Review

Transforming growth factor-β and breast cancer
Cell cycle arrest by transforming growth factor-β and its disruption in cancer

Jeffrey Donovan and Joyce Slingerland

University of Toronto, and Toronto Sunnybrook Regional Cancer Centre and Sunnybrook and Women's College Health Sciences Centre, Toronto, Ontario, Canada

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Abstract

Altered responsiveness to extracellular signals and cell cycle dysregulation are hallmarks of cancer. The cell cycle is governed by cyclin-dependent kinases (cdks) that integrate mitogenic and growth inhibitory signals. Transforming growth factor (TGF)-β mediates G_1 cell cycle arrest by inducing or activating cdk inhibitors, and by inhibiting factors required for cdk activation. Mechanisms that lead to cell cycle arrest by TGF-β are reviewed. Loss of growth inhibition by TGF-β occurs early in breast cell transformation, and may contribute to breast cancer progression. Dysregulation of cell cycle effectors at many different levels may contribute to loss of G_1 arrest by TGF-β. Elucidation of these pathways in breast cancer may ultimately lead to novel and more effective treatments for this disease.

Keywords: breast cancer, cell cycle, cyclin-dependent kinase inhibitor, human mammary epithelial cells, transforming growth factor-β

Introduction

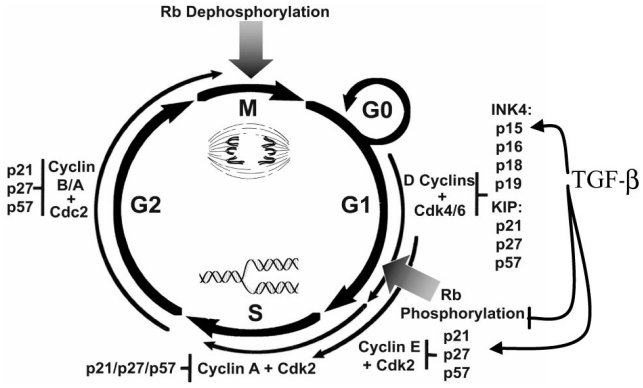
TGF-β is a potent inhibitor of mammary epithelial cell proliferation [1,2] and regulates mammary development *in vivo* [3–5]. Mammary-specific overexpression of TGF-β in transgenic mice can induce mammary hypoplasia and inhibit tumourigenesis [6–8]. Although normal human mammary epithelial cells (HMECs) are exquisitely sensitive to TGF-β [9•], human breast cancer lines require 10-fold to 100-fold more TGF-β to produce an antimitogenic response, and some show complete loss of this effect [10].

Although loss of growth inhibition by TGF-β in human cancers can arise through loss of TGF-β production or through mutational inactivation of the TGF-β receptors and Smad signalling molecules [11,12], these defects are not observed in most arrest-resistant cancer lines. This observation, and the frequent appearance of resistance to more than one inhibitory cytokine in human tumours [13] emphasize the importance of the cell cycle effectors of growth arrest induced by TGF-β as targets for inactivation in cancer.

TGF-β can either lengthen G_1 transit time or cause arrest in late G_1 phase [14]. This cell cycle arrest is usually reversible [15•,16], but in some cases is associated with terminal differentiation [17•,18,19]. Because TGF-β arrests susceptible cells in the G_1 phase, a brief review of cell cycle regulation is presented. This is followed by a review of the multiple and often, complementary mechanisms that contributing to G_1 phase arrest by TGF-β and of how they are disrupted in breast and other cancers.

Cdc = cell division cycle; cdk = cyclin-dependent kinase; CAK = cdk-activating kinase; EGF = epidermal growth factor; HMEC = human mammary epithelial cell; INK4 = inhibitor of cdk4; KIP = kinase inhibitor protein; pRb = retinoblastoma protein; TGF = transforming growth factor.

Figure 1



The cell cycle. Cell cycle progression is governed by cyclin-dependent kinases (cdks), the activities of which are regulated by binding of cyclins, by phosphorylation and by the cdk inhibitors [the inhibitor of cdk4 (INK4) family: p15, p16, p18 and p19; and the kinase inhibitor protein (KIP) family: p21, p27 and p57].

Cell cycle

Cell cycle progression is governed by cdks, which are activated by cyclin binding [20,21] and inhibited by the cdk inhibitors [22,23]. The cdks integrate mitogenic and growth inhibitory signals and coordinate cell cycle transitions [24,25]. G_1 to S phase progression is regulated by D-type cyclin-, E-type cyclin- and cyclin A-associated cdks (Fig. 1). B-type cyclin-associated kinases govern G_2 and M phases. Both E-type and D-type cyclin–cdks contribute to phosphorylation of the retinoblastoma protein (pRb). Hypophosphorylated pRb binds members of the E2F and DP1 families of transcription factors, inhibiting these transcriptional activators and actively repressing certain genes. Phosphorylation of pRb in late G_1 phase liberates free E2F/DP1, allowing activation of genes required for S phase (for review [26]).

Cyclin-dependent kinase regulation by phosphorylation

Cdk activation requires phosphorylation of a critical threonine (Thr160 in cdk2 and Thr187 in cdk4). There are two mammalian kinases with *in vitro* cdk activating kinase (CAK) activity: cyclin H/cdk7 and the protein encoded by the human homolog of the *Saccharomyces cerevisiae CAK1,* called Cak1p (for review [27]). The specific roles of these two kinases are somewhat controversial. CAK is active throughout the cell cycle [20,28], but its access to cyclin-bound cdks is inhibited by p27 [29]. Cdks are also negatively regulated by phosphorylation of specific inhibitory sites [27]. Cdc25 phosphatase family members must dephosphorylate these inhibitory sites for full cdk activation. Cdc25A acts on cyclin E-bound cdk2 and is required for G_1 to S phase progression [30].

Cyclin-dependent kinase inhibitors

Two cdk inhibitor families regulate the cell cycle [22,23]. The inhibitor of cdk4 (INK4) family members ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$) inhibit specifically cdk4 and cdk6. The *p16* gene, or *MTS1* (Multi-Tumor Suppressor 1), was discovered as a tumour suppressor that is deleted in many cancers [31]. Loss of *p15*, located near *p16* on chromosome 9p, may contribute to loss of G_1 arrest by TGF-β (see below).

The kinase inhibitor protein (KIP) family presently consists of three members, $p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. The KIPs bind and inhibit a broader spectrum of cdks than do the INK4s. p21 is low in serum-deprived quiescence, but p21 levels and p21 binding to D-type cyclin–cdk complexes increase in early G_1 phase. In addition to regulating G_1 phase progression, p21 acts to coordinate cell cycle responses to DNA damage [23]. $p27^{Kip1}$ was first identified as a heat stable protein whose binding to cyclin E–cdk2 complexes that was increased by TGF-β, lovostatin, or contact inhibition [32•,33,34•,35•,36]. p27 is high in G_0 and early G_1 phase and decreases during G_1 to S phase progression. p27 degradation by ubiquitin-dependent proteolysis [37] is activated by many different growth factors and may involve ras pathways [38–42]. Although cyclin E–cdk2 phosphorylates p27 on Thr187 leading to its degradation in late G_1 phase [43,44], other kinases may also influence p27 function and/or degradation. The possibility that mitogenic signalling pathways that modulate p27 phosphorylation also oppose Smad activation by TGF-β is the subject of intensive investigation.

Although p21 and p27 inhibit cyclin E–cdk2, they also function in the assembly and activation of cyclin D–cdk4 and cyclin D–cdk6 complexes. KIP-mediated assembly of D-type cyclin–cdks in early G_1 phase may facilitate activation of E-type cyclin–cdks through sequestration of KIPs away from cyclin E complexes [45•,46•].

Mechanisms of cell cycle arrest by TGF-β

TGF-β inhibits phosphorylation of the retinoblastoma protein

Cells are sensitive to TGF-β during a discrete period in early G_1 phase, until they reach a 'restriction point' 6–10 h after G_0 release [47•,48]. When TGF-β is added after this critical time point, cells complete the cell cycle but arrest during the subsequent G_1 phase. Laiho *et al* [47•] observed that TGF-β inhibits pRb phosphorylation when it is added in early G_1 phase. This key observation suggested that TGF-β was acting before the G_1 to S phase transition to inhibit a pRb kinase, and led to the investigation of TGF-β effects on cell cycle regulators. These studies have shown that TGF-β prevents or inhibits G_1 cyclin–cdk activation through multiple mechanisms, leading to pRb dephosphorylation (Fig. 2). E2F activity is also impaired by TGF-β through a decline in E2F mRNA levels [49]. The observation that E2F overexpression can

prevent TGF-β-mediated arrest [49] emphasizes the importance of the effects of TGF-β on pRb and E2F.

TGF-β downregulates c-*myc*

In many cell types, TGF-β causes a rapid inhibition of c-*myc* transcription [2,16,50]. Transcriptional regulation by the c-Myc protein is required for G_1 to S phase progression. Downregulation of c-*myc* by TGF-β is believed to be important for arrest, because c-*myc* overexpression causes TGF-β resistance [2,51•]. Repression of the c-*myc* gene by TGF-β may directly or indirectly contribute to the loss of G_1 cyclins [52,53], to downregulation of Cdc25A [54•] and to the induction of the cdk inhibitor p15 [55•] (see below).

Effects on G_1/S cyclins

TGF-β causes loss of G_1 cyclins in a cell-type-dependent manner. Cyclin A expression is downregulated by TGF-β in most cell types [32•,56•] and a TGF-β-regulated region of the cyclin A promoter has been identified [57]. Effects of TGF-β on cyclin E differ among different cell lines. For example, in HaCat keratinocytes TGF-β decreases both mRNA and protein levels of cyclin A and cyclin E, whereas in HMECs cyclin E mRNA is reduced but protein levels are not [32•,56•]. Although cyclin D_1 levels are decreased by TGF-β in some cell types, this usually occurs late as a consequence of arrest [58,59•].

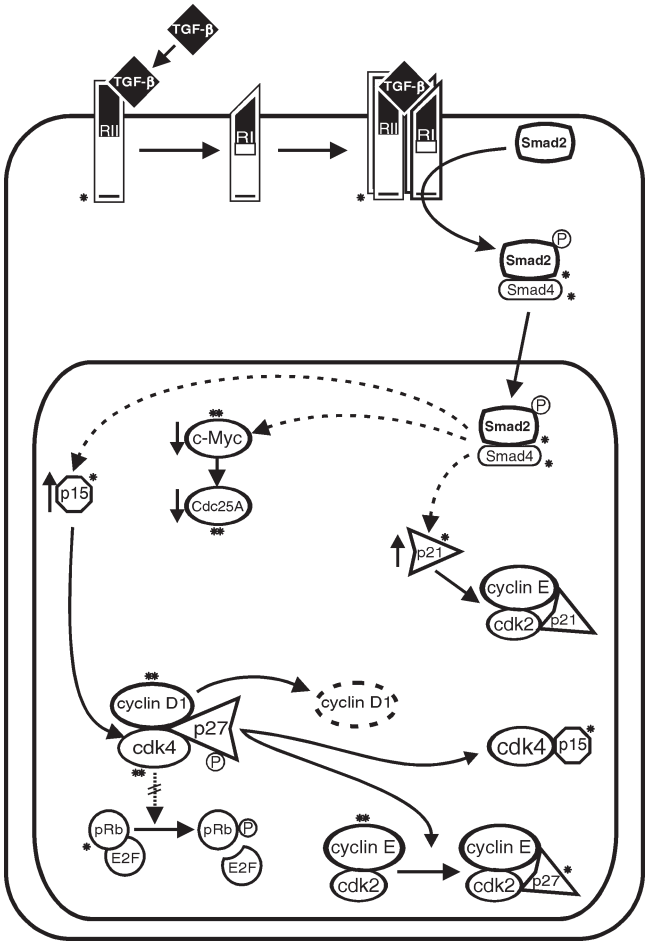
Cooperation between p15 and p27

In epithelial cells, including HMECs, the INK4 and the KIP proteins collaborate to inhibit D-type cyclin–cdks and E-type cyclin–cdks to bring about G_1 arrest by TGF-β [60•,61•]. *p15^{INK4B}* was first cloned as a gene upregulated by TGF-β [62•] and its induction involves an Sp1 site in the promoter [63]. TGF-β induces *p15^{INK4B}* and stabilizes the p15 protein, leading to p15 binding and inhibition of cdk4 and cdk6. Cyclin D_1 and KIP molecules dissociate from cdk4 and cdk6, and p27 accumulates in cyclin E–cdk2 complexes, inhibiting the latter [60•,61•]. A late downregulation of cyclin D_1 and cdk4 follows G_1 arrest. TGF-β appears to actively regulate p27's affinity for its targets, independent of p15 function, favouring p27 accumulation in cyclin E complexes [61•].

Upregulation of p21 expression

In normal HMECs, TGF-β affects neither p21 levels, nor the binding of p21 to target cdks [61•]. In other cell types, TGF-β induction of *p21* plays a role in cdk inhibition [59•,64•,65] and its upregulation is independent of p53 [64•,66•]. The *p21* gene may be a downstream target of Smad4, because transient overexpression of *Smad4* induces *p21* mRNA [67]. Like *p15*, the *p21$^{WAF1/Cip1}$* gene promoter contains Sp1 sites that are regulated by TGF-β in reporter gene assays [63,68]. Other cdk inhibitors, p16, p18, p19 and p57, have not to date been implicated in TGF-β-mediated arrest.

Figure 2



Mechanisms of cell cycle arrest by transforming growth factor (TGF)-β and their deregulation in cancer. TGF-β receptor activation leads to Smad2 phosphorylation. Phosphorylated Smad2 then binds Smad4 and the Smad2–Smad4 complex translocates to the nucleus to modulate transcription. Although *p15* and *p21* genes are induced and c-*myc* and *Cdc25A* repressed by TGF-β, these may not be direct effects of Smad2–Smad4 action (dotted lines). TGF-β inhibits G_1 cyclin–cyclin-dependent kinases (cdks) by increasing p15 binding to cdk4 and cdk6 and by increasing p27 (+/– p21) binding to cyclin E–cdk2, thereby inhibiting retinoblastoma protein (pRb) phosphorylation. *Components of the TGF-β effector pathway that are mutated and/or functionally inactivated in human cancers; **molecules whose activation or overexpression may contribute to TGF-β arrest resistance.

Effects on cdk2 phosphorylation

TGF-β also regulates cdk2 phosphorylation. In Mv1Lu cells, TGF-β inhibits cdk2 in part by inhibiting phosphorylation on Thr160 [32•,34•]. p27 can inhibit CAK access to cyclin-bound cdks *in vitro* [29]. Thus, TGF-β may prevent CAK action by increasing the binding of p27 to cyclin E–cdk2. In HepG2 cells, however, TGF-β inhibits the enzymatic activity of Cak1p [69], indicating an alternative mechanism for the inhibition by TGF-β of Thr160 phosphorylation of cdk2.

Dephosphorylation of inhibitory sites on cyclin E-bound cdk2 is required for G_1 progression and is required for G_1 to S phase progression [30]. In a human breast epithelial line, TGF-β reduced *Cdc25A* expression in association with an increase in inhibitory cdk phosphorylation [54•]. The effect on *Cdc25A* expression may be secondary to the repression by TGF-β of c-*myc*, because in some cell types *Cdc25A* is induced by c-*myc* [70•].

Loss of TGF-β mediated G_1 arrest in cancer

In nontransformed epithelial cells, TGF-β causes G_1 arrest through downregulation of c-*myc*, inhibition of the G_1 cdks and hypophosphorylation of pRb. Overlapping or redundant cell cycle controls assure growth arrest. In malignantly transformed cells, however, this redundancy is often lost and carcinoma-derived cells are usually refractory to growth inhibition by TGF-β [10]. Indeed, in advanced cancers, TGF-β may promote tumour growth and metastatic progression [71]. In this part of the discussion, we review how dysregulation of many different cell cycle mechanisms abrogate TGF-β arrest in cancer (Fig. 2).

Altered cdk inhibitor expression and function

Dysregulation of the INK4 family may contribute to TGF-β resistance in cancer. In human tumours, deletion of *p15* often accompanies *p16* deletion due to their proximity on chromosome 9p [72–74]. Silencing of *p15* through promoter hypermethylation, which is observed in leukaemias, is associated with loss of TGF-β sensitivity [75,76]. In other TGF-β-resistant cells, however, p15 protein levels may increase normally, indicating that, at least in these lines, a functional p15 is not sufficient to mediate arrest by TGF-β [65].

Although p15 and p27 cooperate to inhibit the G_1 cyclin–cdks in normal cells, neither of these cdk inhibitors are essential for G_1 arrest by TGF-β. p15 is clearly not essential for TGF-β-mediated G_1 arrest, because cells bearing *p15* deletions can respond through upregulation of p21 and p27 [59•,65], or downregulation of Cdc25A [54•]. Lymphocytes from p27-null mice can still arrest in response to TGF-β [77]. Nonetheless, the requirement for p27 in arrest by TGF-β may differ in normal and transformed cells. Although inhibition of p27 expression through antisense p27 oligonucleotide transfection did not abrogate TGF-β-mediated arrest in finite lifespan HMECs, it did do so in breast cancer-derived lines (Donovan J, Slingerland J, unpublished data). In normal cells, multiple redundant pathways cooperate to mediate arrest, but in cancer cells the progressive loss of other checkpoints may make p27 indispensable for TGF-β-mediated arrest.

The antiproliferative role of p27 is frequently disrupted in human cancers. Although mutations in *p27* are rare [78,79], accelerated proteolysis causes reduced p27 protein in many cancers, including breast, and may contribute to TGF-β resistance [37,80–82]. Less often, primary tumours may exhibit strong cytoplasmic p27 expression associated with poor prognosis. Cytoplasmic p27 has been observed in some advanced cancer-derived lines [83]. Thus, some cancers may express a stable but inactivated p27. In a TGF-β-resistant HMEC line, we have observed stable cytoplasmic p27 localization, altered p27 phosphorylation and impaired binding of p27 to cyclin E–cdk2 (Ciarallo S, Slingerland J, unpublished data). The elucidation of how of mitogenic signalling pathways alter p27 inhibitor function may prove important insights into mechanisms of TGF-β resistance (see below).

Altered KIP function has also been observed in TGF-β-resistant prostate cancer cells. Although TGF-β caused an upregulation in p21–cdk2 binding, this kinase was not inhibited, suggesting that p21 may not function normally in these cells [84]. Loss of p21 has also been observed in advanced breast cancers in association with a poor patient prognosis [85,86]. As for p27, functional inactivation of p21 could contribute to TGF-β resistance during breast cancer progression.

Cyclin overexpression and TGF-β resistance

Overexpression and/or amplification of the cyclin D_1 gene is seen in up to 40% of breast cancers [87,88] and could contribute to TGF-β resistance. Indeed, cyclin D_1 transfection of an oesophageal epithelial line conferred resistance to TGF-β [89]. Increased cyclin E protein has also been observed in breast cancers [80,90]. Constitutive overexpression of cyclin E does not confer TGF-β resistance in Mv1Lu cells, however (Slingerland J, Reed S, unpublished data). Pathways that link impaired cyclin degradation with loss of cell cycle responses to TGF-β have yet to be elucidated.

Cdk4 gene amplification and activating mutation

Although loss of cdk4 does not contribute significantly to arrest by TGF-β because it occurs after most cells have entered G_1 phase [60•], ectopic *cdk4* expression can abrogate TGF-β-mediated arrest [91•]. The increased cdk4 level may exceed titration by p15 and, in addition, sequestration of KIPs away from cyclin E–cdk2 into newly formed cyclin D–cdk4 complexes may lead to loss of cdk2 inhibition. Overexpression of cdk4 may contribute to TGF-β resistance in human cancers. Amplification of the *cdk4* gene occurs in primary breast cancers [92] and dominant active *cdk4* mutations have been observed in human malignant melanoma [31].

Activation of c-*myc*, and TGF-β resistance

TGF-β arrest-resistant cells often fail to downregulate c-*myc* [65]. Moreover, oncogenic activation of c-*myc*, which is seen in a number of human malignancies, including breast cancer, may impair TGF-β responsiveness through a number of mechanisms.

Overexpression of c-Myc may increase G_1 cyclin levels. c-Myc may regulate indirectly the expression of cyclins D_1, E and A [52,53]. c-Myc induction of cyclin D_1 or cyclin D_2 may lead to the sequestration of p27 and p21 away from cyclin E–cdk2, and thus contribute to cyclin E–cdk2 activation [93,94]. These effects, however, which are best demonstrated in fibroblast lines, may not be relevant to TGF-β resistance in epithelial cells. In Mv1Lu cells, c-*myc* overexpression prevents arrest by TGF-β in part by inhibiting *p15* induction [55•]. c-Myc effects on D-type cyclin expression and cyclin D–cdk4 complex formation were not sufficient to account for loss of the TGF-β response. Thus, repression of *p15* by c-Myc may be important in the arrest-resistant phenotype.

Additional mechanisms link c-Myc with cyclin E–cdk2 activation. Overexpression of c-*myc* can induce a heat labile factor that binds p27 and inhibits its association with cyclin E–cdk2 [95]. This effect is independent of p27 degradation. Although in some cell types cyclins D_1 and D_2 may be the c-*myc*-induced inhibitors of p27 [93,94], in other models the c-*myc*-induced inhibitor of p27 appears to be independent of D-type cyclins [95].

Oncogenic activation of c-*myc* may lead to *Cdc25A* overexpression and loss of TGF-β-mediated repression of *Cdc25A* [54•]. Overexpression of *Cdc25A* is observed in primary breast cancers and is associated with a poor patient prognosis (Loda M, personal communication). The increased Cdc25A may represent one of the checkpoints whose disruption makes subsequent disruption of p27 function more critical during breast cancer progression.

Activation of Ras and its effector pathways and TGF-β resistance

Overexpression of activated Ras has been shown to abrogate the antimitogenic effects of TGF-β [96]. Mutational activation of *ras* is common in many human cancers and may be linked to TGF-β resistance through a number of mechanisms. Activated Ras can interfere with TGF-β signalling by altering Smad2 phosphorylation and signal transduction [97]. Moreover, Ras activation can increase cyclin D_1 levels through both transcriptional and post-translational mechanisms [38,98,99]. Ras activation also accelerates p27 degradation [40,41], in some models requiring coexpression of Myc [39]. Although *ras* mutations are not commonly observed in breast cancer, epidermal growth factor (EGF) and ErbB2 overexpression are, and both activate the Ras effector phosphatidylinositol-3 kinase [100]. Oncogenic activation of different *ras* effector pathways may abrogate p27 function [41,101], contributing importantly to TGF-β resistance.

Regulation of other G_1 events by TGF-β

p53 may play a role in the TGF-β response in some cells. In murine keratinocytes, introduction of mutated *p53* led to TGF-β resistance, and a correlation between *p53* mutation and loss of responsiveness to TGF-β-mediated arrest has been observed in several cancers [102–104].

Constitutive expression of *mdm2* can give rise to TGF-β resistance. Although the Mdm2 protein binds to p53 to mediate p53 proteolysis, the effects of Mdm2 on TGF-β sensitivity appear to be independent of p53 function, because expression of an Mdm2 mutant that failed to bind p53 also conferred resistance [105]. Because overexpression of Mdm2 occurs in about 73% of breast cancers, this too may play a role in TGF-β resistance *in vivo* [85,106, 107].

Conclusion

During the past decade the anatomy of cell cycle regulation has been 'worked out'. TGF-β-induced G_1 arrest occurs through induction of *p15* and *p21* genes, repression of the c-*myc*, *Cdc25A*, *cyclin E* and *cyclin A* genes, and an increase in the association of p15, p21 and p27 with target cdks. Inactivation of G_1 cyclin–cdks leads to pRb dephosphorylation and E2F inhibition. The discovery of the Smads as both transducers of TGF-β signalling and transcriptional regulators has been a major advance in this field. Mitogenic signalling via ras/mitogen-activated protein kinase has been shown to interfere with Smad activation [11]. It will be of interest to ascertain whether cross-talk with Smads can also negatively regulate components of mitogenic signal transduction pathways. How growth factors and mitogenic pathways influence the transcriptional activation, intracellular localization and degradation of cyclins and cdk inhibitors is only beginning to be mapped. As these mechanisms are elucidated, we will be able to move from the myopic view of cyclin–cdk regulation in the nucleus, to a broader three-dimensional view of cell cycle regulation that encompasses extracellular and cytoplasmic signalling pathways. The next frontiers lie in the cytoplasm and at the gateway of the nuclear pore as we begin to elucidate how TGF-β/Smad signalling interfaces with transducers of mitogenic signals to regulate cyclin–cdk activities.

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- • of special interest
- •• of outstanding interest

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Authors' affiliations: Jeffrey Donovan (Department of Medical Biophysics, University of Toronto, and Division of Cancer Research, Toronto Sunnybrook Regional Cancer Centre and Sunnybrook and Women's College Health Sciences Centre, Ontario, Toronto, Canada) and Joyce Slingerland (Departments of Medicine and Medical Biophysics, University of Toronto, and Division of Cancer Research, Toronto Sunnybrook Regional Cancer Centre and Sunnybrook and Women's College Health Sciences Centre, Ontario, Toronto, Canada)

Correspondence: Joyce Slingerland, Division of Cancer Research, Sunnybrook and Women's College Health Sciences Centre, 2075 Bayview Ave, Toronto, Ontario, Canada M3N 3M5.
Tel: +416 480 6100, ext 3494; fax: +416 480 5703;
e-mail: joyce.slingerland@utoronto.ca