REGULATION OF THE CYTOSKELETON: AN ONCOGENIC FUNCTION FOR CDK INHIBITORS?

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Abstract | Cyclin-dependent kinase inhibitors (CKIs) are well known inhibitors of cell proliferation. Their activity is disrupted in many tumour types. Recent studies show that some of these proteins have interesting alternative functions, acting in the cytoplasm to regulate Rho signalling and thereby controlling cytoskeletal organization and cell migration. The upregulation of CKIs in the cytoplasm of many cancer cells indicates that although loss of nuclear CKIs is important for cancer cell proliferation, gain of cytoplasmic CKI function might be involved in tumour invasion and metastasis.

All cancers are characterized by an abnormal control of cell proliferation. This is caused by mutation or misregulation of cell-cycle regulatory genes and proteins[1]. However, tumours progress to an increasingly aggressive phenotype by acquiring an enhanced ability to invade into adjacent tissues and migrate to distant sites[2]. The motility of a cancer cell is governed by the regulators of cytoskeletal dynamics, in particular the Rho family of GTPases[2]. It now seems that these two fundamental features of tumour cells might be mechanistically connected by direct crosstalk between cell-cycle proteins and cytoskeletal regulatory proteins. In particular, the cyclin-dependent kinase (CDK) inhibitors (CKIs) can regulate the Rho signalling pathway and thereby affect functions that are sensitive to cytoskeletal organization, including cell migration. The network of interactions among cell-cycle proteins and elements of the Rho pathway might normally represent a mechanism for coordinating cell proliferation with the structural reorganization of the cytoskeleton, and might become deregulated in cancer cells.

The protein p27 (also known as KIP1; encoded by *CDKN1B*) is a member of the Cip/Kip family of CKIs and is an inhibitor of cell proliferation whose expression is induced by many anti-mitogenic signals[3,4]. p27 exerts its anti-proliferative function by tight association with cyclin and CDKs, preventing them from binding to ATP and thereby blocking their catalytic activity[5]. The importance of p27 in controlling cell proliferation is illustrated by the phenotype of the p27 null mouse[6]. Homozygous deletion of the p27 gene in mice causes increased cell proliferation, increased animal size, and a generalized predisposition to both spontaneous and induced tumorigenesis[6,7].

p27 is also thought to be a tumour-suppressor protein in humans. Many human cancers express decreased amounts of p27 compared with normal cells, and reduced expression correlates with increased tumour aggressiveness and a poor clinical outcome[8–10]. Nevertheless, p27 is an unusual tumour-suppressor protein, as decreased expression of p27 in cancers is almost exclusively due to misregulation of the p27 gene or the protein itself, rather than by mutation of the p27 gene[8–11]. Indeed, p27 is frequently relocated from the nucleus to the cytoplasm in cancer cells, rather than being eliminated entirely[8–10]. Furthermore, increased levels of p27, or its cytoplasmic localization, have been correlated with high tumour grade, poor prognosis and increased metastasis in subsets of carcinomas (breast, cervix, oesophagus and uterus), and in some lymphomas and leukaemia[9,10,12–20].

An explanation for these observations might emerge from recent data showing that p27, in addition to being an inhibitor of cell proliferation when located in the nucleus, is a regulator of cytoskeletal structure and cell migration when located in the cytoplasm[21]. The misregulation of p27 that is observed in human cancers might result from a selection process

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Summary

- Recent evidence points to cell-cycle independent functions for cyclin, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs). In particular, the involvement of these proteins in the regulation of the cytoskeleton and cell migration is emerging.
- In subsets of many human tumour types, the function of CKIs of the Cip/Kip family is altered by relocation to the cytoplasm, rather than through mutation like most other tumour suppressors. The cytoplasmic localization of p21 (CIP1) and p27 (KIP1) is associated with high tumour grade, tumour cell invasiveness and metastasis.
- Whereas the function of CKIs as tumour suppressors is well characterized in the nucleus, they also seem to function in the cytoplasm, where they regulate cytoskeletal functions. This occurs through the modulation of the Rho signalling pathway. This cytoplasmic function could be oncogenic, as inhibition of the Rho pathway can result in increased migratory capacity.
- p27 binds to RhoA and prevents the interaction of RhoA with its activators the guanine-nucleotide exchange factors. Fibroblasts lacking p27 have impaired migration. CIP1 binds to and inhibits Rho kinases (ROCK1 and -2) — downstream effectors of Rho. p57 (KIP2) binds to and targets LIM domain-containing protein kinase (LIMK) to the nucleus, sequestering it in a compartment where it cannot regulate the actin cytoskeleton.
- Rho GTPases regulate the levels and timing of expression of cell-cycle regulators, and cell-cycle regulators also regulate Rho signalling.
- The regulation of the cytoskeleton and cell migration by CKIs might contribute to the process of tumorigenesis. Targeting these functions of CKIs might therefore constitute a new therapeutic strategy.

that eliminates the anti-proliferative function of p27 in the nucleus while retaining its oncogenic function in the cytoplasm. When located in the cytoplasm, CKIs promote assembly and nuclear import of cyclin D–CDK complexes, inhibit apoptosis and might stimulate cell migration. These oncogenic functions might explain why CKIs are often simply mislocalized in cancer cells, rather than inactivated by mutations.

Linking the cell cycle to cell motility

Changes in cell architecture are required for progression through the cell-division cycle. The most dramatic of these is the complete reorganization of the cytoskeleton that is triggered by the cyclin B–CDK1 (also know as p34; encoded by *CDC2*) complex. Cyclin B–CDK1 phosphorylates proteins such as the kinesin-like motor proteins that are required for proper assembly of the mitotic spindle[22,23], as well as the actin-binding protein caldesmon and several regulators of the small GTPases Rho and CDC42 to mediate the rearrangements of the actin cytoskeleton during mitosis[23,24]. Recent data indicate that this link between the cell-cycle machinery and organization of the cytoskeleton is not restricted to mitosis. Cyclin–CDK complexes and CKIs can also regulate cytoskeletal architecture during interphase. In particular, the members of the Cip/Kip family of CKIs have been reported to regulate the Rho signalling pathway. These findings could have implications for physiological processes, such as the coordination of cytoskeletal changes during the different phases of the cell cycle and the regulation of cell migration, but also for pathological events such as the migratory and invasive capacity of tumour cells.

Cyclin–CDK complexes ensure the proper timing of progression from one phase of the cell cycle to the next, and they can become deregulated during tumorigenesis and cancer progression[25]. Cyclin–CDK activity is tightly regulated at several levels: through control of cyclin synthesis and degradation; activating and inhibitory phosphorylation of the CDK subunit; subcellular localization; and inhibition by CKIs (REF. 3). CKIs of the Ink4 family (p15 (INK4B), p16 (INK4A), p18 (INK4C) and p19 (INK4D)) are specific inhibitors of CDK4 and CDK6, whereas CKIs of the Cip/Kip family (p21 (CIP1; encoded by *CDKN1A*), p27 and p57 (KIP2)) have a broader specificity and can bind to all cyclin–CDK complexes[3]. Cyclins, and to a lesser extent CDKs, are frequently overexpressed or the genes encoding them are amplified in several tumour types, whereas CKIs are commonly inactivated. For example, chromosomal deletions, mutations and silencing of the 9p21 locus encoding p16, p15 and p14 (ARF) are common occurrences in human tumours. On the other hand, mutations in p27 and p21 are rarely found in cancer cells, and inactivation preferentially occurs through downregulation of the protein in the nucleus or sequestration in the cytoplasm[3,25]. Increased amounts of both cytoplasmic p27 and p21 have been associated with high tumour grade and poor prognosis[9,12,26].

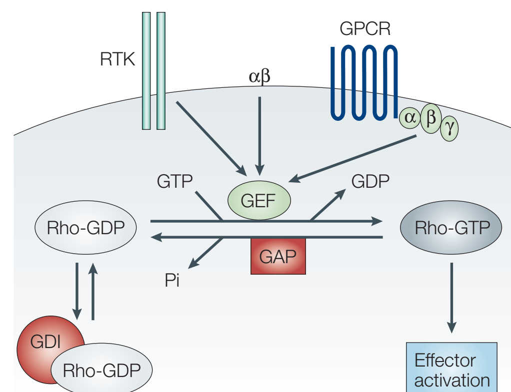
Rho-family GTPases

Signals that induce cell migration originate from cell-surface receptors, including integrins, growth factor receptors and G-protein-coupled receptors, converge to modulate the activity of Rho-family GTPases[2,27,28] (BOX 1). In turn, Rho, Rac and CDC42 control the cytoskeletal reorganizations that are required for migration, which include cell polarization, extension of protrusions, formation of new adhesions at the leading edge, translocation of the cell body, and retraction and release of cell adhesions at the rear of the cell[2]. RhoA promotes the formation of actin stress fibres and focal adhesions through the recruitment and activation of its effectors mammalian diaphanous (mDIA) and the Rho kinases ROCK1 and ROCK2 (FIG. 1). On the other hand, RAC1 promotes the extension of lamellipodia at the leading edge of the cell and the formation of new focal contacts, as well as the disassembly of stress fibres and focal adhesions[2]. So, Rho and Rac have antagonistic and complementary functions during migration, and a careful balance between their activities is needed for efficient cell migration.

In addition to their role in regulating cell migration, Rho-family GTPases control cell proliferation and transformation[29]. Activated *Rac* and *RhoA* function as co-operating oncogenes with *Raf* in transformation assays, and dominant-negative forms of Rho, Rac and CDC42 inhibit Ras-induced cell transformation. CDC42 seems to selectively regulate the adhesion-dependency of cell transformation.

Like CKIs, Rho-related proteins are not usually mutated in tumours, although activating mutations in guanine-nucleotide exchange factors (GEFs) or inactivation of GTPase-activating proteins (GAPs) (BOX 1) have

Box 1 | **Rho-family GTPases**



Signalling from the Rho-family of GTPases regulates a myriad of cellular processes, including the actin cytoskeleton and microtubule network, gene transcription, the activity of various enzymes, and cell-cycle progression. There are currently 20 members of the Rho family[27,28]. Rho-family GTPases are tethered to the membrane where they can be activated by various upstream signals including receptor tyrosine kinases (RTK), integrins (αβ) and G-protein-coupled receptors (GPCR). They alternate between a GDP-bound (Rho-GDP) inactive state, and a GTP-bound (Rho-GTP) active state in which they can interact with and activate various downstream effector proteins (over 60 have been identified so far). Activation (exchange of GDP by GTP) is promoted by guanine-nucleotide exchange factors (GEFs), whereas return to the inactive state (hydrolysis of GTP into GDP) is facilitated by GTPase-activating proteins (GAPs). Rho proteins can also be sequestered in the cytoplasm in their GDP-bound form by guanine-nucleotide dissociation inhibitors (GDIs). Approximately 60 GEFs, 80 GAPs and 3 GDIs are been identified so far, providing a wide variety of mechanisms for regulating Rho-family GTPase activity. There are three archetypical Rho-family members, whose functions are best understood. These include CDC42, which controls sensing of the extracellular microenvironment by the extension of filopodia and the establishment of polarity during directed cell migration; Rac1, which promotes actin polymerization at the cell edges and the extension of the lamellipodia, and provides the driving force for migration; and RhoA, which promotes the formation of actin stress fibres and focal adhesions, and increases actin-myosin contractility.

been reported. Altered expression of Rho proteins (such as RhoC in metastatic melanoma) and their regulators, or the constitutive activation of upstream signalling pathways are commonly observed and can result in increased Rho or Rac protein activation[30]. So, signalling through Rho-family GTPases is an important aspect of cancer development, primarily by modulating cell migration and invasion, but also through the regulation of proliferation and apoptosis[30].

Regulation of the cell cycle by Rho GTPases

Cellular transformation allows for anchorage-independent growth that is commonly associated with altered cytoskeletal structure. The proliferation of most normal cell types is, however, dependent on integrin-mediated cell adhesion to the appropriate substratum. Non-adherent cells arrest in G1 phase with low expression of cyclin D1 (encoded by *CCND1*) and high levels of CIP1 and KIP1, even after exposure to soluble mitogens. Inhibition of actin polymerization mimics the effect of cellular detachment, and recent studies have shown this adhesion/cytoskeleton requirement reflects, in large part, activation of the Rho-family GTPases. How do the Rho-family GTPases regulate cyclin D1 and the Cip/Kip family of inhibitors (FIG. 2)?

Effects on cyclin D1. Rac and, to a lesser degree CDC42, have been reported to stimulate the expression of *CCND1* mRNA in both fibroblasts and epithelial cells[31,32]. The Rac effect has been studied in more detail, with both NF-κB and reactive oxygen species indicated as the responsible Rac effectors. Although the relationship between reactive oxygen species and cyclin D1 has yet to be elucidated, the human and rodent *CCND1* promoters contain functional NF-κB binding sites. Several studies have shown that sustained signalling from extracellular-signal-regulated kinase (ERK) stimulates the production of *CCND1* mRNA[33] in mesenchymal cells. The context in which cells use ERK versus Rac to regulate *CCND1* mRNA levels has yet to be fully defined. Rac also promotes the translation of *CCND1* mRNA in human umbilical vein endothelial cells without significantly affecting the levels of *CCND1* mRNA[34]. So, the ERK and Rac–CDC42 pathways to cyclin D1 seem to be complementary and not merely redundant.

Some reports indicate that activated RhoA activity stimulates *CCND1* gene expression, but the consensus of the data is that RhoA either has a cell-specific effect or is necessary but not sufficient[32]. One possible explanation of the Rho effect comes from the recent finding that induction of cyclin D1 in mid-G1 phase requires the formation of actin stress fibres which, in turn, allows for the sustained clustering of integrins and sustained activation of ERKs[31]. In this model, RhoA affects *CCND1* gene expression by modulating integrin-dependent signalling through the cytoskeleton, rather than directly targeting *CCND1*. Related studies showed that RhoA also inhibits Rac-dependent cyclin D1 expression and that the inhibition requires the nuclear translocation of activated LIM domain-containing protein kinase (LIMK)[35]. So, through its stimulatory effect on ERK signalling and its inhibitory effect on Rac signalling, the Rho-kinase–LIMK pathway determines whether cyclin D1 is expressed and whether the expression is mediated by ERK or Rac. ERK-dependent expression of cyclin D1 requires the presence of actin stress fibres, whereas Rac-dependent expression does not[31]. Ras-transformed cells are highly proliferative but do not have pronounced stress fibres[36], indicating that Rac might mediate cyclin D1 expression in cancer cells.

GEF
Rho-family GTPases cycle between a GDP-bound inactive state, and a GTP-bound active state. Guanine-nucleotide exchange factors (GEFs) facilitate the exchange of GDP for GTP to generate the activated form of the GTPase, which in turn can interact with and activate its downstream effectors.

GAP
GTPase activating enzymes (GAPs) accelerate the intrinsic GTPase activity of Rho family members, returning the proteins to their inactive, GDP-bound state.

GDI
Guanine nucleotide dissociation inhibitors (GDIs) associate with the GDP-bound form of Rho-family proteins and control their cycling between the membrane and the cytosol.

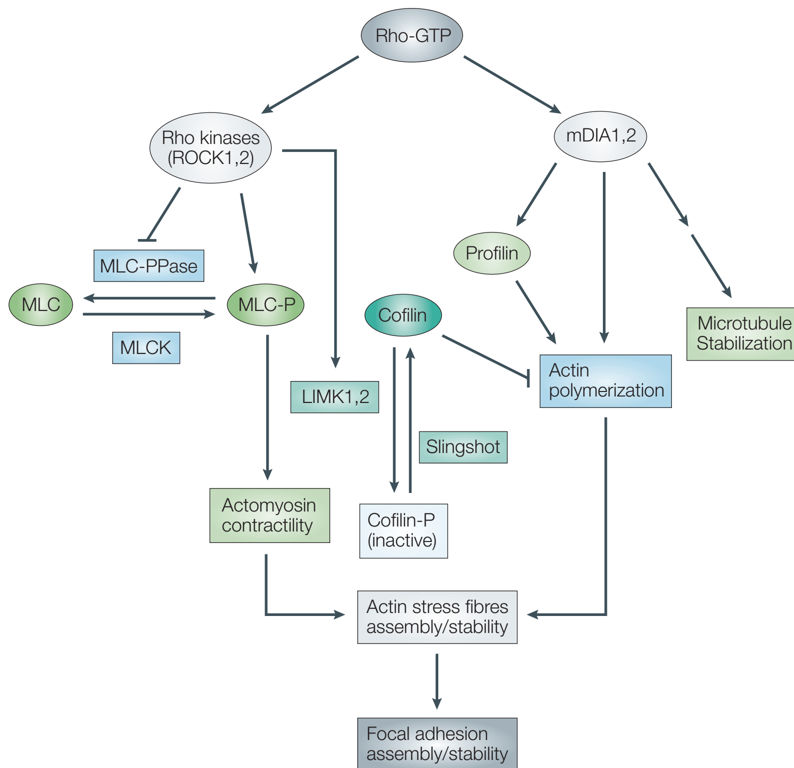


Figure 1 | **Regulation of the cytoskeleton by Rho signalling.** Rho is a crucial modulator of remodelling of both the actin and microtubule cytoskeleton and cell motility through the activation of its effector proteins Rho-kinase (ROCK1 and -2) and mammalian diaphanous (mDIA1 and -2). Activation of mDIA by Rho facilitates actin nucleation and polymerization by the actin-binding protein profilin[27]. mDIA also regulate the formation and orientation of stable microtubules downstream of Rho[61,62]. ROCK1 and -2, alternatively, activate LIM domain-containing protein kinase 1 (LIMK1) and 2 (LIMK2), which phosphorylate and inhibit the actin depolymerization factor cofilin (cofilin-P). This increases actin polymerization and results in the stabilization of actin stress fibres[27]. The phosphatase slingshot dephosphorylates cofilin, which blocks actin polymerization. ROCK1 and -2 also directly phosphorylate myosin light chain (MLC-P), thereby increasing the contractility of myosin, and inhibit MLC-phosphatase (MLC-PPase). This results in increased actomyosin contractility. So, activation of ROCK1 and -2 results in the assembly of actin stress fibres and increased actomyosin contractility, both of which promote integrin clustering, focal adhesion assembly and cell adhesion[2,27].

Effects on p21and p27. RhoA has been implicated in the regulation of p21, and this interaction has been shown to control proliferation of Ras-transformed cells. Olson *et al.*[37] first showed that inhibition of RhoA increased the levels of p21 and blocked Ras-induced transformation. Moreover, RhoA inhibition failed to block proliferation and transformation of p21-null cells. Several studies have shown that oncogenic Ras or Raf lead to a hyperactive ERK signal that increases the expression of the p21 gene[33], and other studies indicate that oncogenic Ras can also stabilize p21 through binding to cyclin D1 (REF. 38). So, Ras and Rho have opposing roles in the regulation of p21, but have complementary roles in regulating cell proliferation — ERK signalling in Ras-transformed cells precludes cell proliferation unless RhoA downregulates p21.

Whereas some studies have not shown strong effects of Rho on the levels of p27, others have reported that RhoA inhibition blocks platelet-derived growth factor (PDGF) and Ras-induced downregulation of p27 (REF. 32). RhoA could stimulate p27 degradation as an indirect consequence of activating the cyclin E–CDK2 complex, but it can also regulate translation of mRNA encoding p27 (REF. 39). The p27 gene is regulated transcriptionally, translationally and by ubiquitin-mediated proteolysis. There are multiple pathways that lead to p27 proteolysis that are dependent on cell cycle position[40,41]. The best characterized proteolytic pathway is mediated by cyclin E–CDK2-dependent phosphorylation of p27 on Thr187, which targets p27 for degradation by the F-BOX PROTEIN SKP2 (REFS 9,41). When spread on fibronectin, endothelial cells required RhoA activity for cell-cycle re-entry to induce SKP2 expression and the subsequent downregulation of p27. RhoA induction of SKP2 expression required mDIA activity but not that of Rho kinase[42].

A few studies have examined the Rho effector pathway that regulates p21 and p27. Several reports indicate that Rho-dependent regulation of p21 and p27 does not require the activity of Rho kinase[31,36,42,43] (FIG. 1). This finding indicates that cells could regulate the actin the cytoskeleton and Cip/Kip proteins through regulation of different Rho effectors. For example, cells that express oncogenic Ras have high levels of RhoA (which represses p21), but they do not have pronounced actin stress fibres because the hyperactive ERK signal that results from oncogenic Ras downregulates the expression of ROCK1 and -2 (REF. 36). So, Ras-transformed cells can proliferate because RhoA levels are high (leading to low levels of p21) and can then migrate because ROCK1 and -2 levels are low.

F-BOX PROTEIN
F-box proteins are subunits of the SCF (Skp1–Cullin1–F box) class of E3 ubiquitin-protein ligases. F-box proteins are adaptors that bind both to protein substrates and to the Skp1–Cullin1 scaffold, and thereby determine the substrate specificity of the complex.

Cip/Kip proteins in Rho pathway regulation

Not only does Rho signalling regulate the timing and expression of cyclins and their inhibitors, but CKIs can also modulate the activity of various components of the Rho pathway by different mechanisms (FIG. 3). This regulation might be evolutionarily conserved, as in budding yeast the CKI Far1 regulates the Rho-family GTPase CDC42. Far1 binds to and sequesters CDC24, a GEF for CDC42, in the nucleus[44]. Phosphorylation and degradation of Far1 in late G1 releases CDC24, which is then exported to the cytoplasm where it regulates cytoskeletal organization and cell polarity[44]. All three mammalian Cip/Kip family members have been reported to inhibit Rho signalling by acting on different proteins in the pathway. p57 was found to interact with LIMK1 without inhibiting its activity, and overexpression of p57 resulted in nuclear localization of LIMK1 (REF. 45) (FIG. 3). This was accompanied by loss of LIMK1-associated stress fibres, indicating that the p57–LIMK1 interaction results in LIMK1 inhibition by sequestering it in the nucleus. Nuclear sequestration of LIMK would inhibit both ERK and Rac-dependent cyclin D1 expression. It remains unclear whether cells that lack p57 have altered actin cytoskeletons or defects in cell proliferation and migration, and whether this function of p57 has a role in tumorigenesis.

Cytoplasmic p21 has been shown to bind and inhibit the Rho kinase ROCK1, promote neurite extension of neuroblastoma cells and hippocampal neurons, and lead to loss of actin stress fibres in NIH3T3 cells[46,47] (FIG. 3). Ras-transformed cells have increased RhoA activity and

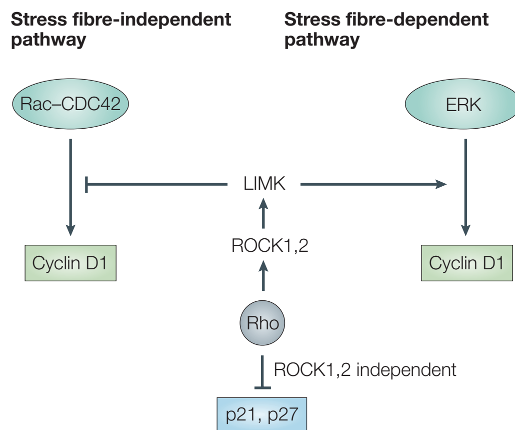


Figure 2 | **Regulation of cyclin-dependent kinase inhibitors and cyclins by the Rho pathway.** Rho-family GTPases regulate mitogenic signalling in the G1 phase of the cell cycle. Rac–CDC42 and extracellular signal-regulated kinase (ERK) function in parallel pathways to stimulate cyclin D1 (encoded by *CCND1*) expression. The activity of Rho–Rho kinase (ROCK1 and -2)–LIMK signalling determines which pathway is used. ERK signalling to activate cyclin D1 is dependent on the formation of actin stress fibres, whereas the Rac–CDC42 pathway is not. During the formation of stress fibres, Rho–ROCK1,2–LIMK signalling promotes ERK-dependent cyclin D1 expression. Conversely, signalling through the Rho–ROCK1,2–LIMK pathway suppresses Rac–CDC42 activation of cyclin D1 expression. So, reducing Rho–ROCK1,2–LIMK signalling would stimulate Rac-dependent cyclin D1 expression. These two pathways might coexist to allow for cyclin D1 expression during remodelling of the actin cytoskeleton, a common event in cellular transformation. Rho, acting independently of ROCK1 and -2, also modulates the cell cycle by regulating expression of p21 (CIP1) and p27 (KIP1). This effect on p21 is mediated by the repression of its transcription. The effect on p27 has not been well studied, but seems to occur at the translational and post-translational levels.

might be expected to have increased actin stress fibres and decreased motility. However, Ras-transformed cells circumvent this problem by decreasing Rho signalling to ROCK1. In some Ras-transformed cells this is achieved by ERK-dependent downregulation of ROCK1 itself (see above). In other Ras-transformed cells, Ras-induced upregulation of p21 uncouples Rho signalling from the formation of stress fibres and focal adhesions by inhibiting ROCK1 (REF. 47). So, oncogenic Ras upregulates p21 through hyperactivation of the ERK pathway, and might induce its cytoplasmic localization by activating phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB)/Akt, which phosphorylates p21 on Thr145 (REF. 48). Once in the cytoplasm, p21 then protects cells from apoptosis[26] and could modulate adhesion and migration through ROCK1 inhibition. Further studies of motility and invasion of cells that have high levels of cytoplasmic p21 are needed to directly confirm that p21 can participate in the acquisition of a migratory phenotype.

The involvement of p27 in the regulation of migration was reported in hepatocellular carcinoma cells, in which transduction of a TAT-p27 fusion protein induced migration[21]. In these cells, hepatocyte growth factor-induced migration correlated with nuclear export of p27 to the cytoplasm through its phosphorylation on Ser10. A region in the carboxy-terminal half of p27 was required to stimulate migration[21]. Further analysis showed that the motility of fibroblasts in p27-null mice were impaired[21,49]. This was probably due to an increase in RhoA activity, as $p27^{-/-}$ cells displayed increased numbers of stress fibres and focal adhesions, and had elevated levels of Rho-GTP[49]. Moreover, Rho-kinase inhibition restored normal migration of p27-null fibroblasts. Overexpression experiments revealed that p27 bound to RhoA, thereby preventing RhoA activation by interfering with RhoA binding to its GEFs[49]. These results indicate that p27 regulates cell migration by preventing Rho activation.

In fibroblasts, loss of p27 impairs cell migration, and in other cell types moderate increases in the level of p27 promote motility. However, it has been reported that overexpression of p27 can negatively regulate migration[50]. It is therefore possible that the effect of RhoA inhibition by p27 varies depending on the degree of inhibition and its context. For instance, different tumour cell lines have distinct requirements for Rho and Rac regarding migration — cells that migrate with an elongated morphology require Rac activity, whereas cells that migrate with a rounded morphology are dependent on Rho–ROCK1 signalling[51]. Moreover, crosstalk between Rho-family GTPases can vary in different biological settings. Depending on the context, Rac can lead to Rho activation or inhibition[27]. Conversely, two RhoA effectors, ROCK1 and mDIA1, have opposing effects on Rho-dependent Rac activation[52]. Rac activation is stimulated by mDIA1, but inhibited by ROCK1 (REF. 52). So until the interactions among cell-cycle proteins and elements of the Rho pathway are studied in more situations, it will be important to avoid generalizations.

The control of CKI subcellular localization by phosphorylation events could represent an important regulatory switch for the activity of these proteins, from nuclear tumour suppressors to cytoplasmic oncogenes. The cytoplasmic localization of p21 and p27 is observed in several tumour types. In the case of p27, several ways to target or retain the protein in the cytoplasm have been described. Mitogenic stimuli triggers p27 phosphorylation on Ser10 by the kinase interacting with stathmin (KIS) and its export from the nucleus[21,53]. Alternatively, PKB/Akt-mediated phosphorylation on Thr157 sequesters p27 in the cytoplasm by impairing nuclear import[12]. PKB/Akt could also phosphorylate p27 on Thr198, which induces its association with 14-3-3 proteins and its export from the nucleus[54]. PKB/Akt has also been reported to phosphorylate p21 on Thr145, causing its cytoplasmic localization[48]. Once in the cytoplasm, p21 protects cells from apoptosis by inhibiting caspase 3 and the apoptosis signal-regulating kinase 1 (ASK1)[26], and could modulate adhesion and migration through ROCK1 inhibition, both of which could actively contribute to tumorigenesis.

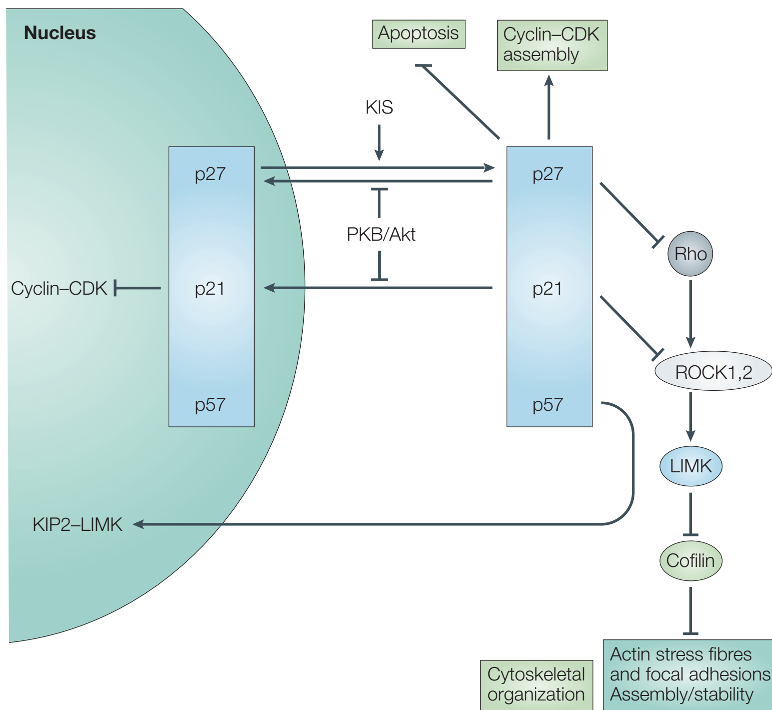


Figure 3 | **Regulation of the Rho pathway and the cytoskeleton by cyclin-dependent kinase (CDK) inhibitors.** When localized to the cytoplasm, Cip/Kip family proteins (p27 (KIP1), p21 (CIP1) and p57 (KIP2)) inhibit the Rho-signalling pathway, affecting cytoskeletal organization and cell motility. Phosphorylation events regulate the shuttling of p21 and p27 between the cytoplasm and the nucleus (green sphere). Phosphorylation of p21 on Thr145 by protein kinase B (PKB)/Akt[48] or Pim1 inhibits the nuclear localization of p21. In the cytoplasm, p21 can protect the cell from apoptosis, as well as bind and inhibit the Rho kinases ROCK1 and -2 to modulate actin cytoskeleton organization[46,47]. Phosphorylation of p27 on Ser10 by the kinase interacting with stathmin (KIS) following mitogen stimulation induces p27 export from the nucleus[21,53]. PKB/Akt-mediated phosphorylation of p27 on Thr198 induces its association with 14-3-3 proteins and its transport to the cytoplasm[54]. Additionally, PKB/Akt-mediated phosphorylation of p27 on Thr157 (a site not conserved in the mouse protein) sequesters p27 in the cytoplasm by impairing nuclear import[12]. Cytoplasmic p27 regulates cell migration by binding to RhoA and preventing its activation by Rho-GEFs. Finally, p57 binds to LIMK1, leading to the nuclear localization of this complex[45]. Whether and how p57 localizes to the cytoplasm is unknown. Overall, Cip/Kip proteins inhibit the Rho pathway at distinct points, the phenotypic consequences of this inhibition is a net decrease in Rho-induced formation of actin stress fibres and focal adhesion.

Cyclins and CDKs regulate cell migration

Aside from members of the Cip/Kip family proteins, p16, cyclin D1, CDK6 and CDK1 have also been reported to regulate some aspects of cell migration. CDK6 and p16 were reported to modulate integrin-mediated spreading and migration[55]. CDK6 promoted the clustering of integrin $\alpha_v\beta_3$ at focal adhesions, whereas p16 inhibited spreading and migration on vitronectin by dissociating $\alpha_v\beta_3$ integrin from focal adhesions[55]. Further work is needed to determine the importance of p16 and CDK6 in the regulation of integrin $\alpha_v\beta_3$.

The mitotic kinase CDK1 has been shown to be upregulated by integrin $\alpha_v\beta_3$ signalling and to increase cell migration in several cancer cell lines[56]. This effect required the association of CDK1 with cyclin B2, as cyclin B2-null fibroblasts show reduced motility, and ectopic expression of cyclin B2 (but not cyclin A or cyclin B1) increased migration[56]. The effect of cyclin B2 and CDK1 on motility was dependent on the CDK1 substrate caldesmon. A mutant form of caldesmon in which all seven CDK1 phosphorylation sites were mutated abolished migration[24,56]. Moreover, CDK1 and caldesmon were co-localized at the lamellipodia following phorbol ester stimulation[56].

Cyclin D1$^{-/-}$ macrophages form an increased number of focal complexes and have increased levels of cortical actin, spreading and adhesion, and decreased motility. This phenotype can be rescued by cyclin D1 re-expression[57]. It remains unclear at this point how cyclin D1 regulates cell morphology and migration and if this is dependent on CDKs. It would be of interest to see if p27 and p21 are involved in this novel function of cyclin D1, as they can facilitate the assembly of complexes between cyclin D and CDK4–6 in the cytoplasm.

These studies indicate that the deregulation of cyclin and CDK expression and activity in cancer cells not only affects proliferation, but also invasiveness. As mentioned earlier, p16 is mutated in a wide range of tumours. Cyclin D1 is frequently overexpressed in many types of cancer, including breast and cervical carcinomas, lymphomas and melanomas. Determining whether cyclin D1 contributes directly to invasion and metastasis, and dissecting the mechanism involved could provide new ways to treat patients with these tumour types. Although cyclin B expression is normally restricted to late S-phase and mitosis, the timing of expression and activation of cyclin–CDK complexes can become deregulated in cancer, such as in the case in glioblastomas[58]. Interestingly, CDK1 expression correlates with high tumour grade and metastasis in prostate and hepatocellular carcinomas[59,60]. So cyclin B CDK1 deregulation promotes not only proliferation and chromosomal rearrangements, but also invasion and metastasis in tumours.

Implications for cancer biology

Tumour invasiveness and metastasis both depend on altered regulation of cell migration. The mechanisms that underlie the characteristics of advanced and clinically aggressive tumours include altered cell–extracellular matrix interactions, disruption of normal cell–cell adhesion, increased production of proteolytic enzymes, and activation of the cell signalling pathways that mediate motility. We have focused this review on the pathways that govern cell motility and their relationships to pathways that control cell proliferation. Indeed, cell-cycle proteins are often altered in tumour cells in a way that is consistent with their recruitment to enhance cell motility.

Recent data indicate the existence of a negative feedback loop between components of the Rho pathway and the Cip/Kip family of CKIs — Rho signalling controls the expression and activities of CKIs, and CKIs negatively regulate various proteins in the Rho pathway. This reciprocal interaction allows for the coordinated control of cellular proliferation with cytoskeletal reorganization and cell migration. In addition, by inhibiting the Rho–ROCK–LIMK pathway, CKIs can also affect Rho-dependent regulation of cyclin D1, p21 and p27. So, during tumorigenesis, deregulation of

these cell-cycle regulators (that is, p21 or p27 mislocalization in the cytoplasm) could result in abnormal Rho signalling and cytoskeletal dynamics. Given the pleiotropic roles of Rho in many aspects of cell regulation, the consequences could range from increased genetic instability, owing to mitotic catastrophes, to increased invasion and metastasis. More work is required to gain a clearer picture of the importance of CKIs and cyclins in cytoskeletal regulation, particularly *in vivo* and during tumorigenesis. Although knockout studies in mice do not provide evidence for a role of Cip/Kip family proteins in cell migration, at least during development, it is clear that developmental plasticity can obscure function in the cyclin–CDK system. Also, owing to functional redundancy, even cell-cycle abnormalities can be subtle and difficult to detect in these mice.

Dissecting cytoplasmic functions from nuclear functions is a new challenge in the study of the Cip/Kip family proteins. Mutations in other cell-cycle proteins have also been considered, mostly in the context of their effects on cell proliferation. However, these proteins, like the CKIs, might also control cytoskeletal organization. Perhaps we should now expand our view of their effects to both normal and tumour cells as well.

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Competing interests statement

The authors declare no competing financial interests.

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