

Perspectives

A Structural Analysis of the Qualitative Networks Regulating the Cell Cycle and Apoptosis

Baltazar D. Aguda*

Christopher K. Algar†

Department of Genetics and Genomics; Boston University School of Medicine; and
Department of Biomedical Engineering; Boston University; Boston, Massachusetts
USA

*Correspondence to: Baltazar D. Aguda; Department of Genetics and Genomics;
Boston University School of Medicine; 715 Albany St., E632; Boston, Massachusetts
02118 USA; Tel.: 617.414.1654; Fax: 617.414.1646; Email: bdaguda@bu.edu

†Permanent address : R.R. #1 Dingwall; Nova Scotia, Canada B0C 1G0

Received 07/07/03; Accepted 07/30/03

Previously published online as Cell Cycle E-publication at:
<http://www.landesbioscience.com/journals/cc/tocnew26.php?volume=2&issue=6>

KEY WORDS

mammalian cell cycle, apoptosis, qualitative networks, computer simulation, kinetic modeling

This research is supported by a grant to B.D.A. from Boston University School of Medicine and the Whitaker Foundation Leadership-Development Program based in the Department of Biomedical Engineering, Boston University.

ABSTRACT

This paper proposes an integration and modular organization of the complex regulatory networks involved in the mammalian cell cycle, apoptosis and related intracellular signaling cascades. A common node linking the cell cycle and apoptosis permits the possibility of coordinate control between the initiation of these two cellular processes. From this node, pathways emanate that lead to the activation of cyclin-dependent kinases (in the cell cycle) and caspases (in apoptosis). Computer simulations are carried out to demonstrate that the proposed network architecture and certain module-module interactions can account for the experimentally observed sequence of cellular events (quiescence, cell cycle and apoptosis) as the transcriptional activities of E2F-1 and c-Myc are increased. Despite the lack of quantitative kinetic data on most of the pathways, it is demonstrated that there can be meaningful conclusions regarding system stability that arise from the topology of the network. It is shown that only cycles in the network graph determine stability. Thus, several positive and negative feedback loops are identified from a literature review of the major pathways involved in the initiation of the cell cycle and of apoptosis.

INTRODUCTION

This paper presents an attempt at organizing the increasingly complex details in the regulation of the mammalian cell cycle and programmed cell death (apoptosis). It begins with observations (discussed below) that certain signaling pathways affecting the cell cycle are linked to pathways affecting apoptosis. These observations are not entirely unexpected as the 'decision' to eradicate a cell should somehow be coupled with how fast cells are dividing (to prevent tumors, for example, if the rate is unduly high) or with how many cells are needed in a given tissue of a developing multicellular organism. Given this cell cycle-apoptosis link, one can then offer a classification of signaling pathways according to the four cases shown in (Fig. 1) Signaling pathways that do not affect the cell cycle and apoptosis are not considered in this figure. Note that the four nodes in (Fig. 1) (symbolized by a circle, a triangle, a square and a hexagon) are generally not identical. Case I, in which both the cell cycle and apoptosis machineries are potentiated, could be exemplified by upstream signaling pathways that activate transcription factors such as E2F-1, c-Myc, c-Fos and others (many proto-oncogenes, in addition to their proliferative role, have the ability to induce apoptosis when overexpressed; see ref. 1 for a review). Case II may involve upstream signaling pathways such as those involving the protein kinase Akt which acts both as a proliferative and a survival (i.e., apoptosis-inhibiting) factor.² The MAPK cascade is another example of a signaling pathway belonging to case II that has both proliferative and anti-apoptotic function.³ Along with case I, case II may also be active in normal cells since apoptosis can be induced under serum starvation (reviewed in ref. 1). Cell cycle checkpoints would trigger signals belonging to case III in which the cell cycle is arrested and apoptosis is triggered. Case IV signals suppress both the cell cycle and apoptosis, a state characteristic of senescent cells. A good example of a case III node would be the tumor-suppressor protein p53. An example of a case IV node would be the CDK inhibitor p21Cip1 which can arrest the cell cycle as well as inhibit apoptosis (reviewed in ref. 4). There are instances where certain combinations of the cases in (Fig. 1) are not separable and an upstream signaling pathway may be common to more than one node. For instance, there are reports (reviewed in ref. 5) that the Ras/MAPK pathway can upregulate both cyclin D1 (proliferative) and p21Cip1 (anti-proliferative) – a mixture of cases II and IV.

The network analysis presented in this paper mainly focuses on case I but will also touch on case II because the latter also involves induction of the cell cycle. The analysis starts with the premise that the complex network is separable into modules. We then

identify the module-module interactions because it is these interactions that are crucial in the coordination between the cell cycle and apoptosis. By coordination we mean the sequence of initiating the cell cycle first and then triggering apoptosis if proliferative factors become deregulated. Examples of these module interactions are shown schematically in (Fig. 2A) (interactions are labeled a, b, b', or c). We will demonstrate that the dynamics of the modular network (Fig. 2A) can explain certain experimentally observed behaviors. Specifically, we are suggesting that transcription factors such as E2F-1 and c-Myc are key members of the node in (Fig 2A). We will provide computer simulations as proofs-of-concept for the ability of the abstract modular network to explain experimentally observed coordination between entry into the cell cycle and of apoptosis. In the next section, we briefly summarize a result from the linear stability analysis of dynamical systems stating that only cycles in the 'qualitative network graph' (defined below) contribute to the stability of the network. This is the reason why we have explored the relevant literature to identify possible cycles in the regulatory networks involved in the initiation of the cell cycle and of apoptosis.

QUALITATIVE NETWORKS

The networks we are considering here are qualitative networks. These are similar to the pathways drawn by molecular biologists when they summarize coupled activation and/or inhibition interactions. Formally, we define a 'qualitative network' (qNET) as a set of modules and their interactions. The word 'qualitative' is used to depict the situation where only qualitative information are known, such as 'X activates Y' or 'W inhibits Z' (these interactions are represented in the network graph as $X \rightarrow Y$ and $W \dashv Z$, respectively). The definition of a 'module' depends on the level of abstraction one is considering; for example, a module could be a specific molecule, a complex, or it could be a set of interacting molecules involved in a subnetwork. Basically, the number of modules depends on how one subdivides the network into units with separable functions. In the present study, these functions include the cell cycle machinery (cyclin-dependent kinases), apoptosis (caspases) and intracellular signaling pathways.

There are already some meaningful conclusions that can be made despite the qualitative nature of qNETs. In the Appendix, we show that network stability is determined by k-cycles in the graph. In many cases one can already predict the stability of a qNET from the existence of destabilizing cycles despite the lack of quantitative kinetic parameters. This is the primary reason why it is useful to identify feedback cycles (positive or negative) in regulatory networks especially when the aim is to predict switching behavior. Switches often arise when there is instability.

KEY COMPONENTS OF THE NODE BELONGING TO CASE

Unless otherwise stated, we will specifically use the term 'node' to mean the middle module in each of the cases shown in Figure 1); the inputs to a node come from upstream signaling pathways and the outputs are pathways to the cell cycle and apoptosis. We shall focus on cases where signaling pathways induce the cell cycle – i.e., cases I and II. We will not consider cases III and IV further.

There are many candidate nodal genes involved in case I of Figure 1. Many genes that are reputed to be proliferative—e.g., c-myc, c-fos, e2f-1, cyclin D1 and viral oncoprotein E1A—are also associated with increased apoptosis in cells in serum-starved cultures.^{1,5} E2F-1, a member of the E2F family of transcription factors, is a particularly good example of case I. E2F-1 is oncogenic as it can stimulate

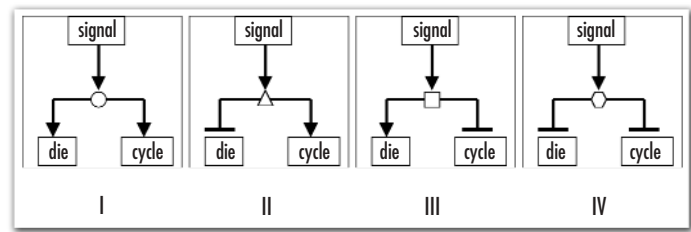


Figure 1. Classification of signaling pathways that impinge on both the cell cycle and apoptosis machineries via different nodes (represented as circle, triangle, square and hexagon). Arrow means activate and hammerhead means inhibit. Cases where the cell cycle and apoptosis are not linked are not covered in this classification.

excessive proliferation when overexpressed. E2F-1 protein can induce the expression of its corresponding gene,⁶ thus establishing a positive feedback loop. Surprisingly, E2F-1 also acts as a tumor-suppressor because its loss of function in mice has been shown to lead to carcinogenesis.^{7,8} E2F-1 seems to provide a crucial contribution to the induction of apoptosis without which cell suicide becomes an insufficient failsafe mechanism to guard against tumorigenic cells.⁹

Another good example of case I node is c-Myc, a transcription factor that is closely linked in a regulatory network involving E2F-1. Evidence has been presented that E2F-1 upregulates c-Myc.¹⁰⁻¹² In turn, c-Myc induces transcription of several cell cycle-related genes including E2F-1, E2F-2 and E2F-3.^{13,14} These observations suggest a synergy in the feedback regulations between the E2F-1 and c-Myc, influencing both the cell cycle and apoptosis; this synergy is the basis of our claim that E2F-1 and c-Myc are key components of the node in case I of Figure 1.

CELL CYCLE AND APOPTOSIS MODULES

We view the core regulatory networks for the initiation of the cell cycle (G_1/S progression) and of apoptosis as separate modules. The cycle module (Figs. 1 and 2A) represents the cyclin-dependent kinase (CDK) regulatory network. The die module is assumed to correspond to the regulatory network involving procaspases and the caspase activation cascade associated with apoptosis. We now briefly discuss some details of these two modules.

The Cell Cycle Module (G_1/S). Although we are referring to the entire cell cycle (all phases) in Figure 2A, we will focus on the G_1/S transition here since we are primarily interested in the initiation of DNA replication. Aguda and Tang¹⁵ have previously analyzed the kinetic origins of an intrinsic switching behavior associated with post-translational interactions among CDK2, the phosphatase Cdc25A and the CDK inhibitor p27Kip1. Several experiments¹⁶⁻¹⁸ have provided evidence that seem to support the Aguda-Tang model; in agreement with this model, we adopt the proposal that the G_1/S module is essentially controlled by the CDK2-Cdc25A-p27Kip1 subnetwork as shown in Figure 3. The most important features of this G_1/S model are:

1. the repression of the E2F transcription factors by the retinoblastoma protein, Rb,
2. inhibition of Rb via its phosphorylation by cyclin D/CDK4 (or CDK6),
3. initial release of E2F leading to activation of cyclin E/CDK2,
4. a positive feedback activation loop between CDK2 and Cdc25A and
5. a mutual inhibition (which is also a positive feedback loop) between p27Kip1 and CDK2.

Justification for these features were discussed by Aguda and Tang.¹⁵

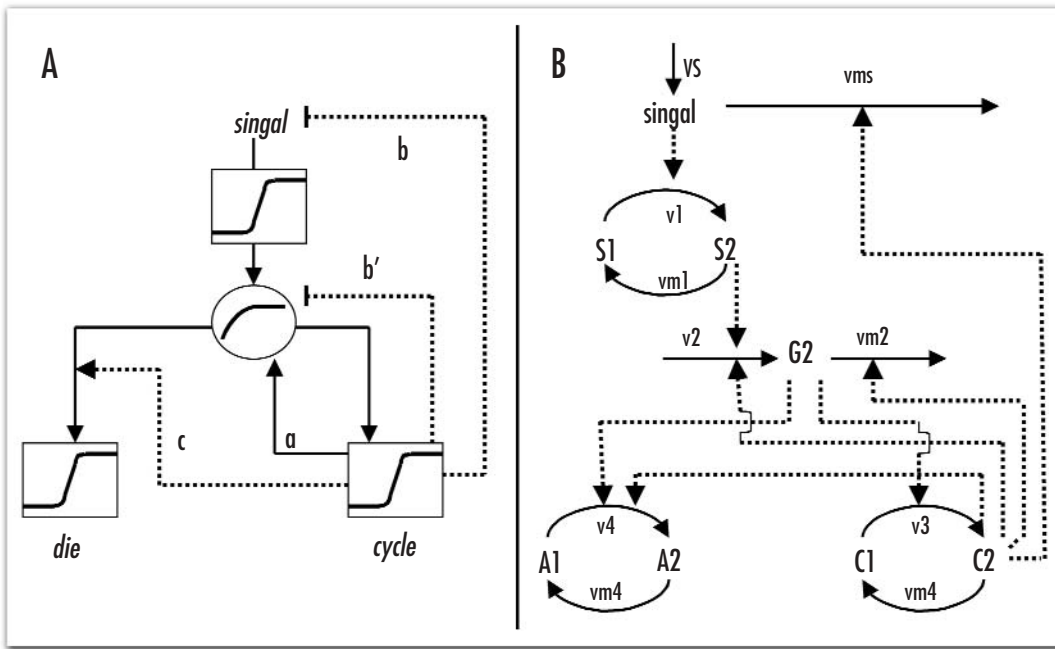


Figure 2. (A) Interactions among the modules in case I of Figure 1. Ultra-sensitive response curves are shown for the signaling, cell cycle and apoptosis modules. A hyperbolic response curve is shown for the middle node. (B) A kinetic model that explicitly implements the qualitative interactions given in (A). A dashed arrow means that the species where the arrow emanates catalyzes (without being consumed) the process where the arrow terminates. See (Table 1) for the rate expressions and parameter values.

It has also been demonstrated that c-Myc induces a pathway parallel to the Rb/E2F pathway that leads to the activation of cyclin E/CDK2.¹⁹ This is depicted schematically in Figure 3 with the (dashed) transcriptional induction of cyclin E, cyclin D and Cdc25A, among others.^{20,21}

The Apoptosis Module (Caspase Cascade). We are proposing that the essential structure of the network for the activation of executioner caspases (e.g., caspase-3, -6, -7) is shown on the leftmost module in (Fig. 3) This structure is consistent with the detailed mathematical model previously analyzed by Fussenegger, Bailey and Varner,²² and discussed by Zheng & Flavell.²³ The structure of the apoptosis module in (Fig. 3) is also consistent with recent experimental results.^{24,25} The module shows two major apoptotic pathways, namely, intrinsic (mitochondrial) and extrinsic (receptor-mediated). The intrinsic pathway involves the formation of the apoptosome

complex composed mainly of procaspase-9, cytochrome c and Apaf-1. The extrinsic pathway involves formation of a death-inducing signaling complex (DISC) upon docking of FasL (Fas Ligand) on death receptors, aggregation of these receptors, recruitment of FADD and procaspase-8 molecules. Coupling between the extrinsic and intrinsic pathways exists, for example, through Bid (activated by caspase-8) which induces release of cytochrome c from mitochondria.

The formation of DISC is crucial for the extrinsic pathway of apoptosis. For the intrinsic pathway, the release of cytochrome c is regulated by pro- and anti-apoptotic Bcl-2 family members. Towards the end of the caspase cascade, active caspases could be actively inhibited by proteins called IAPs (inhibitors of apoptosis). We think that the positive feedback loop between executioner caspases and cytochrome c release is essential in the irreversible switch to apoptosis.²⁶ This is in agreement with a recently proposed view that intrinsic and extrinsic pathways are “conceptually similar pathways in which mitochondria act as amplifiers of caspase activity rather than initiators of caspase activation”.²⁵

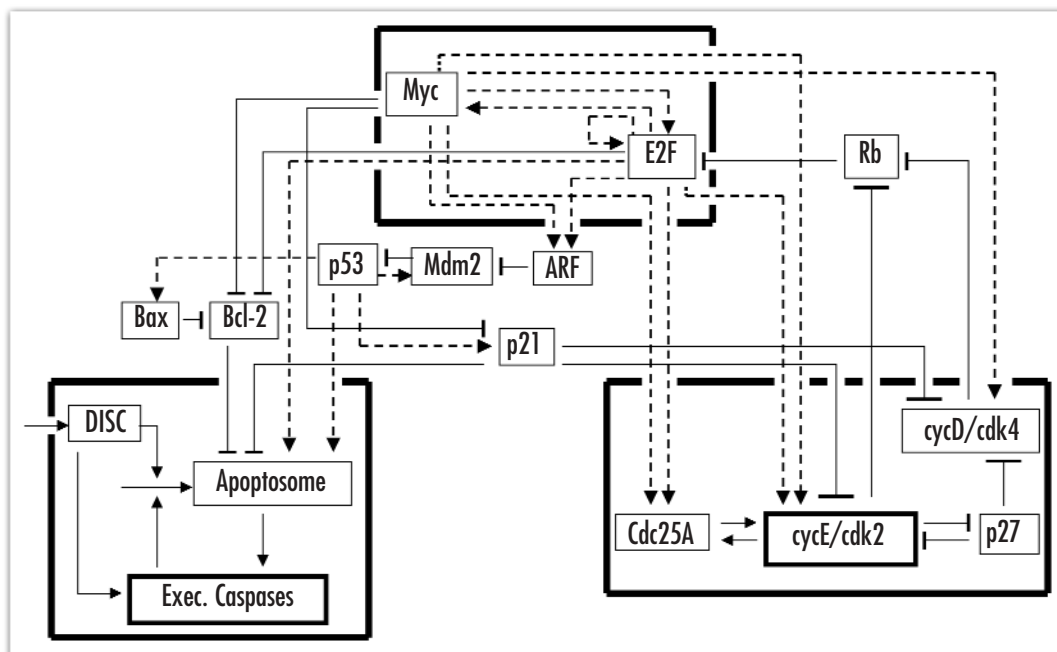


Figure 3. Some details of the node (Myc and E2F), cycle (Cdc25A, cycE/cdk2, cycD/cdk4, p27) and die (DISC, Apoptosome, Executioner Caspases) modules. Interactions between modules are shown as arrows for activation and hammerheads for inhibition (dashed lines at the transcriptional level; solid lines at the post-translational level). See text for descriptions of these interactions.

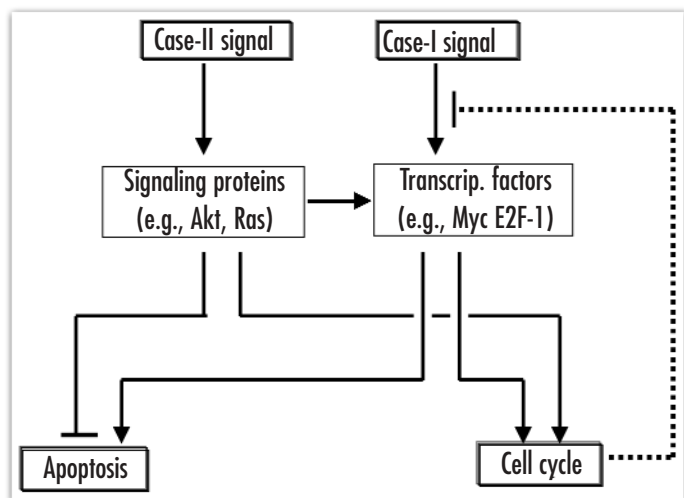


Figure 4. An example of coupling between case-I and case-II upstream signaling pathways of Figure 1.

MODULE INTERACTIONS

Figure 2A shows an elaboration of case I (Fig. 1) that includes the interactions among the modules, namely, a positive feedback loop **a** from the cell cycle machinery to the node, a negative feedback loop **b** from the cell cycle to the mitogen-induced signaling pathway before the node, a negative feedback loop **b'** from the cell cycle to the node and a pathway **c** from the cell cycle that enhances apoptosis. We now discuss the details of these module-module interactions.

Positive Feedback. The positive feedback loop between the cycle module and the node primarily involves the de-repression of E2F transcription factors from the retinoblastoma (Rb) protein due to the latter's phosphorylation by the CDKs. The activation of the E2Fs can be considered autocatalytic in the sense that their release from Rb inhibition leads indirectly to the activation of G₁ CDKs (e.g., CDK4, CDK6, CDK2) which further phosphorylate and inactivate Rb thereby releasing more E2Fs. In addition, there is also a positive feedback loop involved in the expression of E2F-1 since this protein induces expression of its corresponding gene.⁶ The Aguda-Tang model¹⁵ of the G₁/S transition in the mammalian cell cycle demonstrates that various positive feedback loops in the network can be responsible for the timing and sharp switching behavior in CDK activity upon entering S phase. These feedback loops include post-translational interactions among CDK2, the phosphatase Cdc25A and the CDK inhibitor p27Kip1 as depicted in Figure 3.

Negative Feedback. Negative feedback loops in dynamical systems have the ability of generating damped or even sustained oscillations. We suggest that the experimental observations²⁷⁻²⁹ of multiple peaks of Ras and/or MEK/ERK activities indicate the existence of a negative feedback loop shown as **b** in Figure 2A. Indeed, Lee et al²⁹ have proposed that a negative feedback loop emanates from the Rb pathway and targets Ras activation at the level of the guanine-nucleotide exchange factor, but no mechanistic details have been reported so far. The presence of this negative feedback is noteworthy because it could represent a cellular response against excessive stimuli that may endanger the cell. This negative feedback loop also may have the essential function of cutting off the apoptotic pathway before sufficient caspase activity is attained to trigger apoptosis (the feasibility of this idea is demonstrated below using computer simulations). In addition, there are negative feedback loops along signaling pathways

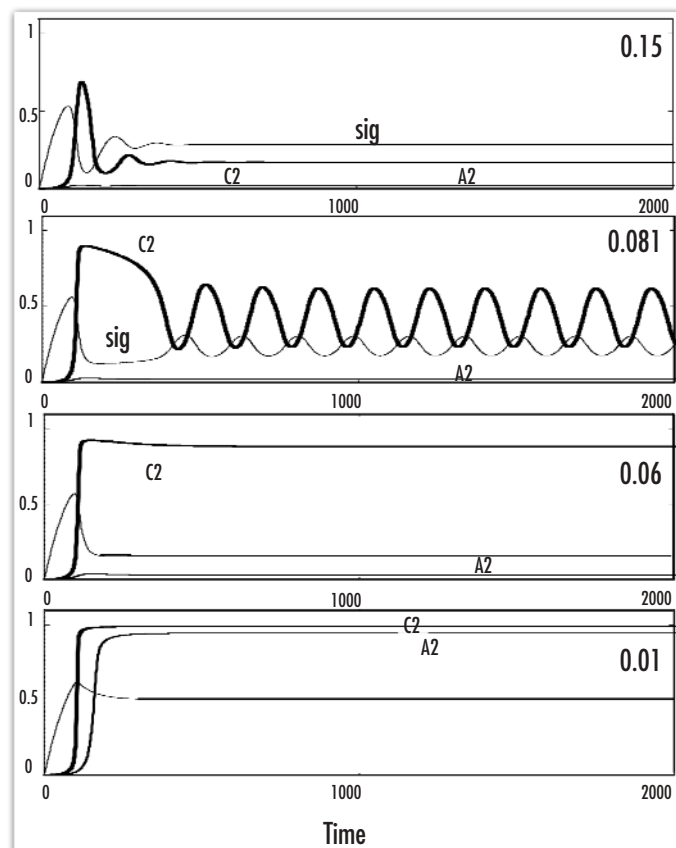


Figure 5. Computer simulations using the kinetic model shown in Figure 2B. The rate expressions and parameters are given in Table 1 except for the values of *ksd2* which are shown on the top right corner of each of the four panels. The time course of the signal (*sig*), *C2* and *A2* are generated from the numerical integration of the differential equations given in Table 1. *C2* corresponds to an active cell cycle factor and *A2* corresponds to an active apoptosis factor. The differential equations in Table 1 were integrated using the BerkeleyMadonna software (<http://www.berkeleymadonna.com>).

such as the Ras/MAPK pathway.³⁰ Another negative feedback loop is shown as **b'** in Fig 2A, from the cell cycle to the node. An example of **b'** would be the phosphorylation by cyclin A/CDK2 of DP-1 (a heterodimer partner of E2Fs) that turns off E2F-1 transcriptional activity.^{8,31}

FROM THE NODE TO APOPTOSIS

Some important pathways between the node and apoptosis are shown in Figure 3. Notable among these is the participation of the tumor suppressor protein p53—via its transcriptional induction of genes, such as Bax, that antagonize Bcl-2. A direct link between apoptosis and the node is the ARF-Mdm2-p53 pathway. Both E2F-1 and c-Myc are known to induce transcription of ARF, a protein that indirectly stabilizes p53. p53 induces transcription of the CDK inhibitor p21Cip1. Recently, along with E2F-1, p53 has also been shown to induce transcription of Apaf-1 and some of the caspases.³²⁻³⁴ It is also interesting to note that p21Cip1 indirectly inhibits apoptosis perhaps via the mitochondrial pathway.⁴ A recent report further suggests that c-Myc induces the switch to apoptosis by repressing the expression of p21Cip1.³⁵ A direct link between the node and apoptosis could involve suppression of the protein and RNA levels of Bcl-2 by c-Myc and E2F-1.³⁶ E2F-1 can also enhance

Table 1. **REACTION RATE EXPRESSIONS AND KINETIC EQUATIONS FOR THE MODEL SHOWN IN FIGURE 2B**

Rate Expressions

$$\begin{aligned}
 v_s &= k_s \\
 v_{ms} &= (k_{sd1} + k_{sd2} \cdot C2) \cdot signal \\
 v_1 &= k_1 \cdot signal \cdot S1 / (KM1 + S1) && \text{where } S1 = Ets - S2 \\
 v_{m1} &= Vm1 \cdot S2 / (KMr1 + S2) \\
 v_2 &= k_2 \cdot S2 + k_{2a} \cdot C2 \\
 v_{m2} &= (km2 + km_{2a} \cdot C2) \cdot G2 \\
 v_3 &= k_3 \cdot G2 \cdot C1 / (KM3 + C1) && \text{where } C1 = Etc - C2 \\
 v_{m3} &= Vm3 \cdot C2 / (KMr3 + C2) \\
 v_4 &= (k_4 \cdot G2 + k_{4a} \cdot C2) \cdot A1 / (KM4 + A1) && \text{where } A1 = Eta - A2 \\
 v_{m4} &= Vm4 \cdot A2 / (KMr4 + A2)
 \end{aligned}$$

Kinetic (differential) Equations

$$\begin{aligned}
 d(signal)/dt &= v_s - v_{ms} \\
 d(S2)/dt &= v_1 - v_{m1} \\
 d(G2)/dt &= v_2 - v_{m2} \\
 d(C2)/dt &= v_3 - v_{m3} \\
 d(A2)/dt &= v_4 - v_{m4}
 \end{aligned}$$

Base Parameter Values

$Ets = 1.0$	$Etc = 1.0$	$Eta = 1.0$
$ks = 0.01$	$ksd1 = 0.01$	$ksd2 = 0.0001$
$k1 = 1.0$	$Vm1 = 1.0$	$KM1 = 0.02$
$KMr1 = 0.02$	$k2 = 1.0$	$k_{2a} = 0.01$
$km2 = 0.01$	$km_{2a} = 0.001$	$k3 = 1.0$
$Vm3 = 1.0$	$KM3 = 0.02$	$KMr3 = 0.02$
$k4 = 0.5$	$k_{4a} = 0.005$	$Vm4 = 1.0$
$KM4 = 0.02$	$KMr4 = 0.02$	

Initial Conditions

$$signal = S2 = G2 = C2 = A2 = 0.0$$

apoptosis, independent of its transcriptional activity, by inhibiting NF-κB through binding with the latter's p65 subunit.³⁷

Cell Cycle-mediated Apoptosis. Enhancement of apoptosis by the cell cycle (c in Fig. 2A) is suggested by reports, for example, linking BAD-mediated apoptosis with Cdc2 and activation of Caspase 2 by cyclin D3.^{38,39} Earlier, Sofer-Levi & Resnitzky⁴⁰ showed that ectopic expression of cyclin D1 can induce apoptosis. These pathways could be interpreted as failsafe mechanisms that kill cells with potential for uncontrolled proliferation.

UPSTREAM SIGNALING PATHWAYS

An attempt to review the broad field of intracellular signal transduction pathways relevant to the initiation of the cell cycle has been made by Aguda;⁴¹ we refer the interested reader to this review for more references. Notable among the signaling pathways are those initiated by the membrane protein Ras and its effector pathways such as MAPK and PI3K/Akt pathways. As summarized in the aforementioned review, direct connections have been made between Ras effector pathways and the regulation (both at the transcriptional and post-translational levels) of G1 cyclin/CDKs such as cyclin D1/CDK4,6 and cyclin E/CDK2. In addition, pathways emanating from tyrosine kinases such as Src and JAKs that lead to the expression of immediate-early genes (e.g., c-myc) which eventually contribute

to the regulation of CDK2 and CDK4 are now being elucidated (reviewed in ref. 41).

It is interesting to consider the combined upstream signaling pathways corresponding to cases I and II of Figure 1. In Figure 4, we show a superposition of these two cases. Oncoproteins Ras and Akt can generate signals that induce proliferation as well as inhibit apoptosis.² An interesting scenario derived from Figure 4 is the way case II-signaling can subjugate case-I signaling. A case-II signal enhances the frequency of the cell cycle with concomitant strengthening of the negative feedback loop (dashed curve in Fig. 4); subsequently, the case-I pathway is weakened along with its pro-apoptotic contribution. In the end, only the case-II signaling pathway is operational.

COMPUTER SIMULATIONS

We now present a proof-of-concept that the modular network structure shown in Figure 2A is capable of generating a situation where there exists an optimal range of signal strengths that induces the cell cycle, suboptimal strengths that lead to quiescence and superoptimal strengths that lead to apoptosis. For our computer simulations, we assume that the input/output response curves of the modules are either ultrasensitive (for the signal, die and cycle modules) or hyperbolic (for the node) as shown in Figure 2A. One way to generate ultrasensitive response curves is to employ the 'zeroth-order ultrasensitive' property of cyclic enzyme reactions when the Michaelis constants are close to zero.⁴² The ultrasensitive character of the MAPK cascade has been demonstrated both by experiments and by kinetic model simulations (e.g., see ref. 43).

A sample implementation of the modular network in Figure 2A is shown in Figure 2B where an explicit kinetic model is depicted. The mathematical details of this model are listed in Table 1. The positive feedback loop a in Figure 2A is represented in the expression of rate v_2 in Table 1. The negative feedback loop b in Figure 2A is included in the degradation rate, v_{ms} , of the signal and the negative feedback loop b' is included in the degradation rate, v_{m2} , of the nodal component symbolized by G2. The cell cycle-dependent pro-apoptotic process c is included in v_4 , the rate of activation of an apoptotic factor A2.

A series of computer simulations for increasing signal strengths (implemented by decreasing a cell cycle-dependent signal-degradation parameter, $ksd2$, involved in the rate v_{ms}) is shown in Figure 5. As $ksd2$ is decreased from 0.15 to 0.01, the peak of the signal increases (curves labeled 'sig' refer to 'signal' in Table 1). No cell cycle oscillations (curves labeled 'C2' referring to C2 in Table 1) are observed at first and then a certain range of $ksd2$ gives sustained oscillations (exemplified by the second panel where $ksd2=0.081$). Apoptosis is eventually triggered when the signal gets sufficiently high as exemplified by curve 'A2' (referring to A2 in Table 1) for $ksd2 = 0.01$ (lowermost panel). Note that the value of km_{2a} is nonzero; if it were set to zero, sustained oscillations can still be achieved by increasing $ksd2$ (simulations not shown; for example, if $km_{2a} = 0$, oscillations occur for $ksd2 = 0.091$; oscillations suddenly disappear for $ksd2 = 0.09$). We have interpreted the oscillations here to represent CDK oscillations involved in the cell cycle. In fact, this simple model is similar to one of the pioneering schematic models of mitotic oscillations proposed by Goldbeter and coworkers.^{44,45} One can also readily demonstrate the importance of the negative feedback loops (b or b' in Fig. 2A) in the sequential timing of the cell cycle and apoptosis. Without a negative feedback loop, apoptosis will always be triggered and no CDK oscillations can be observed.

CONCLUDING REMARKS

A 'top-down' approach was used in this work to organize the complex details of regulatory pathways involved in the cell cycle and apoptosis. The organization is based on a modular network architecture that assumes separable machineries of the cell cycle (cyclin/CDKs), apoptosis (caspases) and upstream signal transduction pathways. We have highlighted reports describing interactions between the modules. The peculiar ability of proto-oncogenes (e.g., c-myc and e2f-1) to induce proliferation as well as apoptosis suggests a common node potentiating both processes. Interactions between modules include a positive feedback loop such as a in Figure 2A, which could serve to trigger the cell cycle ahead of apoptosis and negative feedback loops such as b or b' that could serve to quench the caspase cascade and inhibit apoptosis; such would be the scenario occurring if case I in Figure 1 is the only mechanism available for the cell. As we have demonstrated in our computer simulations, whether or not apoptosis is triggered depends on the strength of the signal (demonstrated by the switch in A2 between the two bottom panels of Fig. 5). Cell survival in this case relies on the existence of negative feedback loops from the cell cycle that attenuate mitogenic signaling. In the event that the normal balance between proliferation and death is compromised, e.g., when cells are hyperproliferating, path c in Figure 2A serves to restore the balance (for without path c, overproliferation also enhances the negative feedback loops b and b' thereby inhibiting, instead of inducing, apoptosis). However, this scenario does not explain the observation that serum-starved cells undergo apoptosis, which leads us to the proposal that cases I and II (in Fig. 1) must both be operational in a normal cell and that these two cases are coupled via the gene expression node (transcription factors in Fig. 4). Are the negative feedback loops (b and b' in Fig. 2A) essential for the inhibition of apoptosis by contributing to survival signals represented by case II in Figure 1? It is clear that the relative strengths of the contributions from cases I and II will influence the outcome of the competition between survival and death.

Although the primary aim of this paper is to explore the general consequences of the interactions between modules representing the cell cycle, apoptosis and upstream signaling, we have also made proposals for the key internal details of these modules. For the cell cycle module, we claim that entry into S phase could be marked by the switch-like activation of cyclin E/CDK2 generated by positive feedback interactions with Cdc25A and p27Kip1.¹⁵ However, the role of CDK2 in S-phase entry has recently been challenged,^{46,47} and further investigation of this issue is warranted. For the commitment to apoptosis, we suggest the hypothesis that the positive feedback loop between the apoptosome and executioner caspases is key to triggering apoptosis. We hope that our analysis of the essential structures of these regulatory networks will contribute to the organization of the increasingly detailed and complex picture of cell proliferation and death. We expect that the detailed qualitative network presented in (Fig. 3) will be useful for future quantitative analyses of the system. In addition, cell differentiation pathways and their links with the cell cycle and apoptosis will be studied using the method of analysis presented in this paper. Blagosklonny⁴⁸ have started paving the way towards this direction.

References

1. Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001; 411:342-8.
2. Testa JR, Bellacosa A. AKT plays a central role in tumorigenesis. *Proc Natl Acad Sci USA* 2001; 98:10983-5.
3. Yu C, Krystal G, Varticovski L, McKinsty R, Rahmani M, Dent P, Grant S. Pharmacologic mitogen-activated protein/extracellular signal-regulated kinase/mEvan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nitrogen-activated protein kinase inhibitors interact synergistically with ST1571 to induce apoptosis in Bcr/Abl-expressing human leukemia cells. *Cancer Res* 2002; 62:188-199.
4. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Molecular Cancer Therapeutics* 2002; 1:639-49.
5. Blagosklonny MV. A node between proliferation, apoptosis and growth arrest. *BioEssays* 1999; 21:704-709.
6. Neuman E, Flemington EK, Sellers WR, Kaelin Jr WG. Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter. *Mol Cell Biol* 1994; 14:6607-15.
7. Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E, Dyson NJ. Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* 1996; 85:537-48.
8. Lees JA, Weinberg RA. Tossing monkey wrenches into the clock: New ways of treating cancer. *Proc Natl Acad Sci USA* 1999; 96:4221-3.
9. Phillips AC, Vousden KH. E2F-1 induced apoptosis. *Apoptosis* 2001; 6:173-82.
10. Hiebert SW, Lipp M, Nevins JR. E1A-dependent transactivation of the human MYC promoter is mediated by the E2F factor. *Proc Natl Acad Sci USA* 1989; 86:3594-8.
11. Thalmeyer K, Synovzik H, Mertz R, Winnacker EL, Lipp M. Nuclear factor E2F mediates basic transcription and transactivation by E1A of the human MYC promoter. *Genes Dev* 1989; 3:527-36.
12. Elliott MJ, Dong YB, Yang H, McMasters KM. E2F-1 up-regulates c-Myc and p14(ARF) and induces apoptosis in colon cancer cells. *Clin Cancer Res* 2001; 7:3590-7.
13. Sears R, Ohtani K, Nevins JR. Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to growth signals. *Mol Cell Biol* 1997; 17:5227-35.
14. Leone G, Sears R, Huang E, Rempel R, Nuckolls F, Park CH, Giangrande P, Wu L, Saavedra HI, Field SJ, Thompson MA, Yang H, Fujiwara Y, Greenberg ME, Orkin S, Smith C, Nevins JR. Myc requires distinct E2F activities to induce S phase and apoptosis. *Mol Cell* 2001; 8:105-13.
15. Aguda BD, Tang Y. The kinetic origins of the restriction point in the mammalian cell cycle. *Cell Prolif* 1999; 32:321-35.
16. Blumberg I, Hoffmann I. Ectopic expression of Cdc25A accelerates the G₁/S transition and leads to premature activation of cyclin-E and cyclin-A-dependent kinases. *Mol Cell Biol* 1999; 19:6183-94.
17. Sandhu C, Donovan J, Bhattacharya N, Stampfer M, Worland P, Slingerland J. Reduction of Cdc25A contributes to cyclin E1-Cdk2 inhibition at senescence in human mammary epithelial cells. *Oncogene* 2000; 19:5314-23.
18. Ekholm SV, Zickert P, Reed SI, Zetterberg A. Accumulation of cyclin E is not a prerequisite for passage through the Restriction Point. *Mol Cell Biol* 2001; 21:3256-65.
19. Santoni-Rugiu E, Falck J, Mailand N, Bartek J, Lukas J. Involvement of Myc activity in a G₁/S-promoting mechanism parallel to the pRb/E2F pathway. *Mol Cell Biol* 2000; 20:3497-509.
20. Collier HA, Grandori C, Tamayo P, Colbert T, Lander ES, Eisenman RN, Golub TR. Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling and adhesion. *Proc Natl Acad Sci USA* 2000; 97:3260-5.
21. Menssen A, Hermeking H. Characterization of the c-MYC-regulated transcriptome by SAGE: Identification and analysis of c-MYC target genes. *Proc Natl Acad Sci USA* 2002; 99:6274-9.
22. Fussenegger M, Bailey JE, Varner J. A mathematical model of caspase function in apoptosis. *Nature Biotechnology* 2000; 18:768-74.
23. Zheng TS, Flavell RA. Death by numbers. *Nature Biotechnology* 2000; 18:717-8.
24. Lassus P, Opitz-Araya X, Lazebnik Y. Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 2002; 297:1352-4.
25. Kumar S, Vaux DL. A cinderella caspase takes center stage. *Science* 2002; 297:1290-1.
26. Green D, Kroemer G. The central executioners of apoptosis: Caspases or mitochondria? *Trends in Cell Biology* 1998; 8:267-71.
27. Meloche S. Cell cycle reentry of mammalian fibroblasts is accompanied by the sustained activation of p44mapk and p42mapk isoforms in the G₁ phase and their inactivation at the G₁/S transition. *J Cellular Physiology* 1995; 163:577-88.
28. Taylor SJ, Shalloway D. Cell cycle-dependent activation of Ras. *Curr Biol* 1996; 6:1621-7.
29. Lee KY, Latha MH, McMahon C, Ewen ME. The retinoblastoma protein is linked to the activation of Ras. *Mol Cell Biol* 1999; 19:7724-32.
30. Denhardt DT. Signal transduction pathways and regulation of the mammalian cell cycle: Cell type-dependent integration of external signals. In: Stein GS, Baserga R, Giordano A, Denhardt DT, eds. *The Molecular Basis of Cell Cycle and Growth Control*. New York: Wiley-Liss, 1999:225-304.
31. Krek W, Xu G, Livingston DM. Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. *Cell* 1995; 83:1149-58.
32. Moroni MC, Hickman ES, Denchi EL, Caprara G, Colli E, Ceconi F, Muller H, Helin K. Apaf-1 is a transcriptional target for E2F and p53. *Nat Cell Biol* 2001; 3:552-8.

33. Nahle Z, Polakoff J, Davuluri RV, McCurrach ME, Jacobson MD, Narita M, Zhang MQ, Lazebnik Y, Bar-Sagi D, Lowe SW. Direct coupling of the cell cycle and cell death machinery by E2F. *Nat Cell Biol* 2002; 4:859-64.
34. MacLachlan TK, El-Deiry WS. Apoptotic threshold is lowered by p53 transactivation of caspase-6. *Proc Natl Acad Sci USA* 2002; 99:9492-7.
35. Vousden KH. Switching from life to death: The Miz-ing link between Myc and p53. *Cancer Cell* 2002; 2:351-352.
36. Eischen CM, Packham G, Nip J, Fee BE, Hiebert SW, Zambetti GP, Cleveland JL. Bcl-2 is an apoptotic target suppressed by both c-Myc and E2F-1. *Oncogene* 2001; 20:6983-93.
37. Tanaka H, Matsumura I, Ezoe S, Satoh Y, Sakamaki T, Albanese C, Machii T, Pestrell RG, Kanakura Y. E2F1 and c-Myc potentiate apoptosis through inhibition of NF-kappaB activity that facilitates MnSOD-mediated ROS elimination. *Mol Cell* 2002; 9:1017-29.
38. Konishi Y, Lehtinen M, Donovan N, Bonni A. Cdc2 Phosphorylation of BAD links the cell cycle to the cell death machinery. *Mol Cell* 2002; 9:1005-16.
39. Mendelsohn AR, Hamer JD, Wang ZB, Brent R. Cyclin D3 activates Caspase 2, connecting cell proliferation with cell death. *Proc Natl Acad Sci USA* 2002; 99:6871-6.
40. Sofer-Levi Y, Resnitzky D. Apoptosis induced by ectopic expression of cyclin D1 but not cyclin E. *Oncogene* 1996; 13:2431-7.
41. Aguda BD. Kick-starting the cell cycle: From growth-factor stimulation to initiation of DNA replication. *Chaos* 2001; 11:269-76.
42. Goldbeter A, Koshland Jr DE. An amplified sensitivity arising from covalent modification in biological systems. *Proc Natl Acad Sci USA* 1981; 78:6840-4.
43. Huang CY, Ferrell Jr JE. Ultrasensitivity in the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci USA* 1996; 93:10078-83.
44. Goldbeter A. A minimal cascade model for the mitotic oscillator involving cyclin and Cdc2 kinase. *Proc Natl Acad Sci USA* 1991; 88:9107-11.
45. Gonze D, Goldbeter A. A model for a network of phosphorylation- dephosphorylation cycles displaying the dynamics of dominoes and clocks. *J Theor Biol* 2000; 210:167-86.
46. Hinds PW. Cdk2 dethroned as master of S phase entry. *Cancer Cell* 2003; 3:305-7.
47. Tetsu O, McCormick F. Proliferation of cancer cells despite CDK2 inhibition. *Cancer Cell* 2003; 3:233-45.
48. Blagosklonny MV. Apoptosis, proliferation, differentiation: In search of the order. *Seminars in Cancer Biology* 2003; 13:97-105.

APPENDIX

Consider a dynamical system described by the following system of differential equations:

$$dX/dt = F(X) \tag{A.1}$$

where X is a vector of the dynamical variables (e.g., chemical concentrations, or enzyme activities), t is time and $F(\cdot)$ is a function of the dynamical variables (usually a nonlinear function). When the system is being analyzed for its stability to small perturbations, it is customary to linearize the equations to give the following time-evolution of the perturbation p :

$$dp/dt = Mp \tag{A.2}$$

where $p = X - X_0$, X_0 being a reference state (usually a steady state) that is perturbed and M is the $n \times n$ Jacobian matrix with components $m_{ij} = [\partial F_i / \partial X_j]_0$ evaluated at $X = X_0$. The stability of the linearized system (Eqn. A.2) is determined by the eigenvalues λ of M . The state X_0 is linearly stable if all eigenvalues have negative real parts; the state is unstable if at least one eigenvalue has a positive real part. The eigenvalues are the roots of the characteristic polynomial $P(\lambda)$ (given below for n independent state variables)

$$P(\lambda) = \det(\lambda I - M) = \lambda^n + \alpha_1 \lambda^{n-1} + \alpha_2 \lambda^{n-2} + \dots + \alpha_{n-1} \lambda + \alpha_n = 0. \tag{A.3}$$

The coefficients α_i of $P(\lambda)$ are functions of the elements of M (the m_{ij} 's) and it turns out that these coefficients can be expressed in terms of k -cycles ($k = 1, 2, \dots$) as follows:

$$\begin{aligned} \alpha_1 &= \sum_i [-C_1(i)] \\ \alpha_2 &= \sum_{i,j} [-C_1(i)][-C_1(j)] + \sum_{j,k} [-C_2(j,k)] \\ \alpha_3 &= \sum_{i,j,k} [-C_1(i)][-C_1(j)][-C_1(k)] + \sum_{i,j,k} [-C_1(i)][-C_2(j,k)] + \sum_{i,j,k} [C_3(i,j,k)] \end{aligned} \tag{A.4}$$

$$\begin{aligned} \text{where } C_1(i) &= m_{ii} && (1\text{-cycles}) \\ C_2(jk) &= m_{jk} m_{kj} && (2\text{-cycles}) \\ C_3(ijk) &= m_{ij} m_{jk} m_{ki} && (3\text{-cycles}) \end{aligned}$$

An example of a 1-cycle would be $C_1(2) = m_{22}$, a 2-cycle example would be $C_2(13) = m_{13} m_{31}$, and a 3-cycle example would be $C_3(214) = m_{21} m_{14} m_{42}$. Since the eigenvalues are determined by the coefficients α_i 's and since these coefficients are determined by k -cycles, we say that the linear stability of the system are determined by k -cycles only. When a k -cycle contributes to the existence of an eigenvalue with positive real part, we say that this cycle is 'destabilizing'; if this k -cycle decreases the possibility of such an eigenvalue, the cycle is 'stabilizing'. We say that "species X_j activates X_i " (graphically represented as $X_j \dashv\vdash X_i$) if $m_{ij} > 0$ and that "species X_j inhibits X_i " (graphically represented as $X_j \dashv X_i$) if $m_{ij} < 0$. A 'qualitative network' (qNET) is a network of these interactions that are described only by the algebraic signs of the m_{ij} 's.