

## Minireview

## Communication between the extracellular environment, cytoplasmic signalling cascades and the nuclear cell-cycle machinery

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**Abstract** In the past decade, we have gained considerable insight into the identities of various cytoplasmic signal transduction cascades and the manner in which they operate in response to changes in the extracellular environment. Moreover, we have begun to understand what the key players are in cell-cycle regulation and how they, in turn, function to promote cell division. A long-standing question, however, has been how communication between signalling routes and the cell-cycle machinery occurs. This review highlights some recent observations that provide possible links between signal transduction and the cell-cycle machinery.

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*Key words:* Signal transduction; Cell cycle; Ras; Rb

## 1. Rb and regulation of cell proliferation

An important property of untransformed, eukaryotic cells is their ability to respond to changes in their extracellular environment, i.e. the local concentration of various factors. These factors include mitogens and anti-mitogens, survival factors, differentiation-inducing factors and cell–cell and cell–substratum interactions. This variety of signals influences a cell's decision to remain (or become) quiescent, to differentiate, to undergo programmed cell death or to enter a cell division cycle and reproduce. In most cases, a cell is responsive to this kind of regulation only in two cell-cycle phases: in quiescence (G0) or in G1, the first gap phase of the cell cycle [reviewed in [1]]. During this time window, extracellular signals are transmitted through cytoplasmic signal cascades which, in turn, induce nuclear processes that eventually determine the type of cellular response. Once a cell has passed a specific point in late G1, termed the restriction point R, it becomes largely refractory to proliferation-stimulating factors: it commits itself to progress until mitosis, even in the presence of growth-restraining signals like the cytokine TGF $\beta$ , contact inhibition, DNA damage or protein synthesis inhibitors [2], reviewed in [3]. The transition from a serum-dependent state to a serum-independent state therefore represents an important event and much effort has been put into identifying the components involved in this decision making.

The product of the retinoblastoma tumor suppressor gene, Rb, likely is intimately involved in this process [3]. Its function is to connect the cell-cycle clock with the transcriptional machinery. Its growth-suppressing activity, exerted during

early G1, is relieved by phosphorylation in mid-to-late G1, thus roughly coinciding with passage of the R point. A critical role for Rb in regulating S-phase entry is further supported by the observation that Rb is sequestered during cellular transformation by various DNA tumor virus oncoproteins [reviewed in [4]] and that the Rb gene carries mutations and deletions in various human tumors [reviewed in [5]]. Together, these observations suggest that in normal cells inactivation of Rb is an obligatory event during G1 progression and entry into S phase, and that in transformed cells absence of functional Rb predisposes to uncontrolled onset of DNA replication.

In view of the importance of Rb during G0/G1, one would predict that physiologic signals influence proliferation, at least in part, by modulating Rb activity. Indeed, TGF $\beta$ -induced G1 arrest was shown already 7 years ago to correlate with accumulation of the hypophosphorylated, growth-suppressive form of Rb [6]. Moreover, the oncoproteins of DNA tumor viruses appeared capable of overriding the growth-inhibitory action of TGF $\beta$ , through their Rb-binding domains [7]. An interesting question then arose as to how Rb, and in particular its phosphorylation, is regulated. Answers to this question not only provide insight into Rb regulation per se, but may also allow identification of a component(s) upstream of Rb in a pathway from extracellular signalling to cell-cycle control. In this respect, (components of) the Rb kinase(s) should, ideally, be responsive to specific proliferation-regulating signals such as serum factors and TGF $\beta$  and, moreover, their activities should be essential for S-phase entry.

## 2. Cyclin D and its regulators: intermediates between mitogenic signalling and Rb

One such protein that seems to meet the criteria outlined above well is cyclin D. First, expression of D-type cyclins (in particular D1 and D2) is highly growth-factor dependent; because of the short half-lives of both the mRNA and the protein, cyclin D levels decline quickly upon mitogen withdrawal [reviewed in [8]]. In fact, a characteristic property that allowed one way of cyclin D identification is its 'delayed early' kinetics during mitogenic stimulation of growth-arrested macrophages or fibroblasts [9,10]. Secondly, cyclin D-dependent kinases (cdk4 and cdk6) can phosphorylate Rb on a number of residues [11,12]. Thirdly, interference with cyclin D1 function by microinjection of antibodies or antisense DNA prevents serum-stimulated fibroblasts from leaving quiescence to enter S phase, when performed before or near the G1/S transition [13,14]. Fourthly, ectopic expression of cyclin D1 shortens the progression from G0/G1 to S phase by sev-

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eral hours [13,15] and, in conjunction with a second oncogene, even allows for transformation of primary cells [16,17]. Finally, the cyclin D-cdk4/6 kinase is inactivated by TGF $\beta$ , by various mechanisms [18–20]. It is, in this respect, of note that cells in cyclin D1<sup>-/-</sup> mice proliferate relatively normally [21], which may be explained in part by a possible redundant role for cyclin D2.

Thus, D-type cyclins fulfil at least some important requirements for acting as regulatory subunits of genuine Rb kinases on one hand and for connecting mitogenic signalling with the regulation of Rb-dependent G1 exit on the other. Therefore, they have been proposed to act as growth-factor sensors, rather than integral components of the cell-cycle clock, such as the (G1/S-specific) E-type cyclin and (S-phase and mitotic) A- and B-type cyclins [8]. If Rb and (one of) its regulator(s) cyclin D are true components of a pathway that is used by extracellular factors to modulate proliferation, one would predict that, in turn, regulators of cyclin D activity may represent additional components in this pathway. Cyclin D-dependent kinase activity is controlled in a complex manner, which may not be surprising in view of its importance for cell proliferation. In addition to careful regulation of cyclin D levels, cyclin D-cdk4 kinase activity is controlled by subcellular localization [14], (de-)phosphorylation [22], translation [23,24] and binding to cdk-inhibitory proteins (CKI) [reviewed in [25–27]].

The rapidly expanding group of small CKIs antagonizes cyclin D-dependent kinase action through protein–protein interactions. They consist of at least two families: Ink4 proteins which specifically inhibit cdk4/6, and Cip1/Kip1 proteins, which are broad-specificity inhibitors of cyclin–cdks [25–28]. Are these inhibitors, in addition to cyclin D, also regulated by extracellular factors? For at least three inhibitors the answer is yes. For example, mRNA and protein abundance of p15<sup>Ink4b</sup>, as well as p21<sup>Cip1</sup>, are induced by TGF $\beta$ , which likely is part of the mechanism by which TGF $\beta$  arrests cells [19,20,29]. Moreover, levels of p27<sup>Kip1</sup> decrease when cells leave a quiescent state; conversely, they rise in response to TGF $\beta$ , cAMP, cell–cell contact and mitogen depletion [30–32]. In the latter case, p27<sup>Kip1</sup> appears to be required for induction of cell-cycle arrest in response to growth-factor withdrawal [32]. By contrast, loss of p27<sup>Kip1</sup> does not seem to lead to reduced sensitivity to TGF $\beta$  [33]. Thus, during the regulation of cell proliferation several extracellular factors likely converge on the Rb pathway, in particular cyclin D and certain CKIs.

### 3. Ras and the Rb pathway

Which cytoplasmic signal-transduction cascades mediate the regulation of the Rb pathway as a function of environmental changes? Small GTPase proteins, like those belonging to the Ras family, monitor and regulate a wide variety of information flows in eukaryotic cells. Ras proteins play critical roles in both controlled and uncontrolled cellular proliferation [reviewed in [34]]. Extracellular stimuli like EGF, PDGF, NGF and CSF-1 activate receptors with intrinsic tyrosine kinase activity which, in turn, leads to an increase in the amount of GTP-bound Ras [reviewed in [35]]. Activation of Ras is mediated by at least two other proteins, Grb2 and SOS, the latter of which is translocated to Ras at the plasma membrane upon mitogenic stimulation [36–38].

The growth-stimulatory effect of oncogenic Ras has been

demonstrated by its ability to induce S-phase entry upon microinjection into quiescent fibroblasts [39]. The requirement for the wild-type Ras counterpart has first been shown with the help of neutralizing antibodies that prevent quiescent fibroblasts from cell-cycle entry by serum or several growth factors [40]. Interestingly, Ras activity appears to be required at all stages prior to the G1/S transition, as was suggested first by data from Mulcahy et al. [40]. Later, similar observations were made with a temperature-sensitive K-Ras mutant [41] and with the combined use of antibody microinjection and cell-cycle inhibitors [42]. Recently, Ras was shown to be active during mid G1, as judged by a novel assay in which activated Ras is isolated from cell extracts by virtue of its affinity for the Ras-binding domain of Raf1 [43].

Is there communication between Ras-dependent signalling cascades and the Rb pathway? Studies on TGF $\beta$  and cAMP suggest, at the least, functional correlations. First, in Mink lung epithelial cells TGF $\beta$ -induced G1 arrest parallels both an increase in GDP-bound Ras [44] (although conflicting results have been reported [45]), and a modulation of the Rb pathway described above. The induction of G1 arrest can be prevented by activated Ras [44,46–48]. TGF $\beta$  arrests cells in late G1; when anti-Ras antibodies are microinjected just prior to the release from TGF $\beta$ , cells do not enter S phase, underscoring the observation that Ras activity is not only required for the G0/G1 transition, but also for passage of the G1/S boundary [44]. cAMP, on the other hand, has also been shown to inhibit the Ras pathway, specifically the Ras-dependent activation of Raf1 [49] and MAP kinase [50], along with regulating p27<sup>Kip1</sup>, as discussed above.

There are, in addition to the correlations discussed here, more examples that suggest a role for Ras as an upstream regulator of the Rb pathway. For instance, expression of active Ras shortens the G1 interval, which is accompanied by an increase in levels of cyclin D1, but not cyclin E or Cdk4 [51–55]. However, in the absence of growth factors, Ras cannot induce cyclin D-dependent kinase activity, probably due to the presence of high levels of p27<sup>Kip1</sup>, which can be overcome only in the presence of plasma factors [55]. Similar observations are made when resting T-cells are stimulated with antigen and IL2, leading to cyclin synthesis and removal of p27<sup>Kip1</sup>, respectively [56,57]. In addition, in microinjection experiments the adenovirus E1A oncoproteins can override the requirement for Ras during entry into S phase [58]. Since the transforming activity of E1A can be attributed to its ability to sequester a number of cellular proteins, including Rb, also these results are compatible with (yet do not demonstrate) a model in which Ras and the Rb pathway communicate with one another.

We have recently obtained direct evidence for this hypothesis [59]. Using both an interfering Ras<sup>Asn17</sup> mutant [60,61] and microinjection of neutralizing Ras antibodies [40,42], we observed that inactivation of Ras in cycling fibroblasts and myoblasts leads to G1 arrest only in the presence of functional Rb. Inactivation of Ras causes a decline in cyclin D1 protein levels, accumulation of the hypophosphorylated, growth-suppressive form of Rb and G1 arrest. Upon disruption of Rb function, either genetically (in Rb<sup>-/-</sup> cells) or biochemically (by ectopic expression of D cyclins or a downstream target for Rb, the E2F-1 transcription factor) cells lose their ability to stop proliferating in response to Ras inactivation. In contrast, inactivation of Ras in quiescent cells prevents growth-factor

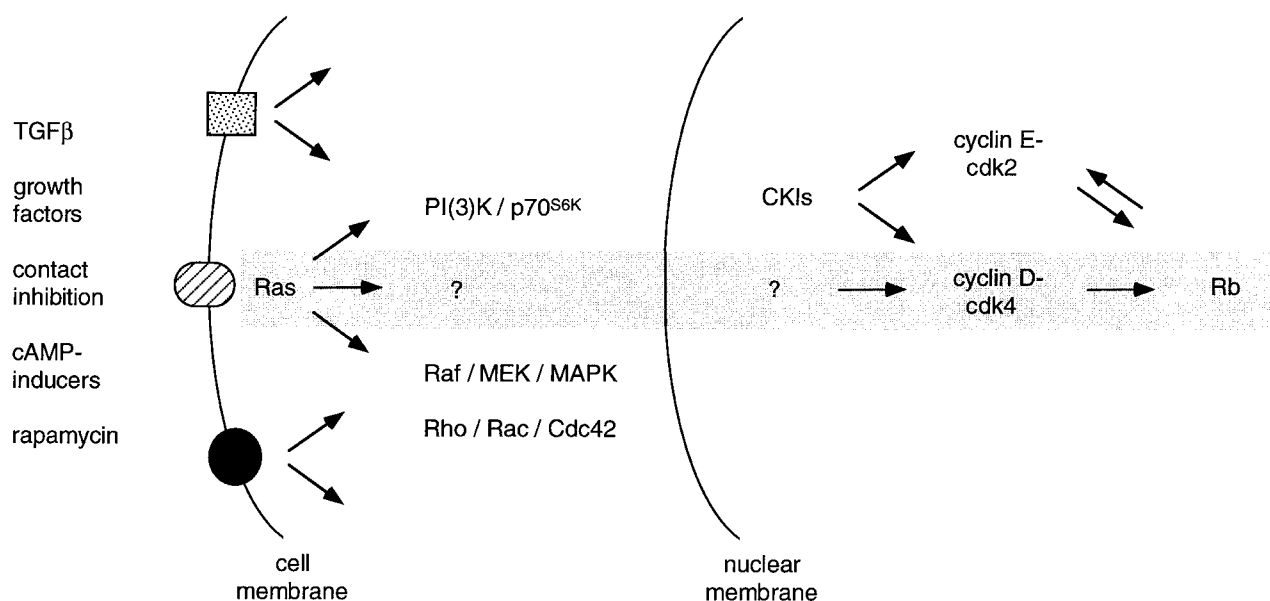


Fig. 1. Schematic representation of communication between extracellular factors and cellular proteins. Extracellular factors modulate the activities of membrane-bound proteins, including receptor tyrosine kinases (indicated by square, ellipse and circle). These signals are subsequently transduced through various cytoplasmic signalling cascades, in part in a Ras-dependent manner. In turn, components of the nuclear cell-cycle machinery are influenced, which results in the alteration of the activities of G1- and G1/S-specific cyclin-dependent kinases and their inhibitors (CKIs). The relevance of this type of communication can for example be illustrated by the signal transduction from Ras to cyclin D and Rb (shaded box): this communication route is important for adequate growth regulation (in particular growth inhibition), for disruption of the cyclin D-Rb pathway results in cellular failure to respond to downregulation of Ras activity during G1. For further details see text.

induction of both immediate-early gene transcription and G0 exit in an Rb-independent manner. These results are in keeping with the observation that  $p16^{\text{Ink4a}}$  (an Rb-dependent growth inhibitor [62–65]) inhibits S-phase entry induced by an activated Ras allele [66]. Moreover, they are consistent with the recent demonstration that TGF $\beta$  fails to inhibit the proliferation of Rb $^{-/-}$  fibroblasts [67]. In sum, accumulating evidence suggests that the Rb pathway may indeed form a critical target for various Ras-dependent (anti-)mitogenic signals.

#### 4. Ras effectors and the Rb pathway

The use of dominant negative mutants like Ras $^{\text{Asn17}}$  has revealed the involvement of Ras in a number of different pathways, including those constituted by Raf1/MAP kinase kinase (MEK)/MAP kinase/RSK [68,69], PI(3)K [70], Rho/Rac/Cdc42 [71], and, as shown recently, KSR1 [72] and references therein). Which of these Ras effectors feeds into Rb-dependent cell-cycle progression?

Most of these pathways have been implicated in regulation of G0/G1 progression. For example, kinase-deficient mutants of Raf1, MEKs and MAPKs can block Ras-induced mitogenic signalling and transformation [73–77], as do dominant-negative mutants of Rac, Rho and Cdc42 [71,78–80] and deregulated expression of MAP kinase phosphatase [81,82]. Consistent with this, the use of Ras effector-loop mutants has revealed that, in addition to Raf1, multiple cellular factors contribute to the transforming potential of Ras [83]. Moreover, simultaneous stimulation of distinct Ras effector pathways, involved in either membrane ruffling or MAP kinase activation, are required for Ras-induced DNA synthesis [84].

Specific inhibitors of PI(3)K block growth factor- or IL2-induced DNA synthesis [85–87]. It is, in this respect, not clear

whether PI(3)K signalling requires Ras function, for conflicting results have been reported on the hierarchy of Ras and PI(3)K [70,88,89]. The  $p70^{\text{S6K}}$  kinase is a PI(3)K effector [90], and can be activated independently of Ras [91], and by Rho family proteins [92]. Microinjection of polyclonal antibodies to  $p70^{\text{S6K}}$  also prevents G1 progression [93], as does an inhibitor of  $p70^{\text{S6K}}$ , rapamycin [94–97]. Rapamycin prevents resting T cells from entering the cell cycle upon IL2 treatment, but only when rapamycin is added up to a point in G1, before Rb is phosphorylated [98]. Interestingly, this immunosuppressant has also been shown to prevent IL2-induced degradation of  $p27^{\text{Kip1}}$  during T-cell mitogenesis [56,57], the relevance of which has been underscored with the use of  $p27^{\text{Kip1-/-}}$  fibroblasts which show reduced sensitivity to rapamycin-induced G1 arrest [99]. Finally, polymerized collagen-induced inhibition of proliferation of arterial muscle cells has recently been shown to correlate with suppression of  $p70^{\text{S6K}}$  and induction of  $p21^{\text{Cip1}}$  and  $p27^{\text{Kip1}}$  [100]. Thus, cross-talk between (in part Ras-dependent) cytoplasmic cascades and Rb-dependent G1 exit may involve signal transducers like PI(3)K and  $p70^{\text{S6K}}$  on one hand and CKIs such as  $p27^{\text{Kip1}}$  and  $p21^{\text{Cip1}}$  on the other.

Yet another, more direct, connection between Ras signalling and cell-cycle control is suggested by the interaction between Raf1 and Cdc25A [101]. Cdc25A dephosphorylates regulatory threonine and tyrosine residues on cdks [102,103], which may be modulated upon interaction of Raf1 with Cdc25A. Cdc25A and B cooperate in oncogenic transformation with either oncogenic Ras or genetic loss of Rb [104]. The resulting suggestion that, at least in this respect, activation of Ras and loss of Rb are functionally equivalent, is in agreement with the observation that inactivation of Rb renders cells insensitive to downregulation of Ras activity, as discussed. The latter result suggests a model in which Ras and Rb lie on a common pathway. However, a prediction that Ras

activity and Rb deficiency would be interchangeable in transformation cooperation assays in general appears incorrect, as becomes clear from the observation that p16<sup>Ink4a</sup>−/− fibroblasts can be oncogenically transformed by an activated Ras allele alone [105]. This result, in turn, would suggest that Ras and Rb are components of cooperating, rather than overlapping, pathways. This apparent inconsistency could perhaps be explained by the possibility that the roles of Ras in normal versus neoplastic cell proliferation may be only partly overlapping. This may, for example, be due to the differences in duration of Ras activity (periodic versus constitutive, respectively), something that has been proposed previously to be important for differential Ras/MAP kinase-dependent signalling [reviewed in [106]].

Ras activity is required as soon as cells leave G0 upon mitogenic stimulation, up to late G1. Are the Ras effectors during exit from quiescence identical to those during G1 progression and G1 exit? The answer to this question is not known, but some observations can be taken into consideration. On one hand, it has recently been clearly shown that Ras is activated in mid-G1, upon release from either mitosis (in HeLa cells) or quiescence (in NIH 3T3 cells) [43]. This is consistent with previous findings that Ras activity is necessary at least twice during exit from G0 and G1, as described above. Probably even more interesting is the observation that the second peak of Ras activity does not seem to correlate with that of MAP kinase activity, nor with that of Shc phosphorylation and Shc–Grb2 complex formation [43]. These data suggest that the activation of Ras during G1 is not mediated by a Shc/Grb2-dependent pathway and, in addition, that the receiver of the mitogenic signal is not the MAP kinase pathway. On the other hand and as outlined above, it has been shown for various Ras effectors (e.g. Raf1, MAP kinase and MEK) that their activities are required for colony outgrowth of normal cells and for Ras transformation [73,75,82], although in some strains of NIH 3T3 cells, Raf/MAP kinase-dependent pathways do not contribute to Ras transformation [107]. Thus, at this time it is unclear whether overlapping, or rather distinct, Ras-effector pathways are involved in promoting exit from G0 and from G1 (see Fig. 1).

## 5. Signal transduction and gene expression

Ultimately, many signals transduced by cytoplasmic protein cascades during exit from G0 are translated into modulation of immediate-early gene expression which, in turn, leads to alterations in cell-cycle control. Examples of transcription factors that are induced upon Ras activation are Jun and Ets-1; their activities are important since dominant negative mutant forms of either protein block Ras transformation [108–110]. In addition, Jun and Fos, but not Myc, rescue a block to PDGF-induced DNA synthesis imposed by Ras<sup>Asn17</sup> [111]. This suggests that transcription factors like AP-1 and Ets-1 link Ras signalling to the transcriptional machinery which, in turn, triggers a second wave of gene expression, namely that of the delayed-early genes like D-type cyclins. Moreover, these results place Ras and Myc in two separate pathways leading to DNA synthesis.

The cyclin D genes obviously are attractive candidates to be subjected to transcriptional regulation by immediate-early gene products. Numerous laboratories have therefore attempted to unravel the mechanism and identify the transcrip-

tion factors responsible for the delayed-early expression of the cyclin D genes in response to mitogens. The cyclin D1 promoter contains several elements, including binding sites for AP-1, Ets-2, Myc, and CREB/ATF [52,112]. In addition, it has been shown that Ras, p42/44 MAP kinase, p38 HOG/MAP kinase, AP-1 family proteins and Rb can regulate the cyclin D1 promoter [51,52,112,113]. Conflicting results have been reported on the requirement for protein synthesis for full activation of the cyclin D gene [10,114,115]. Together, the data suggest that transcriptional elements within the cyclin D1 promoter respond to a complex variety of signalling pathways and transcription factors. It will, in this respect, be of interest to determine whether the transcription factors and promoter elements involved in cyclin D1 regulation are the same during exit from G0 and in cycling cells.

Recently, a potential transcriptional target for Myc has been identified, namely the Cdc25A gene [116]. This gene can be activated through Myc-binding elements, while Cdc25A expression is required for Myc-induced apoptosis [116]. Together with the Raf1–Cdc25A interaction described above, these data suggest that Cdc25A is regulated by both Ras- and Myc-dependent signalling routes. If Cdc25A, indeed, is activated by Ras–Raf1 signalling, these observations raise the question why Cdc25A cooperates with Ras in transformation [101,104]. A possible explanation for this would be to assume that Ras/Raf1 activation of Cdc25A needs to be accompanied by (Myc-dependent) induction of Cdc25A levels. Another connection between Myc and the cell cycle may be provided by observation that Myc can override a p27<sup>Kip1</sup>-induced growth arrest. The mechanism by which this occurs is proposed to involve sequestration of p27<sup>Kip1</sup> by an as yet unidentified protein, leading to activation of G1 cyclin–cdks [117].

Two other connections between signal transduction and transcriptional regulation of a cell-cycle component have been suggested. First, STAT1 proteins appear to induce p21<sup>Cip1</sup> transcription through specific binding sites [118]. This correlates with induction of G1 arrest in response to ionizing radiation, for STAT1-deficient cells arrest upon radiation only after reintroduction of a STAT1 allele. Secondly, the cytokine-responsive transcription factor NF-κB has been shown to bind the p300 coactivator which, in turn, binds the cyclin E–cdk2 kinase [119]. This results in stimulation of NF-κB-dependent transcription by p300, but only if cyclin E-associated kinase activity is prevented by expression of either p21<sup>Cip1</sup> or an interfering cdk2 mutant.

## 6. Conclusion and perspectives

In this review, we have discussed a number of observations that provide new insight into a long-standing problem: how cytoplasmic signal transduction cascades are linked to the nuclear cell-cycle machinery. Probably the most far-reaching conclusion is that it seems that at least a majority of signalling routes seems to control cell proliferation through acting on components of the Rb pathway, in particular cyclin D-dependent kinases and their CKIs. For example, the observation that cells lacking functional Rb, fail to stop proliferating in response to inactivation of Ras suggests that, indeed, there is close communication between ‘classical’ signalling and cell-cycle pathways. It will, therefore, be challenging to look for novel Ras targets that may function specifically during G1, but not G0. In this respect, kinetic studies, as performed by

Taylor and Shalloway [43], on potential (Ras) target proteins serve as informative and relatively reliable tools in addition to the common 'block-and-override' experiments.

Do all signal transduction routes converge on Rb? Probably not. This is for example illustrated by the observation that Rb-deficient fibroblasts require serum factors, albeit at a reduced level, for their proliferation [59,120,121]. This indicates that other pathways, in addition to the Rb pathway, need to be stimulated simultaneously to ensure cell-cycle progression. A candidate pathway may be one that regulates the activity of the cyclin E-cdk2 kinase. In contrast to cyclin D-cdk4, inactivation of which leads to cessation of proliferation only in the context of functional Rb, disruption of cyclin E-cdk2 function inhibits proliferation in an Rb-independent manner [25,122,123]. As outlined above, this may not be completely accounted for by the observation that cyclin E appears to be a transcriptional target of Rb/E2F [124–128]. Thus, although cyclin E is transcriptionally regulated by Rb/E2F (as well as post-transcriptionally, i.e. by CKIs like p27<sup>Kip1</sup>), additional, Rb-independent, signals are required for (full) activation of the cyclin E cytochrome kinase, necessary for the onset of DNA replication. Finally, another cell-cycle kinase that may operate independently of Rb is cdk3, since a dominant-negative cdk3 mutant can induce G1 arrest and inhibit E2F transcriptional activity in Rb-independent manners [122,129].

In conclusion, although Rb undoubtedly is a key player that regulates cell-cycle progression in response to (the majority of) mitogenic signals, Rb-independent pathways may operate in concert with Rb to trigger additional events that are essential for exiting G1 and entering S phase. Therefore, it will be of interest to identify not only novel components of G1-specific (Ras-dependent) signalling routes that regulate Rb, but also pathways that regulate cell proliferation in parallel to the Rb pathway.

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