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Report

Underlying principles of cell fate determination during G_1 phase of the mammalian cell cycle

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Abbreviations: Cdk, cyclin dependent kinase; CKI, CDK inhibitor; PI3K, phosphatidylinositol 3-kinase; GF, growth factors; Str, stresses

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Upon their exit from mitosis, mammalian cells enter a G_1 phase during which they acutely sense all sorts of environmental stimuli. On the basis of these signals that they first need to decipher and integrate, they decide whether to undergo division, differentiation, senescence or apoptosis. We questioned whether, despite the complexity of the G_1 regulatory network, simple organizing principles might be identified that could explain how specific input signals are converted into appropriate cell fates. For this purpose, we formulated a mathematical model of the G_1 regulatory network using a simplified description of activities linked to signal transduction, cell growth, cell division and cell death. Bifurcation analysis of the model revealed the existence of multistability between several attractor states corresponding to G_0-arrest, G_1-arrest, S-phase entry and apoptosis cell fates. We further unravelled interlinked feedback and feedforward loops within the G_1 regulatory network that drive the signal-dependent transition between G_0 arrest and the other cell fates. Initially, exit from G_0 and progression in early G_1 entail growth factor-dependent activation of an upstream positive feedback loop that activates the cell-growth machinery. Once ribosome synthesis is restored in G_1, a competition develops between a downstream positive feedback loop, which, upon activation, triggers S-phase entry, and stress-activated pathways that promote G_1 arrest. If S-phase entry prevails over G_1 arrest, cells are sensitized to apoptosis due to stress-induced activation of pro-apoptotic pathways or repression of pro-survival pathways. Thus, the choice between the four possible cell fates in the G_1 phase relies on the flexibly interlinked growth-activatory and division-activatory modules, certain components of which have antagonistic effects on pathways involved in driving apoptosis and G_1 arrest. The final outcome ultimately depends on the context-dependent coordination between the cell-growth and cell-division processes.

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Introduction

In unicellular organisms, the issue whether a cell would divide primarily relies on its growth capacity which, in turn, depends on the concentration of nutrients in its environment. Thus, single cells generally initiate division when they reach a critical size that reflects a rate of macromolecular synthesis sufficient to execute accurately major cell cycle events.[1-3] The situation is very different in multicellular organisms, especially mammals, in which the rate of cell division strongly depends on the context. Thus, cell division actively takes place during development and tissue regeneration but rarely in most fully-developed organs despite the fact that the organism's cells are suffused with an abundance of nutrients. This is because, in adult tissues, local and systemic controls operate to restrain cell division in order to maintain tissue homeostasis.[4] In fact, mammalian cells that exit mitosis enter a G_1 phase during which they are snowed under a flood of conflicting signals which must be deciphered and integrated before the cells become committed either to divide or not and, eventually then, differentiate, senesce or die.[5-7] Failures in this process may lead to cancer.[7,8] G_1-phase regulating signals include soluble macromolecules as well as insoluble structures bound to the surface of neighboring cells or to the extracellular matrix and various forms of exogenous and endogenous stresses. Depending on the type, strength and timing of the signals, cells either commit to divide or shift into reversible or irreversible out-of-cycle states: (i) G_0 arrest (or quiescence), which is reversible; (ii) G_1 arrest, which can be reversible or irreversible and may ultimately drift toward either a senescent or a differentiated state; (iii) programmed cell death (or apoptosis).

Because of the complexity of G_1 signaling network involved in a cell's response to the manifold environmental stimuli that it senses, it is difficult to determine how a given input signal directs commitment to a particular cell fate. However, because this apparently inextricable network in fact operates to maintain tissue homeostasis, we expect that simple rules exist to govern the connection between input signals and cell fates. In an attempt to capture these rules, we use a modeling approach that has been exploited over the last decade to gain insights into the dynamic mechanisms that underlie the cell cycle.[9] G_1-phase regulation in mammalian cells has already inspired a broad spectrum of models essentially based on interactions between key cell cycle regulators: (i) the retinoblastoma (Rb) protein; (ii) the cyclin-Cdk complexes; (iii) the E2F transcription factors; and (iv)

the Cdk inhibitors (CKIs).[10-18] Our model extends the scope of such G_1-phase models by integrating the contribution of selected signal transduction pathways and of cell growth, which are likely to play a crucial role in cell fate determination during G_1-phase progression. In particular, the involvement of two families of G_1 regulatory signals were considered: (i) mitogenic factors that activate the interactive Ras/Erk/Raf and PI3K/Akt/mTOR signal transduction pathways;[19] and (ii) stresses (e.g., DNA damage, hypoxia, nutrient deprivation, oncogenic signals) that activate the p53 pathway.[20] Both impinge on the activity of cell cycle as well as cell-growth regulators.

Considering the absence of quantitative data on kinetics and the inherent complexity of the G_1 regulatory pathways, we adopted a simplified description of activities linked to signal transduction, cell growth, cell division and cell death. Such a coarse-grained model is amenable to bifurcation analysis and numerical simulations and enabled us to identify how the attractors in the G_1 regulatory network model depend on network architecture and the control parameters of the model. Our working hypothesis is that the attractor states represent various cell fates.[21] Indeed, the G_1 regulatory network model exhibits four attractor states referred to as G_0 arrest, G_1 arrest, S-phase entry and apoptosis. The occurrence of multistability between these states indicates the presence of positive feedbacks that enforce cell transition from the G_0-arrested state to one of the other states in response to appropriate stimuli. First, we depicted the transition between the state of G_0 arrest and that of S-phase entry as an event that is triggered by the sequential activation of two positive feedback loops by growth factors: a first loop drives partial phosphorylation of Rb and initiation of ribosome synthesis while a second loop leads to the completion of Rb phosphorylation and to the accumulation and activation of both cyclin E-Cdk2 and E2F transcription factors. Next, we examined how the stress-induced p53/p21 pathway may interfere with this two-step progression between G_0 arrest to S-phase entry. In early G_1, p21 levels are sufficient to antagonize the activity of the cyclin E-Cdk2 complex and preclude full activation of the second positive feedback loop, thus giving rise to an intermediate stable state, namely G_1 arrest. Later in G_1, the p21 levels become insufficient to antagonize cyclin E-Cdk2 activity, thus enabling p53 and E2F factors to cooperate in the initiation of cell-death programs. Based on these informations, we designed a toy model of the G_1 regulatory network, which recapitulates the simple rules that underlie the connection between input signals and cell fates.

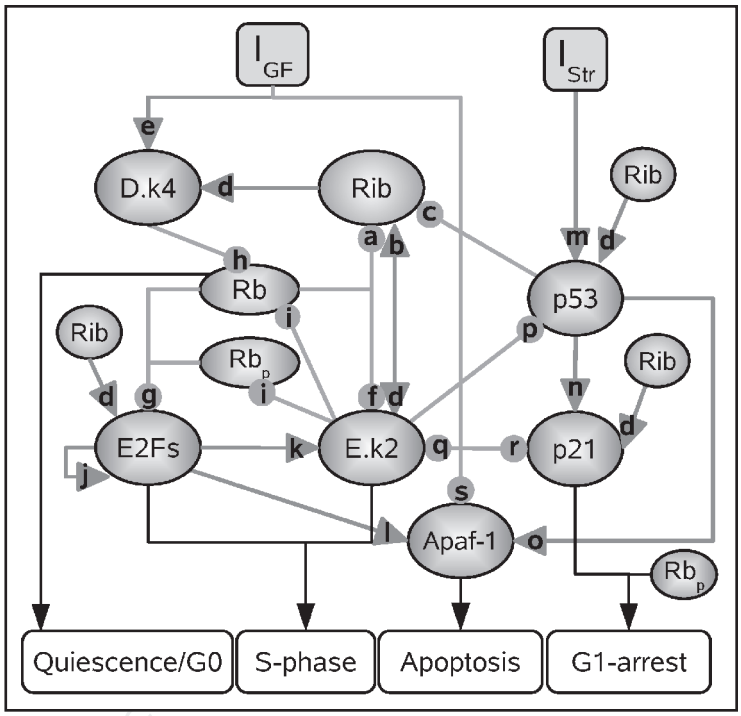


Figure 1. Molecular network regulating G_1-phase progression in mammalian cells. Gray arrowhead lines (vs light gray roundhead lines) indicate positive (vs negative) regulations of the G_1 signaling network that are mentioned in the text using alphabetic letters. Black arrowhead lines point to specific downstream biological programs such as quiescence/G_0, S phase, apoptosis and G_1 arrest (reversible or irreversible). Upstream, the G_1 regulatory network integrates input signals emanating from two main families of signaling pathways: (i) *IGF* refers to factors (mitogenic, growth and survival factors) that activate the interacting Ras/Erk/Raf and PI3K/Akt/mTOR signaling pathways and (ii) *IStr* refers more specifically in this study to p53-activating stresses. (Abbreviations: E.k2, cyclin E-Cdk2; D.k4, cyclin D-Cdk4,6; Ap, Apaf-1; E2F, activator E2Fs; Rib, Ribosomes; Rb_p, hypophosphorylated Rb).

Model

Figure 1 presents a simplified depiction of the mammalian G_1 regulatory network, which includes key interactions between cell cycle regulators that have been experimentally identified over the last two decades[22] and incorporated into our model. This G_1 regulatory network model was formulated as a set of ordinary differential equations listed in Table 1.

Ribosomes and cell growth regulation. Ribosomes are the central parts of the protein-synthesizing machine of the cell. They consist of two dissociable subunits that are produced in the nucleolus and exported from the nucleus into the cytoplasm where they are assembled into functional ribosomes. Ribosome biogenesis is a highly complex and energy-expending process that requires the interaction of a diversity of cellular components, including the three RNA polymerases Pol I/II/III, ribosomal proteins, ribosomal RNAs (rRNAs) and small molecules, in various cell compartments.[23,24] Thus, there are many levels at which ribosome synthesis could be regulated. A critical regulatory element is the phosphoprotein Rb, which is not only a powerful repressor of cell cycle-regulating gene transcription but also acts as a potent block to rRNA Pol I transcription by interacting with and inactivating the nucleolar transcription initiation cofactor UBF25 (Fig. 1, pathway a). Another likely regulatory element is the kinase cyclin E-Cdk2, which has been shown to participate in nucleolar re-assembly and restoration of ribosome biogenesis after mitotic exit[26,27] (Fig. 1, pathway b). Finally, rRNA synthesis and pre-RNA processing are downregulated in response to various forms of exogenous and endogenous stress, notably via p53-dependent mechanisms[26,28,29] (Fig. 1, pathway c). Many events could affect the initiation and rate of ribosome synthesis to some degree; however, for the purpose of our model, we developed a qualitative description of cell-growth regulation by assuming that the rate of ribosome synthesis primarily depends on the levels of these three entities: (i) unphosphorylated Rb; (ii) cyclin E-Cdk2 activity; and (iii) p53 (Equation 1, Table 1). Newly synthesized ribosomes, in turn, catalyze protein synthesis (Fig. 1, pathway d).

Table 1 Differential equations and parameters for mammalian G_1 regulatory network model

$\frac{d[\mathrm{Rib}]}{dt} = R_0 + (R_{max} - R_0) + \frac{[\mathrm{E.k2}]^2}{([\mathrm{E.k2}]^2 + C_{\mathrm{E.k2}\to\mathrm{Rib}})(1 + C_{\mathrm{Rb}\to\mathrm{Rib}}[\mathrm{Rb}]^2)(1 + C_{\mathrm{p53}\to\mathrm{Rib}}[\mathrm{p53}])} - d_{\mathrm{Rib}}[\mathrm{Rib}]$	(1)
$\frac{d[\mathrm{D.k4}]}{dt} = [\mathrm{Rib}]\ (k_{\mathrm{D.k4}} + k_{\mathrm{GF}\to\mathrm{D.k4}}\, I_{\mathrm{GF}}) - d_{\mathrm{D.k4}}\,[\mathrm{D.k4}]$	(2)
$\frac{d[\mathrm{E2F}]}{dt} = [\mathrm{Rib}](k_{\mathrm{E2F}} + \frac{[\mathrm{E2F}]}{k'_{\mathrm{E2F}} + [\mathrm{E2F}]}) - (d_{\mathrm{E2F}} + d_{\mathrm{Rb}\to\mathrm{E2F}}\,([\mathrm{Rb}] + [\mathrm{Rb}]_P))[\mathrm{E2F}]$	(3)
$\frac{d[\mathrm{E.k2}]}{dt} = \frac{[\mathrm{Rib}]}{C_{\mathrm{Rb}\to\mathrm{E.k2}} + 1}(k_{\mathrm{E.k2}} + k_{\mathrm{E2F}\to\mathrm{E.k2}}\,[\mathrm{E2F}]) - (d_{\mathrm{E.k2}} + d_{\mathrm{p21}\to\mathrm{E.k2}}[\mathrm{p21}])\,[\mathrm{E.k2}]$	(4)
$\frac{d[\mathrm{p53}]}{dt} = [\mathrm{Rib}](k_{\mathrm{p53}} + k_{\mathrm{Str}\to\mathrm{p53}}\, I_{\mathrm{Str}}) - (d_{\mathrm{p53}} + d_{\mathrm{E.k2}\to\mathrm{p53}}[\mathrm{E.k2}])\,[\mathrm{p53}]$	(5)
$\frac{d[\mathrm{p21}]}{dt} = [\mathrm{Rib}](k_{\mathrm{p21}} + k_{\mathrm{p53}\to\mathrm{p21}}\,[\mathrm{p53}]) - (d_{\mathrm{p21}} + d_{\mathrm{E.k2}\to\mathrm{p21}}[\mathrm{E.k2}])\,[\mathrm{p21}]$	(6)
$\frac{d[\mathrm{Ap}]}{dt} = [\mathrm{Rib}](k_{\mathrm{p53}\to\mathrm{Ap}}[\mathrm{p53}] + k_{\mathrm{E2F}\to\mathrm{Ap}}[\mathrm{E2F}] + k_{\mathrm{E2F.p53}\to\mathrm{Ap}}\,[\mathrm{E2F}][\mathrm{p53}]) - (d_{\mathrm{Ap}} + d_{\mathrm{GF}\to\mathrm{Ap}}\, I_{\mathrm{GF}}[\mathrm{Rib}])\,[\mathrm{Ap}]$	(7)
$[\mathrm{Rb}] = \frac{k'_{\mathrm{Rb}}\, Rb_{tot}}{(k'_{\mathrm{Rb}} + (k'_{\mathrm{Rb}} + d_{\mathrm{E.k2}\to\mathrm{Rb}}[\mathrm{E.k2}])(d_{\mathrm{D.k4}\to\mathrm{Rb}}[\mathrm{D.k4}] + d_{\mathrm{E.k2}\to\mathrm{Rb}}\,[\mathrm{E.k2}])/k_{\mathrm{Rb}})}$	(8)
$[\mathrm{Rb}_P] = [\mathrm{Rb}]\ \frac{d_{\mathrm{D.k4}\to\mathrm{Rb}}[\mathrm{D.k4}] + d_{\mathrm{E.k2}\to\mathrm{Rb}}\,[\mathrm{E.k2}]}{k_{\mathrm{Rb}}}$	(9)
k_X / $k_{Y\to X}$: Intrinsic/Y-dependent synthesis/activation rate of X	
d_X / $d_{Y\to X}$: Intrinsic/Y-dependent degradation/inactivation rate of X	
$C_{Y\to X}$: Intrinsic/Y-dependent control of the synthesis/activation rate of X	
$d_{E2F}=d_{D.k4}=d_{E.k2}=d_{p21}=d_{p53}=d_{Ap}=d_{Rib}=1$, $R_0=1$, $R_{max}=1$, $C_{Rb\to Rib}=5$, $C_{E.k2\to Rib}=0.0005$, $C_{p53\to Rib}=1$	
$k_{D.k4}=0.1$, $k_{GF\to D,k4}=0.9$, $k_{E2F}=0.1$, $k'_{E2F}=0.11$, $d_{Rb\to E2F}=10$, $\theta_{E2F}=0.5$, $C_{Rb\to E,k2}=10$, $k_{E.k2}=0.1$	
$k_{E2F\to E.k2}=0.9$, $d_{p21\to E.k2}=20$, $k_{p53}=0.1$, $k_{Str\to p53}=0.9$, $d_{E.k2\to p53}=5$, $k_{p21}=0.1$, $k_{p53\to p21}=0.9$, $d_{Ek2\to p21}=40$	
$k_{p53\to Ap}=0.5$, $k_{E2F\to Ap}=0.5$, $d_{p53.E2F\to Ap}=5$, $d_{GF\to Ap}=0.5$, $\theta_{Ap}=0.5$, $d_{D.k4\to Rb}=20$, $d_{E.k2\to Rb}=50$, $k_{Rb}=k'_{Rb}=Rb_{tot}=1$	

Cyclin D-Cdk4,6 complexes as extracellular growth factor sensors. Exit of cells from G_0 entails continuous mitogenic stimulation, which upregulates *cyclin D* mRNA levels and facilitates the formation, accumulation and activation of cyclin D-Cdk4,6 complexes. The increase in cyclin D-Cdk levels in response to interactions between mitogens and cell-surface receptors requires activation of the leading member of the Ras superfamily of small GTPases. Three major Ras effector pathways, mediated by Erk, PI3K and the Rho subfamily of small GTPases, cooperate in this process.[19] By contrast, activation of certain other signaling pathways (e.g., those involving cell-adhesion molecules or TGFβ) inhibits cyclin D-Cdk complex formation by inducing the expression of CKIs of the Ink4 family, which compete with the D-type cyclins for binding to Cdk4,6.[30] For the sake of simplicity, we consider that these conflicting signals combine to ultimatily generate a single input *IGF* that modulates cyclin D-Cdk levels (Equation 2, Table 1 and Fig. 1, pathway e). It should not be ignored, however, that there are many pathways that could regulate the rate of cyclin D-Cdk accumulation.

Rb regulation at the crossroad of G_1-phase signaling pathways. The first identified member of the retinoblastoma (Rb) protein family acts as a central pivot in the G_1 regulatory network, operating at the endpoint of signaling cascades which convey information concerning the extracellular environment and at the startpoint of

genetic cascades which elicit diverse responses to these inputs.[31,32] Indeed Rb, on the one side, is a principal phosphorylation substrate of cyclin D,E-Cdks but, on the other side, obstructs gene expression by two distinct mechanisms: (i) it binds to and blocks the transactivation domain of transcription factors, notably the E2F factors, which positively regulate RNA polymerase II (Fig. 1, pathways f and g); (ii) it mobilizes chromatin-modifying factors, and consequently it directly represses transcription, especially of rRNA and tRNA polymerases Pol I and Pol III (Fig. 1, pathway a). Rb can exist in three different phosphorylated forms, each of which exerts unique activities:[33] (1) unphosphorylated, Rb acts as a general transcriptional repressor inhibiting the activity of all three RNA polymerases;[25] (2) when partially (hypo) phosphorylated by cyclin D-Cdks (Fig. 1, pathway h), it loses its ability to directly repress transcription, especially that of rRNAs and *cyclin E*; (3) when hyperphosphorylated by cyclin E-Cdk2 (Fig. 1, pathway i), Rb dissociates from the E2F factors, enabling them to stimulate the transcription of genes involved in both cell division and cell death. In our model, phosphorylation/dephosphorylation reactions are assumed to be fast enough while the half-life of Rb is assumed to be long enough[34] so that the three forms are always in equilibrium (Equations 8 and 9, Table 1).

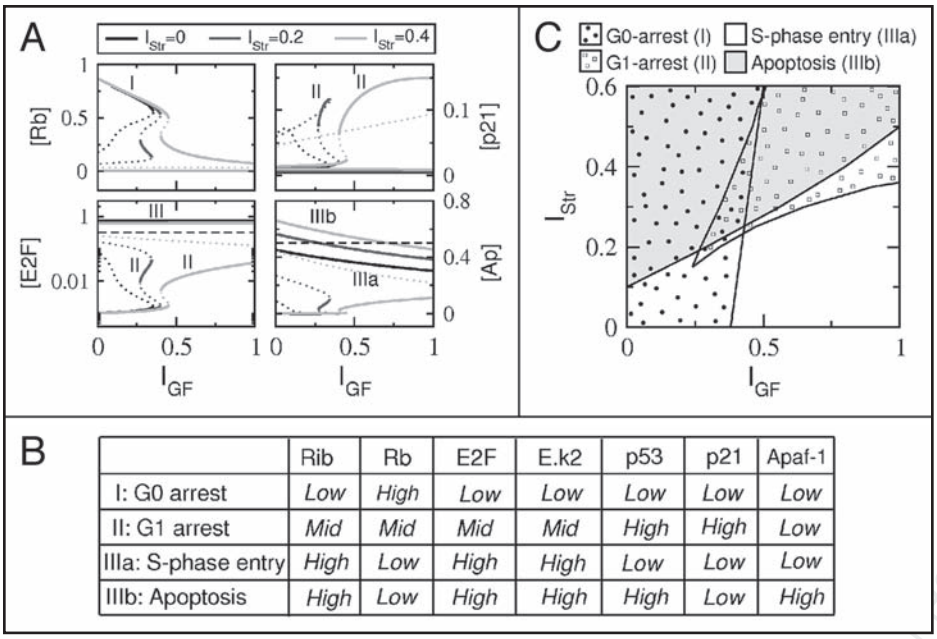
Regulation of activator E2Fs. There are two subclasses of E2F transcription factors, termed activator E2Fs (E2F1,2,3) and repressor E2Fs (E2F4,5). Activator E2Fs occur in cycling cells, where they induce transcription of a cohort of cell cycle regulatory as well as pro-apoptotic genes; notably they upregulate their own expression (Fig. 1, pathway j) as well as that of *cyclin E* (Fig. 1, pathway k). In contrast, repressor E2Fs primarily occur in quiescent and terminally-differentiated cells.[35] Rb is a powerful repressor of activator E2Fs and, thus, of cell cycle-regulating gene transcription. It is important to recognize that cyclin D-Cdk-mediated Rb phosphorylation does not dissociate the activator E2Fs, which would promote S-phase entry, a function which is strictly controlled by cyclin E-Cdk2 complex activity. Equation 3 (Table 1) recapitulates these regulations of activator E2Fs. Our model also assumes that DNA replication is initiated and S-phase entry takes place when the level of activator E2Fs reaches a threshold. E2F-mediated apoptosis involves many target genes of the activator E2Fs, some of which are transcriptional targets or pro-apoptotic cofactors of p53 as well[36-39] (Fig. 1, pathway l).

The stress-sensitive p53/p21 module. The tumor suppressor protein p53 is a ubiquitous transcription factor that not only upregulates the expression of genes involved in cell cycle arrest (e.g., p21) and in apoptosis (e.g., Bax, Apaf1), but also represses Pol I and Pol III transcription, and thus can interfere with ribosome biogenesis and cell growth[20] (Fig. 1, pathways c, n and o). The transactivation function of p53 is finely tuned to its nuclear level of expression due to the formation of a negative loop with the product of one of its target genes, Mdm2, which binds p53 and thereby inhibits its transcriptional activity and facilitates its nuclear export and degradation. p53 nuclear levels dramatically increase in response to a wide variety of stress signals (e.g., DNA damage, depletion of nucleotide triphosphates, hypoxia, nutrient deprivation, cell-cell contact, oncogenic signals, cell deformation) that alleviate its negative regulation by Mdm2. p53 nuclear exclusion, in turn, is facilitated by cyclin E-Cdk2 activity (Fig. 1, pathway p). For simplicity, we shall assume that p53 passively accumulates in the nucleus in the presence of stress (Fig. 1, pathway m) even though the negative feedback loop between Mdm2 and p53 should build up a non-linear p53 response (Equation 6, Table 1).

Stress-induced p53 nuclear accumulation and activation during the G_1 phase triggers cell cycle arrest that is mediated in great part by $p21^{Cip1}$, which belongs to the Cip/Kip family of CKIs. Like the two other family members ($p27^{Kip1}$, $p57^{Kip2}$), $p21^{Cip1}$ binds to and inhibits the activity of all cyclin-Cdk1,2 complexes (Fig. l, pathway q). Interestingly, it also binds to cyclin D-Cdk complexes and facilitates their nuclear import, but does not impair their kinase activity.[40] p21, in turn, is quickly downregulated upon phosphorylation by the cyclin-Cdk1,2 complexes, which indicates a strong mutually antagonistic interaction between these two components (Equation 7, Table 1; Fig. 1, pathway r). The expression of various Cip/Kip proteins is induced by different signals.[41] Our model focuses on p21, which was the first identified[42] and is the most thoroughly studied member of the Cip/Kip family, perhaps because it is the product of a main target gene of p53,[43] and a major mediator of p53-dependent cell cycle arrest. However, given their relatively high degree of homology with p21, it is likely that other Cip/Kip proteins could function similarly, albeit in different contexts.

Cyclin E-Cdk2 regulation. We recapitulate here all the processes described above, which either regulate or are regulated by the activity of cyclin E-Cdk2 complexes. On the one hand, unphosphorylated Rb and the activator E2Fs repress and upregulate, respectively, the expression of *cyclin E* mRNAs (Fig. 1, pathways f and k), which is prerequisite for the appearance of cyclin E-Cdk2 complexes, while p21 inhibits cyclin E-Cdk2 kinase activity (Equation 4, Table 1). On the other hand, cyclin E-Cdk2 hyperphosphorylates Rb, facilitates p53 nuclear export and downregulates p21 (Fig. 1, pathways i, p and r). Furthermore, cyclin E-Cdk2 kinase activity reinstates nucleolar assembly and pre-rRNA processing, which eventually leads to the restoration of ribosome synthesis (Fig. 1, pathway b).

Apoptosome-mediated initiation of cell death. Apoptosis refers to a form of programmed cell death, which results from the activation of caspases, a family of proteases that irreversibly cleave cellular proteins, generating dramatic physiological and morphological changes incompatible with cell survival. Apoptosis may be triggered via two main routes:[44] (i) an extrinsic pathway that is initiated upon binding of specific extracellular ligands to cell-surface death receptors, which mediate instructive apoptosis; (ii) an intrinsic pathway that is activated in response to endogenous stress signals which lead to the release of cytochrome c from mitochondria and subsequently drives the formation of apoptosomes and activation of caspases. Apoptosome formation is driven by the oligomerization of Apaf-1 (a caspase-9 cofactor) in response to cytochrome c binding in the presence of dATP or ATP. The intrinsic pathway is regulated at many levels by activator E2Fs and p53,[36,37] both of which transactivate Apaf-1,[45] (Fig. 1, pathways l and o). In turn, apoptosis is opposed by the activity of the PI3K/Akt pathway, which operates at both the transcriptional and translational levels via a multiplicity of mechanisms to promote cell survival[46] (Fig. 1, pathway s). Equation 7 (Table 1) formulates, in a simplified way, how Apaf-1 activation depends on the balance between the pro-apoptotic activities of the E2F factors and p53 vis-à-vis the anti-apoptotic effects of growth factors and survival factors. It is assumed that, when Apaf-1 reaches a threshold level, caspase-3 is irreversibly activated and induces apoptosis.[47]



B

	Rib	Rb	E2F	E.k2	p53	p21	Apaf-1
I: G0 arrest	*Low*	*High*	*Low*	*Low*	*Low*	*Low*	*Low*
II: G1 arrest	*Mid*	*Mid*	*Mid*	*Mid*	*High*	*High*	*Low*
IIIa: S-phase entry	*High*	*Low*	*High*	*High*	*Low*	*Low*	*Low*
IIIb: Apoptosis	*High*	*Low*	*High*	*High*	*High*	*Low*	*High*

Figure 2. The four steady-state attractors of the G_1 regulatory network. (A) Plots depicting the steady-state levels of Rb, E2F, p21 and Apaf-1 as a function of *IGF* for three different values of *IStr* ranging from 0 to 0.4, which reveal three branches (I–III) of stable solutions (full lines indicate stable states and dotted lines indicate unstable states; dashed lines indicate $\theta E2F$ and θAp, respectively). (B) Qualitative and relative estimation of the amounts of selected species in the four identified stable steady states of the G_1 regulatory network. (C) Overlapping stability domains of the four stable steady states of the G_1 regulatory network as a function of the intensities of the *IGF* and *IStr* signals. Coding : S-phase entry (white region), apoptosis (gray region), G_0 arrest (black dots) and G_1 arrest (empty squares).

Results

Cell fates as attractor states of the G_1 regulatory network. To begin with, we present a static picture of the behavior of the G_1 regulatory network predicted by our model. The bifurcation diagrams in Figure 2A show how the steady state levels of selected species (E2F, Apaf-1, Rb and p21) depend on the two main control parameters, *IGF* and *IStr*. There are clearly three separate classes of solution branches that differ in their content of the selected species. In one of the branches (III), the Apaf-1 level varies such that it could be either below (IIIa) or beyond (IIIb) a threshold at which a cell-death program is triggered. Thus, it is possible to qualitatively identify four distinct stable steady states that arise depending on cellular content of selected species, including ribosomes (Rib), unphosphorylated Rb, activator E2Fs (E2F), cyclin E-Cdk2 (E.k2), p53, p21 and Apaf-1 (Fig. 2B). We assume that these four classes of steady states correspond to the physiological states of G_0 arrest, G_1 arrest, S-phase entry and apoptosis. Their overlapping stability domains are shown in Figure 2C: (i) the G_0-arrest state is stable when the intensity of *IGF* signals is low enough and typically exhibits high Rb content but low contents of all other species; (ii) the state of S-phase entry is stable when the intensity of *IStr* signals is low enough and the intensity of *IGF* signals is high enough and it is characterized by high contents of ribosomes as well as of cyclin E-Cdk2 and E2Fs; (iii) the G_1-arrest state is stable when the intensities of *IGF* and *IStr* signals are high enough and typically displays high p53 and p21 levels, an intermediate ribosome supply, and relatively low levels of cyclin E-Cdk2 and E2Fs; (iv) the state of apoptosis is stable when the intensity of *IGF* signals is low enough and the intensity of *IStr* signals is high enough and is characterized by high levels of Apaf-1 along with high levels of cyclin E-Cdk2, E2Fs and p53. The existence of multistability between these distinct states allows us to predict that the final outcome depends not only on the actual signal intensities, but also on the history of the signal intensity changes.

It is important to recognize that the physiological states of G_0 arrest, G_1 arrest, S-phase entry or apoptosis do not have, strictly speaking, 'steady' properties. In fact, the physiological G_1-arrest state is transitory as G_1-arrested cells ultimately drift toward diverse differentiated or senescent states or even return to the cell cycle. Likewise, cells do not stay confined to S phase as they usually progress toward later cell cycle phases. However, for the purpose of our model, it is valid to consider these transient states as steady so long as we focus on the G_1 regulatory network dynamics and do not incorporate specific events activated downstream from cyclin E-Cdk2, E2Fs, Rb or p21.

In the following sections, we describe how our model predicts the possible mechanisms by which context-dependent multistability drive these different cell fates during the G_1 phase.

From G_0 arrest to S-phase entry: activating two positive feedbacks and crossing one irreversible transition. We first investigated how growth factors that stimulate both cell growth and cell division (*IGF*) drives G_1 regulatory network dynamics in the absence of p53/p21-activating signals (*IStr* = 0). In the absence of growth-factor stimulation, postmitotic cells exhibit high levels of unphosphorylated Rb as well as a generally moderate pool of ribosomes while the levels of cyclin-Cdk and of activator E2Fs (thereafter called E2Fs) are nearly nil (Fig. 3A). Physiologically, this stable state corresponds to G_0 arrest. A moderate level of *IGF* does not significantly modify the G_1 regulatory network activity but larger amounts can drive the network into a steady state characterized by a substantial pool of ribosomes as well as of hyperphosphorylated Rb, E2Fs and selected cyclin-Cdk complexes (cyclin D-Cdks and cyclin E-Cdk2). This state corresponds to S-phase entry in which [*E2F*] exceeds the critical value $\theta E2F$. The bifurcation diagram (right, Fig. 3) depicts how the stable G_0-arrest state disappears when *IGF* exceeds a critical amount.

Like the *IGF* strength, the duration of suprathreshold growth-factor stimulation is a determining factor in cell exit from G_0. If, indeed, growth factors are withdrawn before a specific time in G_1, the cell can readily return to the G_0-arrest state (Fig. 3B). If, however, growth factors are withdrawn beyond that specific time, the cell is unable to return to G_0 and it inevitably continues to drift toward the state of S-phase entry (Fig. 3C). This specific 'point of no-return' in the G_1 phase has been called the restriction point.[48,49] In terms of dynamical systems theory, it corresponds to the unstable boundary that separates the attractors of G_0-arrest and of S-phase entry in the absence of growth factors.

The irreversible switch between the states of G_0 arrest and S-phase entry entails the existence of a strong positive feedback loop within the G_1 regulatory network that could generate bistability between the two states. Our model enables us to identify the origin of this feedback, which actually results from a cascade of at least two intimately linked positive feedback loops. The bifurcation diagrams of Figure 4 emphasize the critical role played in this process by the differential capacity of cyclin D-Cdks and cyclin E-Cdk2 to phosphorylate Rb. For *dE.k2→Rb* = 0, the main operational G_1 regulatory module comprises cyclin D-Cdks, hypophosphorylated Rb, and a moderate pool of cyclin E-Cdk2 and of ribosomes ('cyclin D-Cdks/Rb/cyclin E-Cdk2/Ribosome loop'): cyclin D-Cdks partially phosphorylate Rb, relieving the Rb-dependent repression of rRNA and *cyclin E* transcription and, thereby, reinstate ribosome and protein synthesis (operating with the pre-existing pool of ribosomes and the low-level of emerging cyclin E-Cdk2 activity). The resulting increase in overall protein translation, in turn, contributes to raising the levels of all cyclin-Cdks. Such a multiple-path positive feedback loop can induce bistability between the G_0-arrest state and an 'active' state with increased levels of ribosomes and cyclin-Cdks (Fig. 4A; light gray line). However, as long as cyclin E-Cdk2 does not efficiently phosphorylate Rb, the rise in cyclin E-Cdk2 and E2F levels is relatively modest because cyclin D-Cdk-dependent Rb phosphorylation does not induce E2F release from the Rb-E2F complex efficiently. In contrast, at large *dE.k2→Rb* values, the levels of cyclin E-Cdk2 and E2Fs increase significantly (Fig. 4A; dark gray and black lines) in response to the activation of a positive feedback loop which drives cyclin E-Cdk2-dependent hyperphosphorylation of Rb, thereby liberating the E2F factors and enabling them to boost their own synthesis as well as that of cyclin E ('cyclin E-Cdk2/Rb/E2F loop'). Thus, the steady state concentration of cyclin E-Cdk2 exhibits a discrete transition from low to high values as *dE.k2→Rb* increases (Fig. 4B).

In summary, activation of the cyclin D-Cdks/Rb/cyclin E-Cdk2/Ribosome loop enables to restore ribosome biogenesis and protein synthesis, which does not require a high level of cyclin E-Cdk2 activity. On the other hand, activation of the cyclin E-Cdk2/Rb/E2F loop is necessary to promote S-phase entry. Thus, early activation of the cyclin D-Cdks/Rb/cyclin E-Cdk2/Ribosome loop contributes to the later activation of the cyclin E-Cdk2/Rb/E2F loop because cyclin E-Cdk2 accumulation depends on both the translational cell capacity and Rb phosphorylation status. It should be possible, however, to uncouple the two loops by changing parameters other than *dE.k2→Rb*. Indeed, any process that would straightaway prohibits or promotes cyclin E or E2F accumulation would consequently repress or activate the cyclin E-Cdk2/Rb/E2F loop.

Stress-induced G_1 arrest versus apoptosis. The pivot of the cyclin E-Cdk2/Rb/E2F loop is the activity of the cyclin E-Cdk2 complex, which controls the release of E2F factors that activate cell cycle-regulating gene transcription and promotes S-phase entry. Therefore, negative regulation of cyclin E-Cdk2 by physiological inhibitors such as Cip/Kip proteins (e.g., p21) are expected to interfere with G_1-phase progression toward S-phase.

We applied our model to investigate how p53-activating stresses modify G_1 regulatory network dynamics in the presence of growth factors that otherwise impel cell exit from quiescence (Fig. 5). Exposure to a mild stress intensity (*IStr* = 0.3), which results in rates of p53 and p21 accumulation that are insufficient to stop the activation of the cyclin E-Cdk2/Rb/E2F loop, only delays G_1-phase progression and entry into S phase (Fig. 5A). However, exposure to a more severe stress intensity (e.g., *IStr* = 0.4), results in higher rates of p53 and p21 accumulation that stop activation of the cyclin E-Cdk2/Rb/E2F loop and halt G_1-phase progression at a steady state that is distinct from G_0 arrest or S-phase entry (Fig. 5B). The bifurcation diagram depicted in Figure 5B (right) depicts how, in this case, a new steady state branch appears and coexists with the two other stable branches corresponding to G_0 arrest and S-phase entry. This new branch, which we call the 'G_1-arrest' state, is characterized by low levels of E2Fs and cyclin E-Cdk2 ([*E.k2*] ~ 0.01) as occurs in the situation where *Cp21→E.k2* = 0 and *dE.k2→Rb* = 0 (Fig. 4B). This suggests that G_1 arrest results from a failure to activate

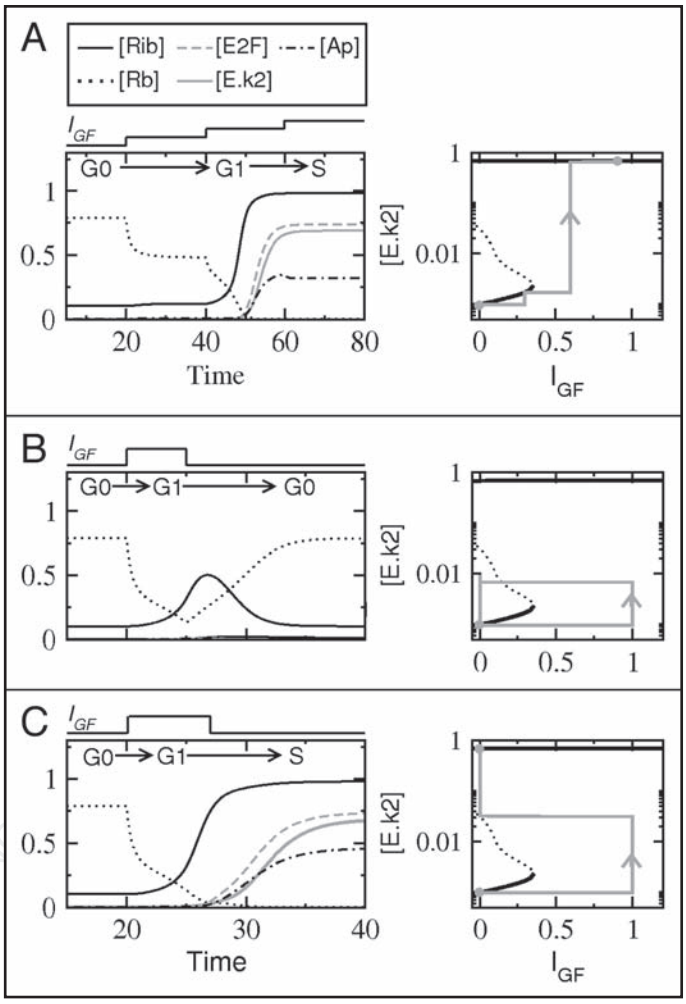


Figure 3. S-phase entry requires a minimal strength and duration of growth-factor signals. Results from simulation and bifurcation analysis of the G_1 regulatory network dynamics in the absence of stress signals (*IStr* = 0). Left: Plots of concentration changes over time of selected components of the G_1 regulatory network in response to a step or pulse of *IGF*. Right: Corresponding plots of the cyclin E-Cdk2 concentration changes as a function of *IGF* (gray lines), as compared to its steady-state level (full lines indicate stable states and dotted lines indicate unstable states): (A) Successive increasing *IGF* steps: 0, 0.3, 0.6 and 0.9. (B) A 'short' pulse of growth factors (*IGF* = 1) is applied from *t* = 20 to *t* = 25. (C) A 'long' pulse of growth factors (*IGF* = 1) is applied from *t* = 20 to *t* = 27.

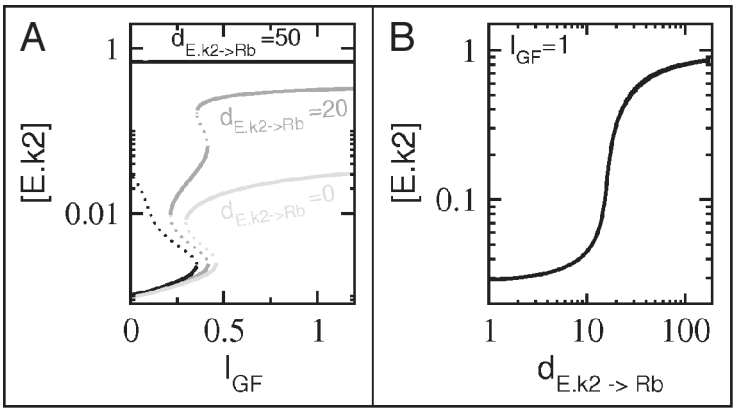
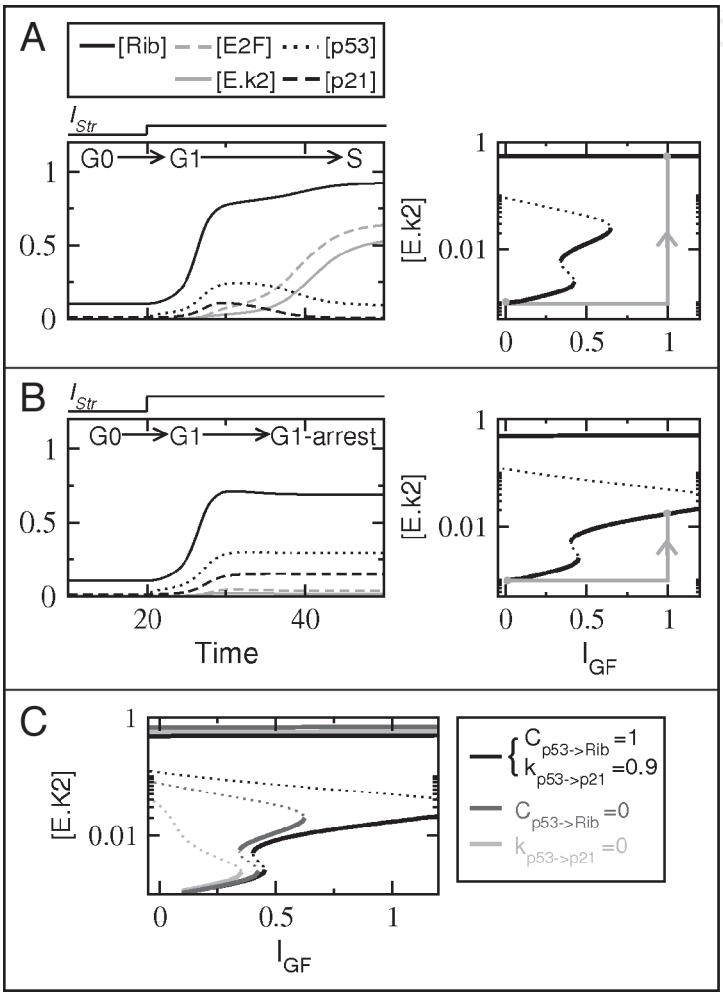


Figure 4. Coupled growth-activatory and division-activatory positive feedback loops. Bifurcation diagrams demonstrating the presence of two intricately entwined positive feedback loops within the G_1 regulatory network (full lines indicate stable states and dotted lines indicate unstable states). (A) Steady-state levels of cyclin E-Cdk2 as a function of *IGF* for three values of *dE.k2→Rb* (light gray line: 0; dark gray line: 20; black line: 50). (B) Steady state level of cyclin E-Cdk2 as a function of *dE.k2→Rb* in the presence of growth-activatory signals (*IGF* = 1).



the cyclin E-Cdk2/Rb/E2F loop. The exact mechanism of failure is depicted on the bifurcation diagram obtained as a function of *IGF* for different values of *kp53→p21* and of *Cp53→Rib* in the presence of stress (*IStr* = 0.4) (Fig. 5C). Following stress-induced p53 activation, the branch corresponding to the G_1-arrest steady state inflates proportionately to the extent of p53-dependent p21 induction or downregulation of ribosome synthesis. Thus, two distinct processes cooperate to induce G_1 arrest in response to p53-activating stresses: (i) p21-dependent cyclin E-Cdk2 inhibition, which results in the requirement for a larger ribosome supply to support activation of the cyclin E-Cdk2/Rb/E2F loop and (ii) p53-dependent downregulation of ribosome synthesis.

Because the G_1 regulatory network model exhibits bistability between the states of G_1 arrest and S-phase entry, we predict that the time in G_1 phase at which the stress signal is applied would have a critical impact on cell fate. Figure 6 depicts the differential effect of stress (*IStr* = 0.4) exposure at different times after growth factor stimulation. When the stress is applied at time *t* = 26, inhibition of the cyclin E-Cdk2 complexes is sufficient to drive G_1 arrest (Fig. 6A). However, when the stress is applied slightly later (*t* = 28), it is unable to sufficiently repress cyclin E-Cdk2 activity to block the surge in E2F factor concentrations, which otherwise precipitate entry into S phase (Fig. 6B). In the later case, however, p53 continues to accumulate in the nucleus of late-G_1/S-phase cells, where it can activate pro-apoptotic target genes. As a result, the simultaneous presence of high levels of p53 and E2Fs leads to increased apoptosome activity in late G_1/S-phase cells, which eventually surpasses the threshold required to trigger cell-death programs (Fig. 6C). In summary, E2F factors and p53 cooperate to trigger apoptosis when p53-dependent p21 accumulation in G_1 phase fails to arrest cell cycle progression by preventing activation of cyclin E-Cdk2 above the threshold necessary to free E2F factors.

Bypassing the *Rb* module. In the previous section, we established that the selective inhibition of cyclin E-Cdk2 by stress-induced CKIs is able to stop activation of the cyclin E-Cdk2/Rb/E2F loop and create a stable G_1-arrest state. The G_1-arrest state especially differs from the G_0-arrest state with respect to its ribosome content. Moreover, it is not incompatible with continued cell growth in the absence of cell division as long as Rb continues to prevent the liberation of E2F factors. Thus, the probability that a cell arrests in G_1 and eventually drifts toward a differentiated or senescent state depends on the presence of a functional *Rb* module. In the absence of Rb, E2F factors would be constitutively activated and

Figure 5. Stress-induced G_1 delay and arrest. (A and B) Simulation and analysis of G_1 regulatory network dynamics in the presence of various steps of stress (A: *IStr* = 0.3; B: *IStr* = 0.4). Left: Plots of the concentration changes over time of selected components of the G_1 regulatory network in response to *IGF* and *IStr* steps, simultaneously applied at time *t* = 20. Right: Corresponding plots of the changes in cyclin E-Cdk2 concentration as a function of *IGF* (gray lines), as compared to its steady-state level (full lines indicate stable states and dotted lines indicate unstable states). (A) a moderate step of stress delays S-phase entry while (B) a slightly larger step induces G_1 arrest (characterized by high levels of p53 and p21 and low levels of E2F and cyclin E-Cdk2). (C) Bifurcation diagrams as a function of *IGF* in situations in which stress application results in (i) p53-dependent induction of p21 only (dark gray: *Cp53→Rib* = 0; *kp53→p21* = 0.9), (ii) p53-dependent downregulation of ribosome synthesis only (light gray: *Cp53→Rib* = 1; *kp53→p21* = 0) or (iii) both (black: *Cp53→Rib* = 1; *kp53→p21* = 0.9).

E2F-dependent cell cycle-regulating gene transcription and ribosome synthesis would simultaneously take place after mitotic exit in the absence of cyclin D-Cdk complexes. As shown in Figure 7A, *Rb*-negative (*Rb*$^-$) cells are unable to undergo G_0 arrest because cyclin E-Cdk2 and E2F factors accumulate even in the absence of growth-factor signals (*IGF* = 0). As a result, *Rb*$^-$ cells become sensitized to apoptosis because, in the absence of growth factors, they fail to experience levels of anti-apoptotic factors sufficient to overcome the pro-apoptotic activity of E2F factors. This scenario of apoptosis does not require the presence of stress signals such as those that activate p53 functions. Instead, it proceeds from a premature activation of E2F factors, which leads to a precipitate loading of pro-apoptotic factors that is not countered at the time by a large enough cell provisioning of survival factors from the environment. The absence of a G_0-arrest state and the enhanced sensitivity to apoptosis associated with the loss of *Rb* function is depicted in the phase diagram of Figure 7B. E2F or *cyclin E* upregulation should a priori have the same effect as *Rb* loss, considering that E2F factors induce *cyclin E* expression and that cyclin E-Cdk2 complexes can substitute for the cyclin D-Cdk complexes to relieve *Rb*-dependent repression of RNA Pol I and III transcription.

A toy model of the G_1 regulatory network. Dissecting the G_1 regulatory network allowed us to extract the main feedback/feedforward structure that underlies the context-dependent switch between, on one side, the state of G_0 arrest, and, on the other side, the states of S-phase entry, G_1 arrest or apoptosis: the growth factor-stimulated cyclin D-Cdks/Rb/cyclin E-Cdk2/Ribosome loop triggers a competition between the cyclin E-Cdk2/Rb/E2F loop and the stress-activated p53/p21 pathway. We have designed a toy model based on this feedback/feedforward structure to verify whether such a simple organization is necessary and sufficient to reproduce the qualitative dynamic properties of the G_1 regulatory network. The model contains four variables, each of them represents the 'activity' of one particular module of the G_1 regulatory network: the cyclin D-Cdks/Rb/cyclin E-Cdk2/Ribosome module (*M1*), the cyclin E-Cdk2/Rb/E2F module (*M2*), the p53/p21 module (*M3*) and the apoptosome module (*M4*). The internal dynamics of *M1*, *M3* and *M4* contains one positive feedback loop. *IGF* switches on *M1* while *IStr* switches on *M3* and *M4*. *M1* activates *M2* and *M3* while *M2* and *M3* inhibit each other and *M2* activates *M1* and *M4* (Fig. 8A and Table 2). Using an appropriate and carefully-selected set of parameters, the dynamics of the model displays a phase diagram closely similar to that of the original model (compare Figs. 8B and C with 2A and B). Hence, the toy model preserves the bifurcation structure of the original model, which supports the view that the modular organization of the G_1 regulatory network depicted in Figure 8A portrays critical roles in determining cell fate during the G_1 phase. Such a match, however, is very sensitive to parameter changes, which are likely to induce gradual rather hysteretic transitions between steady states, or even prevent such transitions. We therefore conclude that the complexification of more realistic G_1 regulatory networks would contribute to reinforce the robustness of their switching behavior against mild change in kinetics parameters.

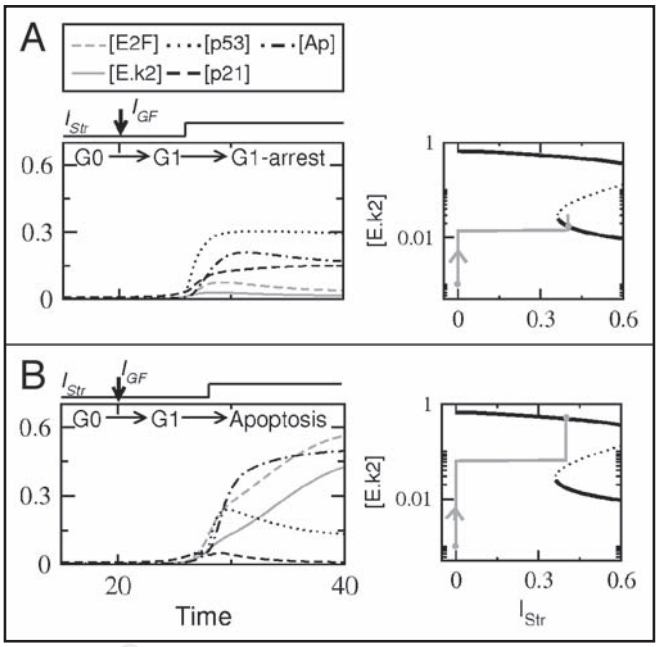


Figure 6. E2Fs and p53 cooperate in promoting apoptosis. Simulation and analysis of G_1 regulatory network dynamics in the presence of an *IStr* step elicited at various times. Left: Plots of the concentration changes over time of selected components of the G_1 regulatory network in response to a *IGF* step applied at time t = 20 and *IStr* step applied later at various time. Right: Corresponding plots of the changes in cyclin E-Cdk2 level as a function of *IGF* (gray lines), as compared to its steady-state level (full lines indicate stable states and dotted lines indicate unstable states). (A) A stress step (*IStr* = 0.4) applied at time *t* = 26 induces G_1 arrest with high levels of p53 and p21. (B) The same stress step applied at time *t* = 28 induces apoptosis with high levels of E2F, p53 and Apaf-1.

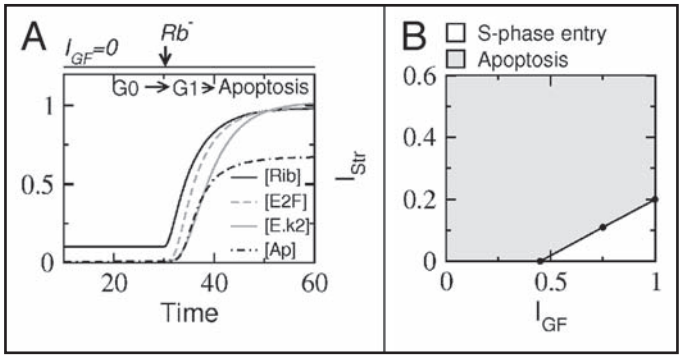


Figure 7. Bypassing the Rb module leads to premature S-phase entry and promotes apoptosis. (A) Concentration changes over time of selected components of the G_1 regulatory network following the disruption of *Rb* function ([*Rb*] = [*Rbp*] = 0) at time *t* = 30 , in the absence of growth-activatory signals (*IGF* = 0). The G_1 regulatory network switches from the G_0-arrest state to apoptosis ([*Ap*] > θ*Ap*). (B) The phase diagram of an *Rb*-deficient cell shows a large apoptotic region while the G_1-arrest and G_0-arrest domains totally vanish.

Discussion

Uncoupling cell growth and cell division. Our findings underscore that simple organizing principles can explain how the mammalian G_1-phase regulatory network dictates the issue whether a cell would divide or shift into out-of-cycle states such as G_0 or G_1 arrests or apoptosis in response to environmental stimuli. These principles depend on the unique organization of the G_1 regulatory network, which consists of two temporally separable but flexibly coupled positive feedback loops: an upstream 'growth-activatory loop' and

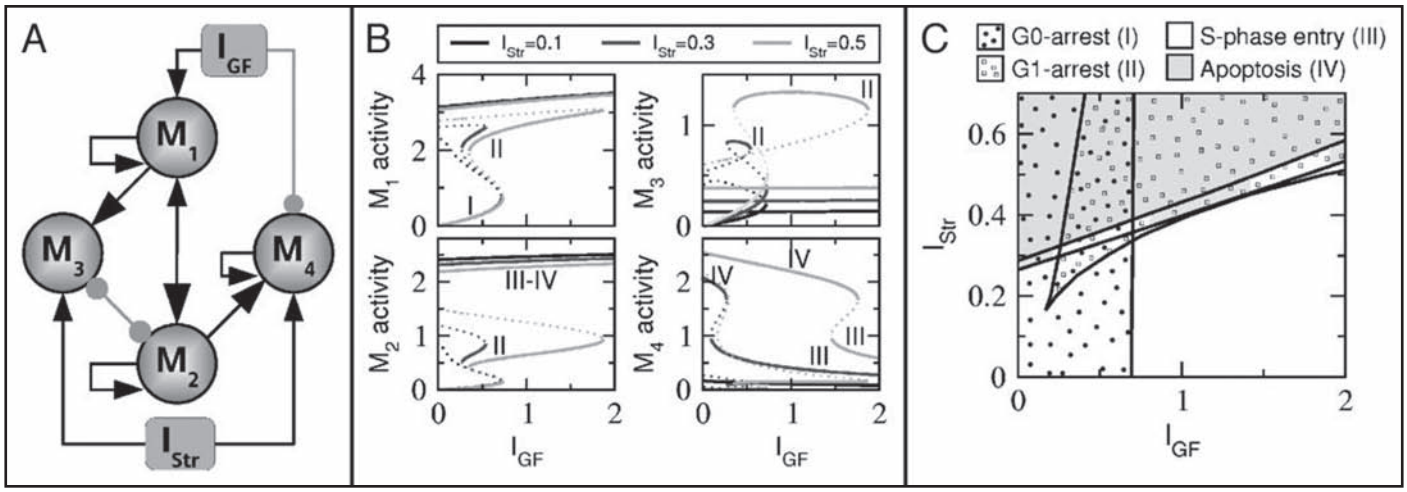


Figure 8. Toy model of the G_1 regulatory network. (A) Reduced representation of the G_1 regulatory network as a set of interactive modules. (B) Steady-state levels of the M1, M2, M3, M4 activities for three values of *IStr* (0, 0.4, 0.8). (C) Phase diagram corresponding to the toy model, the equations of which are displayed in Table 2.

Table 2 Differential equations for the toy model of the G_1 regulatory network

$d\,M_1/dt = (2\,I_{GF} + 3M_2) - 5M_1 + 4M_1^2 - M_1^3$	(1)
$d\,M_2/dt = M_1 - (5 + M_3)M_2 + 4M_2^2 - M_2^3$	(2)
$d\,M_3/dt = M_1(I_{Str} + 0.2) - (1 + M_2^2)M^3$	(3)
$d\,M_4/dt = 3M_2\,I_{Str}/(1 + 0.5I_{GF}) - 5M_4 + 4M_4^2 - M_4^3$	(4)

a downstream 'division-activatory loop', certain components of which have antagonistic effects on pathways involved in apoptosis in G_1-arrest (Fig. 9A).

Cells that exit mitosis are exposed during the G_1 phase to a multitude of stimuli and stresses that differ in their capacity to activate or inhibit these two loop mechanisms. Thus, cell fate determination during the G_1 phase is dictated by the specific combination of stimuli and stresses that the cell senses. G_1-specific regulatory signals impinge on the two loops principally at the level of two well-defined cyclin-dependent protein kinases, cyclin D-Cdk4,6 and cyclin E-Cdk2, that differ in their timing of apparition, their mode of regulation and their substrate specificity. In the absence of signals that positively or negatively regulate cyclin E-Cdk2 activity, inactivation or activation of the growth-activating loop merely triggers the inactivation or activation of the division-activating loop, giving rise to the states of G_0-arrest or S-phase entry, respectively (Fig. 9B and C). But, activation of stress-response pathways that directly affect cyclin E-Cdk2 activity may alter the tight coordination between growth and division that is required to initiate timely cell division. For instance, selective inhibition of cyclin E-Cdk2 by p53/p21-activating stresses in the presence of growth-factor stimulation leads to the emergence of high-energy G_1-arrest states (Fig. 9D). By contrast, selective cyclin E-Cdk2 upregulation (e.g., via *Myc* overexpression or *Rb* loss of function) in the absence of sufficient levels of growth and survival factors would favor apoptosis (Fig. 9E). Thus, our modeling study supports the view that the inherent ambivalence of signaling pathways, which, on one side, could be activated by a multiplicity of conflicting signals and, on the other side, could drive opposing cellular responses, lays the foundations for cell fate diversification in mammalian cells.[5,50-52]

This antagonistic relationship between cell-growth regulators, which facilitate G_1-arrest and pro-survival pathways and cell division regulators, which facilitate apoptosis and strongly compete with components of G_1-arrest pathways, possibly originates from the ancestral device elaborated by unicellular systems to coordinate their growth and division. Indeed, in order to quickly reproduce under the best of conditions, unicellular organisms have evolved cell-size and DNA-damage checkpoints that enable them to delay cell cycle entry to allow enough 'time to grow' and enough 'time to repair', respectively.[3,53] Multi-celled organisms, however, have developed more sophisticated mechanisms. Notably, they created new cell fates for their unit cells, such as apoptosis or permanent G_1 arrest, to allow, for instance, the shaping of organs during development and the safeguarding of tissue homeostasis in adulthood.[4,54] The G_1 regulatory checkpoints inherited from unicellular systems have probably been relaxed and made more flexible in metazoan cells in order to adapt to new constraints imposed by the necessity of preserving the viability of the organism as a whole. Owing to this flexibility, specific environmental signals may now either precipitate cell division in the absence of sufficient prior cell growth, thus triggering apoptosis, or delay cell division, possibly forever, despite continued cell growth, thus inducing G_1 arrest.

Exiting from G_0 arrest. G_0 arrest (or quiescence) usually describes a collection of low-energy states in which specific sets of genes involved in repressing the transcription of rRNA and *cyclins* are activated.[55] This low-metabolism state can be induced in vitro by serum starvation, which deprives cells of mitogens and growth factors. Conversely, re-exposure of cells to these factors triggers G_0 exit following activation of a positive feedback loop (the cyclin D-Cdks/Rb/cyclin E-Cdk2/Ribosome loop), which primarily functions to relieve *Rb*-dependent repression of rRNA and *cyclin* transcription and restore ribosome biogenesis. The restriction point[48,49] coincides with the time point in the G_1 phase when cyclin E-Cdk2 kinase activity reaches the critical level necessary to achieve

hyperphosphorylation of Rb and activate a second positive feedback loop (the cyclin E-Cdk2/Rb/E2F loop), which primarily functions to promote growth factor-independent cell cycle progression. An elegant study combining experiments and modeling provides convincing evidence of the implication of the second loop in this function.[56] Our findings predict that the critical level of cyclin E-Cdk2 activity required to cross the R point is much lower than that required to enter S-phase. This would be difficult to detect experimentally, which may explain why it has been previously stated that R point transit precedes cyclin E-Cdk2 accumulation.[57]

Shifting to G_1 arrest. The relief of *Rb*-dependent repression of rRNA, ribosomal protein and cyclin synthesis associated with early G_1-phase progression is necessary but not sufficient to activate the second positive feedback loop, whereby the cyclin E-Cdk2 complex frees E2F factors and boosts its own production. Indeed, any event that interferes with the accumulation and/or activation of cyclin E-Cdk2 complexes could consequently prevent activation of the cyclin E-Cdk2/Rb/E2F loop and irreversible transit into S phase. This is what occurs in the presence of p53/p21-activating stresses that induce G_1 arrest via a combination of two distinct processes: (i) p21-dependent inhibition of cyclin E-Cdk2; and (ii) p53-dependent downregulation of ribosome synthesis. Thus, G_1-arrest states would emerge following exposure of cells to various forms of cytotoxic as well as genotoxic stresses that induce the accumulation of CKIs in the Cip/Kip family, which directly inhibit cyclin E-Cdk2 complex activity. In sharp contrast to the G_0-arrest state, G_1-arrest states require mitogens and growth factors.[52] Our model, however, does not discriminate between (i) reversible G_1 arrest from which the cell can readily exit to return into a viable cell cycle and (ii) irreversible (or permanent) G_1 arrest, which may ultimately drift toward a senescent or a differentiated state.

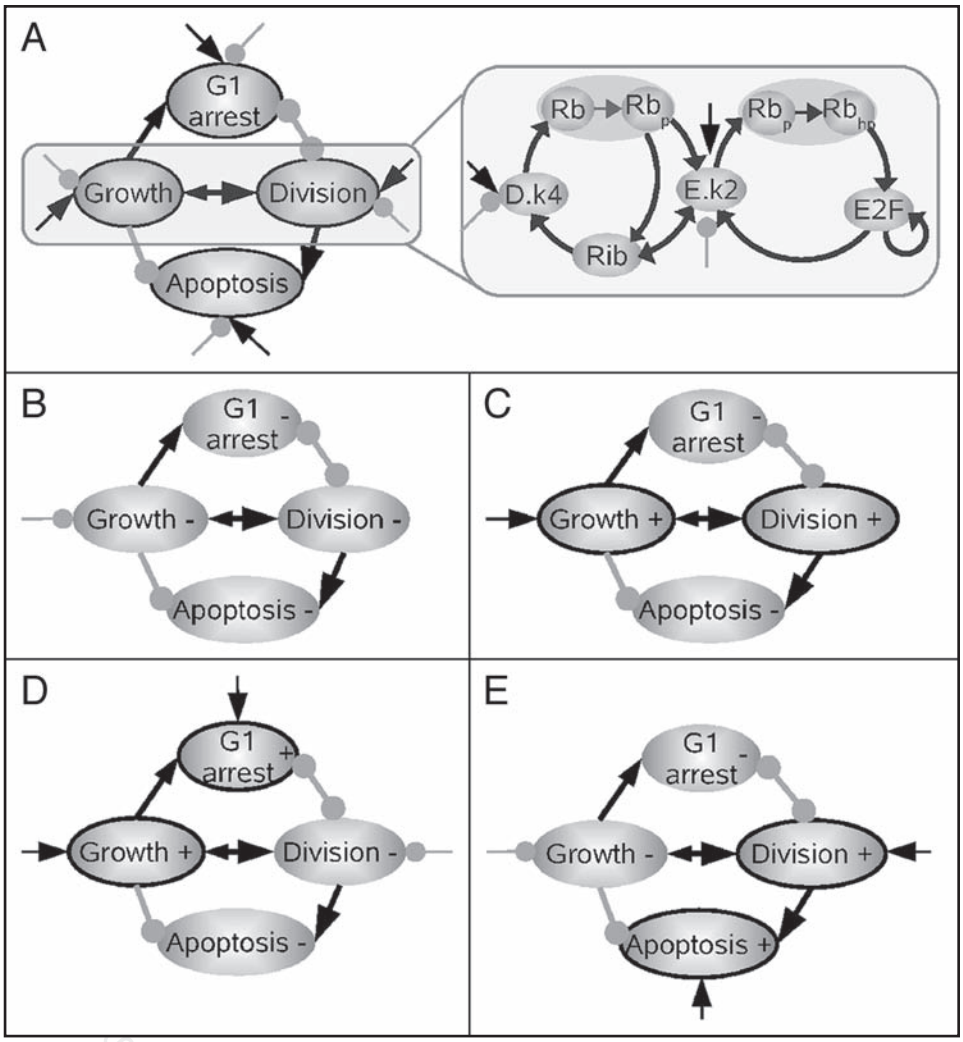


Figure 9. Cell fates associated with various patterns of growth-division relationship. (A) The positively coupled growth-activatory and division-activatory modules have antagonist effets on stress-activated modules involved in driving apoptosis and G_1-arrest. These core modules comprise a multiple path positive feedback loop (Abbreviations: E.k2, cyclin E-Cdk2; D.k4, cyclin D-Cdk4,6; E2F, activator E2Fs; Rib, Ribosomes; Rb_p, hypophosphorylated Rb; Rb_{hp}, hyperphos-phorylated Rb). (B) Inhibition of the growth-activatory module prevents activation of the division-activatory module, thus producing a G_0-arrest state. (C) Activation of the growth-activatory module stimulates the division-activatory module, thus triggering S-phase entry. (D) Selective inhibition of the division-activatory module associated with stimulation of the growth-activatory module leads to a G_1-arrest state. (E) Selective activation of the division-activatory module without sufficient prior stimulation of the growth-activatory module promotes apoptosis.

In principle, stress-induced CKI-dependent G_1 arrest should be reversible/irreversible if the events arising during breaks in the cell cycle were transitory/persistent and would vanish/persist after stress removal. The nature and intensity of the division-inhibitory stress signal(s) as well as their timing of application should be determining factors in the drive toward reversible or irreversible G_1 arrest, senescence or differentiation. In the case of irreversible outcomes, our model predicts that the impact of the G_1 arrest-promoting signals would not be restricted to simply inducing CKI accumulation and subsequent CKI-dependent inhibition of the cyclin E-Cdk2 complex activity. The p53 tumor suppressor protein may serve as a paradigm on this subject.

DNA damage, which typically produces irreversible p53 responses (irreversible G_1 and G_2 arrests or apoptosis) operates by activating a set of protein kinases (ATM, ATR, Chk1,2) that phosphorylate both p53 and Mdm2 at multiple sites, irreversibly disrupting p53-Mdm2 interaction.[58] In contrast, oncogenic stresses activate p53 function by inducing the expression of the Arf protein, which binds to Mdm2 to mitigate its antagonistic effects on p53 without disrupting the p53-Mdm2 interaction.[59] Arf-dependent p53/p21-activating stresses could thereby trigger reversible G_1 arrest. The fact that Arf/p53-dependent oncogenic *Ras*-induced senescence is readily reversed by *Myc* overexpression[60] is consistent with this prediction. In the case of p53-activating stresses, it appears that the reversibility of

p21-dependent G_1 arrest depends on whether nuclear p53 accumulation and activation as a transcription factor is reversible.[58,61,62] p21, however, can also be upregulated via p53-independent pathways, for example during differentiation.[63-66] Therefore, in order for our model to take into account how cells are driven into different fates following initial p53-dependent cell cycle arrest, we must include potential concurrent mechanisms of CKI activation as well as the intricate p53 interaction network with its numerous coregulators such as Arf, Mdm2, ATM, Chk1, and so on.

Shifting to cell death. Once a cell has been exposed to sufficient levels of mitogens and growth factors to bypass both G_0 and G_1 arrests, it can still initiate DNA replication or die because E2F transcription factors can initiate both of these processes. Our model anticipates that two distinct situations favor apoptosis.

First, even in the absence of p53-activating stresses, cells must acquire a sufficient load of anti-apoptotic signals to overcome the pro-apoptotic activity of E2F factors and progress into S-phase. Anti-apoptotic cues are provided in great part by growth factor-dependent activation of the PI3K/Akt pathway, but also (at least in the case epithelial cells) by their physical attachment to neighboring cells or to the extracellular matrix.[4,46,67] Thus, premature activation of the cyclin E-Cdk2/Rb/E2F loop resulting from defective cell adhesion and/or growing capacity should favor cell death over S-phase entry. Our model depicts how this scenario occurs upon loss of *Rb*. In *Rb*- cells, the cyclin E-Cdk2/Rb/E2F loop is constitutively activated so that *Rb*- cells, which lack a restriction point control, can not retire in the G_0 state while they are unable to develop a rate of macromolecular synthesis sufficient to support cell division. This explains why they are prone to undergo apoptosis in response to growth-factor deprivation. Indeed, it is well known that *Rb*- cells in culture exhibit accelerated rates of S-phase entry and apoptosis. Moreover, the ratio between the rates of apoptosis and S-phase entry increases with decreasing serum concentration.[68] The observation that *E2F1* loss suppresses apoptosis and inappropriate S-phase entry in *Rb*- mouse embryos further argues that apoptosis in *Rb*- mouse embryos is driven by *E2F1* upregulation.[69]

Second, in the presence of p53-activating stresses, nuclear p53 accumulation may lead to activation of a set of pro-apoptotic p53 target genes and thereby increase the pro-apoptotic load of the cell. p53 has indeed been reported to induce the expression of approximately 5,000 target genes.[70] A few of these (e.g., p21) mediate its cell cycle arrest functions. However, many of them mediate its pro-apoptotic activities by participating in both the extrinsic and intrinsic apoptotic pathways.[44] Our analysis predicts that a transitional point divides the G_1 phase in two domains in which p53-activating stresses generate opposite cell fates: G_1 arrest would occur upstream from this point, and apoptosis, downstream from it. This transitional point coincides with the time when cyclin E-Cdk2 activity reaches the critical level sufficient to bypass p21-dependent inactivation and activate the positive cyclin E-Cdk2/Rb/E2F feedback loop, allowing thereby the explosive rise in activator E2F levels. Beyond this point, both p53 and E2F are predicted to be present at relatively high concentrations in the nucleus where they would cooperate to increase the load of pro-apoptotic signals above a threshold capable of driving completion of a cell-death program, despite the possible presence of an abundance of growth and survival factors.[38,39,71]

Limits and perspectives of the model. By definition, models are an abstraction of reality because they require simplifying assumptions to deal with constraints imposed by the technical tools, the state of scientific knowledge and the modeler's questions. Our coarse-grained model of the G_1 regulatory network of mammalian cells is based on a simplified description of activities linked to signal transduction, cell growth, cell division and cell death. Admittedly, it neglects a multitude of variant pathways that could significantly contribute to signal-specific and cell-type-specific events. We focused on selected members of the Rb, E2F, p53 or Cip/Kip families but realize that other members of these families may have redundant and/or complementary functions, depending on the cellular context.[8] The model does not exhaustively take into account either the ubiquituous interactions between cell-growth and cell-division processes. Notably, it ignores the competition between growth-related protein mRNAs and division-related protein mRNAs for monopolizing the translational cell machinery, which most likely contributes to establishing an antagonistic relationship between cell growth and cell division.[72] It also ignores the possible impact of the growth-dependent increase in cell size on the rate of nucleocytoplasmic transport and, then, on the rate of appearance of active cyclin-Cdk complexes in the nucleus.[73] Furthermore, the model focuses on p53-activating stresses without stipulating the particular type of stress involved (DNA damage, ribonucleotide depletion, cell adhesion, oncogenic signals, telomere shortening, and so on), even though it is known that each type of stress utilizes unique mechanisms to activate p53 function. Finally, p53-independent stress signals are not considered in our model.

Yet, our simplified model provides a sound description of how the organization of the mammalian G_1 regulatory network as a set of flexibly interlinked positive feedkback loops enables cells to integrate two streams of opposite input signals and offers them the alternative between four diverging fates. The utmost important dynamic feature associated with these positive feedbacks is the emergence of multistable states, which offer cells the possibility to switch, eventually irreversibly, from one state to another.[74] Switching behavior is a well-recognized biological phenomenon associated with cell cycle progression[13,75,76] as well as cell differentiation[21,77] and biochemical signaling.[78] From an evolutionary perspective, the model provides a valuable framework for understanding the evolution toward multicellularity that is marked by a magnitudinous jump in cell-fate multiplication and diversification despite the fact that many core processes that participate in cell fate determination, especially cyclin-Cdk-based cell cycle, are highly conserved from yeast to mammals.[53,79] Finally, our model stresses the importance of the *Rb* and *p53* modules in context-dependent cell-fate determination and, thereby, in the safeguarding of tissue homeostasis and organism's defence against cancer.[8]

Material and Methods

Numerical methods. The differential equations used to simulate the G_1 regulatory network model were integrated using the second-order Runge-Kutta scheme with fixed-time step: $t = 0.01$. Bifurcation diagrams were constructed with AUTO2000.[80]

Assumptions of the model. To simplify the equations, several important assumptions were made: (i) The variables are protein concentrations. Because of their rapid turnover, mRNAs levels are assumed to be always at steady states.

(ii) The effects of many regulations, through which one component triggers the activation/synthesis or inactivation/degradation of another at either the transcriptional or translational level, have been linearized.

Only the E2F-dependent synthesis of the E2F factors and the regulation of ribosome synthesis exhibit saturation kinetics described by Hill functions.

(iii) Regarding ribosome synthesis, we assume linear dynamics controlled by effective synthesis and degradation rates. This is certainly a rough approximation since the real dynamics of ribosome concentration is intrinsically non-linear. Indeed, the cell growth rate depends on the ribosome concentration and the dilution rate associated with cell volume expansion is not smaller than the ribosome degradation rate. That is, we assume that the dilution rate associated with cell volume expansion is much smaller than the degradation rates of proteins.

Choice of the kinetic parameters. The choice of the parameters is mostly arbitrary because of the lack of data regarding the rate constants of the physiological reactions that participate in the G_1 regulatory network and, also, because we were interested by the phenomenological features of the network dynamics.

(i) First, we have taken the same intrinsic degradation rate for all proteins (except for the Rb proteins) which has been set to 1, thereby setting the intrinsic timescale of the system.

(ii) Second, since the variables and parameters of the model are dimensionless, a large subset of parameters has been adjusted in order to normalize the maximum values of all concentrations to 1 (except for Apaf-1, which has been normalized to 6), in the situations where *IGF* and *IStr* are ranging from 0 to 1.

(iii) Third, the remaining kinetic constants have been adjusted so that the dynamical behavior of the G_1 regulatory network would give rise to four distinct cell fates characterized by their relative contents of selected species. Such constraint is loose enough to be satisfied over a relatively large range of parameters, among which we made an arbitrary choice. All parameter values are shown in Table 1 and their possible changes in the course of the study are specified in the captions.

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