al. 1995; Ferrando et al. 1996; Alessandrini et al. 1997). Moreover, given what is presented here, a complete elimination of p27^{Kip1} might not confer as much of a growth advantage to tumor cells as would p27^{Kip1} relocation from the nucleus and to the cytoplasm. A distinguishing feature of malignant cells is their capacity to invade surrounding normal tissues and migrate through the blood and lymphatic systems to distant organs. Metastatic tumors are thought to select for both increased invasive growth and

cell migration. Thus, the cytoplasmic role of p27^{kip1} in promoting cell migration and the absence of a tumor selection for complete loss of p27^{kip1} begins to look like a gene living a dual life in cancer, one as a nuclear tumor suppressor and the other as a cytoplasmic metastatic oncogene. Clearly much more work on p27^{kip1} is required before we connect these two dots.

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Genes Dev. 2004, **18**:

Access the most recent version at doi:[10.1101/gad.1205304](https://doi.org/10.1101/gad.1205304)

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