

Kick-starting the cell cycle: From growth-factor stimulation to initiation of DNA replication

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The essential genes, proteins and associated regulatory networks involved in the entry into the mammalian cell cycle are identified, from activation of growth-factor receptors to intracellular signal transduction pathways that impinge on the cell cycle machinery and ultimately on the initiation of DNA replication. Signaling pathways mediated by the oncoproteins Ras and Myc induce the activation of cyclin-dependent kinases CDK4 and CDK2, and the assembly and firing of pre-replication complexes require a collaboration among E2F, CDK2, and Cdc7 kinase. A proposed core mechanism of the restriction point, the major checkpoint prior to commitment to DNA synthesis, involves cyclin E/CDK2, the phosphatase Cdc25A, and the CDK inhibitor *p27Kip1*.

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The cell division cycle can be viewed as a process of unfolding the genetic blueprint for the construction and maintenance of an organism. With the remarkable recent advances in molecular biology, it may be feasible in the near future to compose a sufficiently detailed mechanism of the cell cycle to an extent that would allow us to simulate its real-time dynamics on a computer. The major players and pathways of interactions are rapidly being identified and the time has come to start encoding this information in the form of quantitative kinetic models that could enhance our understanding and control of this complex and intrinsically nonlinear process. In this review, we focus on the important issue of how cells enter the proliferative state upon stimulation by growth factors. Our goal is to identify, based on solid experimental evidence, the essential components of the dynamical system in preparation for future mathematical modeling; these components include intracellular signal transduction pathways that trigger the cell cycle machinery and ultimately the unwinding of the DNA duplex to initiate replication.

I. INTRODUCTION

Most of the cells in our body are in a nondividing quiescent state but some continually proliferate and die such as those of the skin, of epithelial tissues, and stem cells in the bone marrow. Some cells, like those of the breast and uterus, although normally quiescent, can also be induced to proliferate in response to specific physiological stimuli. Tissue regeneration, as in the case when part of the liver is excised, is an important example of a process wherein cells reenter the cell cycle due to removal of signals that inhibit proliferation.¹ Terminally differentiated cells such as heart muscle cells, nerve cells, and neurons do not possess the

ability to regenerate. However, it has been shown recently² that nuclei of some heart muscle cells can be induced to divide with an appropriate stimulus, at least in a cell-free system; this demonstration could pave the way to strategies for regeneration of cardiac muscle. On the other hand, transformed cells that proliferate uncontrollably and form tumors would have undergone certain genetic mutations that abrogate cell cycle checkpoints rendering the cells unresponsive to homeostatic signals. In both cases, whether starting or arresting the cell cycle, one needs to know how extracellular stimuli and intracellular signal transduction pathways are coupled with the cell cycle machinery. This paper is an attempt to sketch a picture of this complex process, from activation of membrane receptors by growth factors to the unwinding of the DNA duplex at the start of replication. The topic covers broad fields in molecular cell biology and it is not my intention to merely summarize existing authoritative reviews of the impressive progress in these fields; instead, I will try to present an interpretation of the known or postulated network of pathways by highlighting those that could dominantly affect the dynamics of the entire process and, in particular, identifying features of the network that possess intrinsic switching behavior. The complexity of the pathways of signal transduction and cell-cycle regulation is overwhelming that a mathematical and computer-based analysis is now required to arrive at a system-level understanding and control of the cell cycle; identification of the essential components of the system and how they interact, which is a primary objective of this review, is a necessary step in this direction.

II. THE G1/S CELL CYCLE MACHINERY

At the very least, the cell cycle can be defined as the alternation between the process of replicating the chromosomes and the process of segregating the duplicate chromosomes into two homologous sets. The first process requires DNA synthesis and is said to occur during *S* phase, while the second process is carried out by a sophisticated molecular

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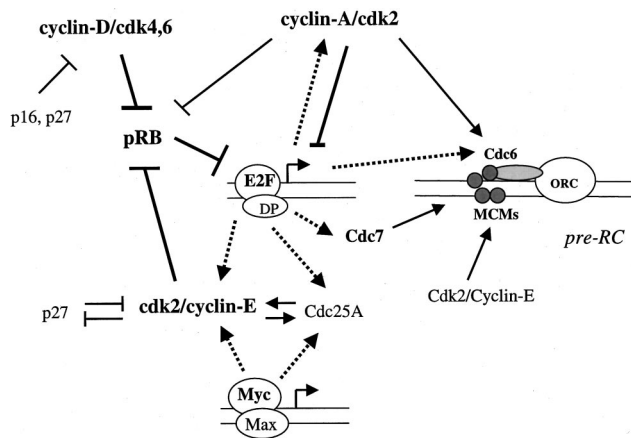


FIG. 1. An overview of the major players and pathways involved in the $G1-S$ transition of the mammalian cell cycle. The $G1/S$ cyclins include cyclins D, E, and A which bind their respective cyclin-dependent kinase partners CDK4,6 and CDK2 as shown. The transcription factors E2F and Myc (associated with their partners DP and Max, respectively) are shown bound to DNA and stimulate expression of various genes as indicated with dashed arrows. Active retinoblastoma protein, pRB, binds to E2F and represses the latter's transcriptional activity; the cyclin-CDK complexes inactivate pRB by phosphorylation. CDK inhibitors such as p16 and p27 are shown. The activation of cyclin E/CDK2 is shown to be regulated by the phosphatase Cdc25A and the inhibitor p27. The assembly and activation of pre-replication complexes (pre-RCs) involve the collaboration of E2F, CDK2 and the kinase Cdc7. ORC=origin-recognition complex; MCMs =minichromosome-maintenance proteins MCM2-7.

machinery operating during mitosis or M phase. The standard eukaryotic cell cycle goes through the sequence of phases, namely, $G1$, S , $G2$, and M , where $G1$ and $G2$ are the intervening gaps between S and M phases. An overview of the impressive progress in elucidating the molecular mechanism of the cell cycle is provided in a review article of Pines.³ Figure 1 gives a network diagram of the major players of the cell cycle machinery in $G1/S$ and should be consulted by the reader during the discussion below. A list of important abbreviations used is given in the Appendix.

A. Regulation of protein kinases that drive the cell cycle

Associated with the transitions through the phases of the cell cycle are the sequential expressions of proteins called cyclins. The major mammalian cyclins have been grouped into types called cyclins D, E, A, and B. Levels of D-type cyclins increase upon exposure to growth factors, followed by increases in cyclins E and A. These cyclins bind with, and are absolutely required for the activation of, a group of enzymes called cyclin-dependent kinases (CDKs). These CDKs drive the cell cycle "engine" by catalyzing the phosphorylation of serine and/or threonine residues of their protein substrates. In the $G1$ and S phases, the most important mammalian CDKs are CDK4, CDK6, and CDK2. Complexes of cyclin D/CDK4 and cyclin D/CDK6 are activated early in $G1$ while active complexes of cyclin E/CDK2 appear in mid-to late- $G1$ followed by cyclin A/CDK2 (reviewed in Ref. 3); thus, cyclin D/CDK4 (or CDK6) complexes are considered

as "growth-factor" sensors. The link between cyclin D synthesis and intracellular signal transduction pathways will be discussed in more detail below.

In addition to binding with cyclins, activation of CDKs also requires phosphorylation of certain threonine residues, a task carried out by CAK (for CDK-Activating Kinase, itself a complex composed of cyclin H, CDK7, and MAT1). On the other hand, certain kinases (e.g., Wee1 and Mik1) can phosphorylate these CDKs on specific tyrosine and/or threonine residues resulting in the inhibition of CDK activity; removal of these inhibitory phosphate groups is carried out by a family of Cdc25 phosphatases (isoforms Cdc25A, -B, and -C exist in mammalian cells; of these, Cdc25A is the most important member in $G1$). As will be discussed in more detail below, various experimental results point to Cdc25A as a crucial target of intracellular signal transduction pathways and as a key player in the $G1-S$ transition.

The activity of CDKs is also inhibited by binding with small proteins called CKIs (for CDK Inhibitors) of which there are two families: the INK4 family (p16, p15, p18, p19) and the CIP/KIP family (p27, p21, p57). INK4 members are specific inhibitors of CDK4 and CDK6, while the CIP/KIP proteins are universal CDK inhibitors. Protein levels of p27 are generally high in quiescent cells and bringing these levels down has been recognized as one of the key prerequisites for S -phase entry (reviewed in Ref. 4). The regulation of p27 levels by intracellular signal transduction pathways will be discussed in more detail in Sec. III.

B. Transcriptional regulation of S -phase entry

The activation of CDK4,6 is normally followed by the activation of CDK2; this sequential activation is linked to the transcription of $G1$ -related genes including cyclin E, cyclin A, Cdc25A, and those required for the assembly and activation of DNA pre-replication complexes, replication complexes and polymerases. This transcriptional link is mediated primarily by members of the E2F family of transcription factors.^{5,6} The transcriptional activity of E2F is regulated by various factors that include (a) binding with DP proteins, (b) phosphorylation of E2F or its DP partner that affects the dimer's ability to bind DNA and stability of E2F against proteolysis, and (c) binding with "pocket" proteins, namely, the retinoblastoma protein (pRB), p107 and p130. Besides regulating E2F-dependent gene expression, pRB exerts a global influence on gene expression through its recruitment of a histone deacetylase (HDAC) whose activity induces a chromatin structure that is repressive to transcription.⁷⁻⁹ Another important transcription factor is Myc whose role in $G1/S$ progression has been shown to parallel that of E2F.¹⁰⁻¹² Myc forms a heterodimer with Max and Myc's transcriptional activity could be repressed by binding of the pocket protein p107.¹² Myc stimulates the expression of cyclin E, cyclin A and Cdc25A, all of which contribute to the activation of CDK2. Furthermore, Myc has been suggested to exert global effects on gene expression by inducing chromatin remodeling in the neighborhood of Myc binding sites on DNA.¹³

Transcription factors that are expressed immediately af-

ter mitogenic stimulation may have functions beyond transcriptional induction of specific *S*-phase genes. It is also thought that they contribute to remodeling chromatin structure by recruiting co-activators possessing histone acetyltransferase (HAT) activity or by recruiting chromatin remodeling factors to replication origins. HAT activity induces a more open chromatin that increases the likelihood of initiating transcription. An example of a transcriptional co-activator is CBP/*p300* which possesses an intrinsic HAT activity and has been shown to be required for *G1/S* transition in some mammalian cell lines.¹⁴ Of significance is the demonstration that CBP/*p300* HAT activity is regulated in a cell cycle-dependent manner; HAT activity is maximal at a time point preceding the *G1/S* transition.¹⁴ CBP/*p300* is a co-activator for the transcription factors E2F, Myc, and *p53*. Indeed, some of these transcription factors may also play a direct role in initiating replication; for example, some replication origins in higher eukaryotes are located in promoter regions of genes such as *c-myc*, and replication near the *c-myc* promoter can indeed be induced in a model replication system (specifically, the negative supercoiling introduced by transcription induced the unwinding of the DNA duplex at the origin sequence).¹⁵

Another intriguing observation is that the CDK-activating kinase, CAK, has been found to be a component of protein complexes involved in the basal transcription machinery and that CAK can phosphorylate RNA polymerase II;¹⁶ thus, CAK can also provide a direct link between cell cycle control and transcription.

C. The *pRB* pathway

Almost all human cancers are associated with some defect in the *pRB* pathway¹² which is loosely defined as the network regulating the activity of the retinoblastoma protein (*pRB*) (see Fig. 1). *pRB* is considered a tumor-suppressor which, as we have stated above, restrains E2F transcriptional activity. In early *G1*, *pRB* is in a hypophosphorylated (active) form that binds and inhibits E2F; entry into *S*-phase requires hyperphosphorylation of *pRB* so that E2F's activity is derepressed allowing the expression of E2F's target *S*-phase genes. It is important to note that there are various feedback loops involving E2F and CDK2 (see Fig. 1). One example of a positive feedback loop is the following: E2F induces transcription of cyclins E and A which then help activate CDK2 that, in turn, phosphorylates *pRB* thereby derepressing E2F. Initial stages of *pRB* phosphorylation are carried out by cyclin D/CDK4,6 and, because of the positive feedback loop involving E2F and CDK2, hyperphosphorylation is driven by the rapid increase in CDK2 activity. We will discuss below that the positive feedback loop mentioned above may not be sufficient to explain the switching behavior expected of the *G1* checkpoint that triggers entry into *S*-phase; the involvement of the phosphatase Cdc25A and the CDK inhibitor *p27* has been suggested to be crucial.⁴

D. The restriction point

The restriction point (R point) marks the transition from mitogen-dependent to mitogen-independent cell cycle pro-

gression in the animal cell cycle.¹⁷ It is a central issue in our present discussion and its very existence could point out the key to understanding the kinetics and control of the initiation of DNA replication. When the R point is "crossed" in mid-to late-*G1*, the cell is committed to replicate its DNA. The preponderance of experimental evidence supports the idea that the activation of CDK2, specifically cyclin E/CDK2, to a sufficient threshold level is necessary to trigger *S*-phase entry (reviewed in Ref. 4). More recent findings point to a necessary collaboration between E2F and cyclin E/CDK2 because certain E2F-target gene products are required for the formation of pre-replication complexes.¹⁸

We have recently suggested and demonstrated by computer simulations⁴ that there could be two important ingredients of a switch associated with the R point: (a) The positive feedback loop between Cdc25A and cyclin E/CDK2, and (b) the mutually antagonistic interaction between *p27* and cyclin E/CDK2 (see Fig. 1). The positive feedback loop in (a) involves the Cdc25A phosphatase activating CDK2 by removing inhibitory phosphate groups and, in turn, CDK2 activates Cdc25A by phosphorylation; however, the existence of this positive feedback loop is still controversial.¹⁹ The antagonistic interaction between *p27* and CDK2 involves, on the one hand, the binding of *p27* to Cyclin E/CDK2 thereby inhibiting the latter's kinase activity and, on the other hand, CDK2 phosphorylates *p27* leading to the proteolytic degradation of *p27* (see Ref. 4 for review). Two important observations have been reported supporting the picture above: (1) in *pRB*-negative cells, a switching behavior in CDK2 activity could still be observed,²⁰ and (2) overexpression of Cdc25A accelerates the *G1/S* transition and shows simultaneous activation of Cdc25A and cyclin E/CDK2.²¹ The core mechanism proposed here has some attractive kinetic properties attributable to an R point switch and it could be expected that signal transduction pathways inducing entry into *S* phase would target this core mechanism; this indeed is the case, as we discuss below.

III. SIGNAL TRANSDUCTION PATHWAYS

Most intracellular signal transduction pathways that are known to induce cell proliferation do so by eliciting two major events in *G1*: One is increased cyclin D synthesis leading to the activation of CDK4, and the other is the activation of cyclin E/CDK2. Experimental evidence has shown that the activation of cyclin D/CDK4 is not absolutely required for the activation of cyclin E/CDK2 as certain signaling pathways mediated by Myc can regulate the expression of proteins that affect CDK2 activity, namely, Cdc25A and cyclin E, as well as downregulate the CDK inhibitor *p27*.¹¹

Quiescent cells, when exposed to growth factors, reenter the cell cycle by rapidly expressing "immediate-early" (IE) genes such as Fos, Jun, Ets, and Myc, to name a few. Both the Ets and AP1 transcription factors (Fos and Jun being components of AP1) induce cyclin D1 expression (reviewed in Ref. 22). Myc induces the expression of Cdc25A, cyclin E, cyclin A (Ref. 10), and also CDK4 (Ref. 23). Using cDNA microarrays, Iyer *et al.*²⁴ monitored the IE genes that are rapidly expressed when quiescent human fibroblasts are

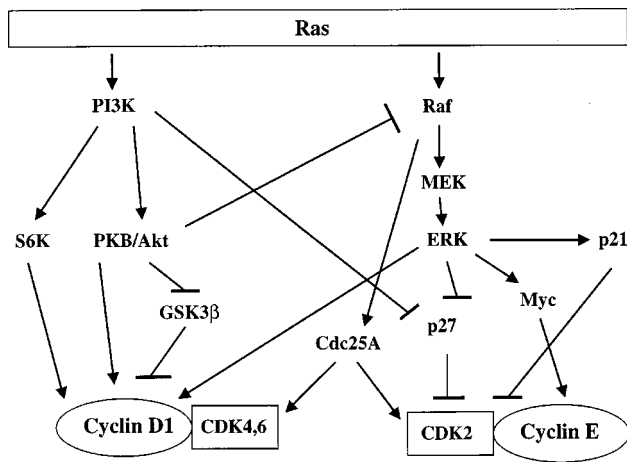


FIG. 2. Two major Ras-effectors, PI3K and Raf, located upstream of intracellular signaling pathways that impinge on cyclin D1, cyclin E, CDK4,6 and CDK2. Stimulatory pathways are shown as arrows while inhibitory pathways are indicated with hammerheads. Abbreviations: CDK=cyclin-dependent kinase; PI3K=phosphatidy inositol 3-kinase; S6K=ribosomal S6 protein kinase; PKB=protein kinase B; GSK3 β =glycogen synthase kinase 3 β ; ERK=extracellular signal-regulated kinase (also known as MAPK, for mitogen-activated protein kinase); MEK=MAPK/ERK kinase (also known as MAPKK, for MAPK Kinase). Figure adapted from Refs. 22 and 26.

stimulated by serum to enter the proliferative state; it was found that these IE genes include those encoding transcription factors and signal transduction proteins.

In this section, we will consider signal transduction pathways initiated at receptors in the plasma membrane that are activated upon binding of growth factors. Two well-characterized groups of these membrane receptors are the receptor tyrosine kinases (RTKs) and the G protein-coupled receptors (GPCRs). For a recent review, the reader is referred to Ref. 1. For some examples of the various growth factors that can activate an important signaling pathway, the MAP kinase pathway to be discussed below, the reader is referred to Ref. 25. In general, multiple signaling pathways may emanate from one type of membrane receptor, these pathways may converge downstream, and there could be cross-talks (inhibitory or activating) among pathways; the challenge is to understand how this complex network of signaling pathways is processed by the cellular system to enter the proliferative state.

A. Signals regulating cyclin D/CDK4 activity

Many studies point to the involvement of the Ras family of small GTPases in the upregulation of cyclin D1 synthesis. A summary of the important pathways from Ras to cyclin D1 is given in Fig. 2 (a figure adapted from Refs. 22 and 26). Three Ras-effector pathways are shown that lead to the synthesis or stabilization of cyclin D1: (1) The mitogen-activated protein kinase (MAPK) cascade involving the sequence Raf/MEK/ERK, (2) the PI3K/S6K pathway, and (3) the PI3K/Akt pathway that can induce transcription of cyclin D1 or stabilization of cyclin D1 by inhibiting GSK-3 β (glycogen synthase kinase 3 β), the kinase that phosphorylates cyclin D1 causing this cyclin's degradation (reviewed in Ref. 22). It has been claimed that activation of PI3K is necessary

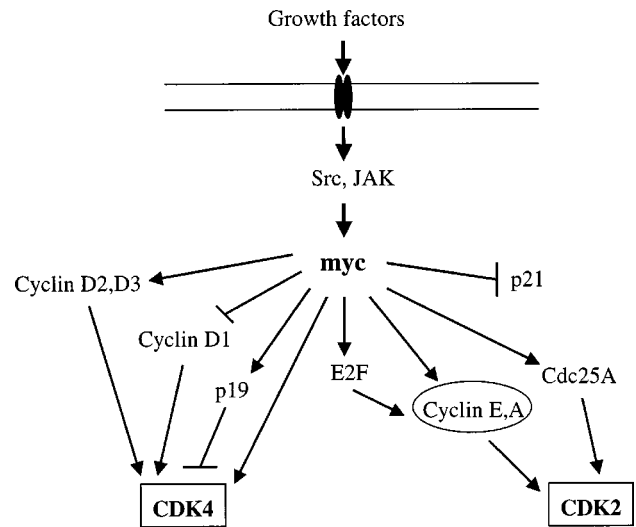


FIG. 3. Expression of the immediate-early gene *c-myc* after activation of growth-factor receptors and tyrosine kinases *Src* or *JAK*. *Myc* targets are shown to include D-type cyclins, cyclins E and A, E2F, Cdc25A, and CDK inhibitors *p19* and *p21*, all of which ultimately affect the activities of the G1 CDKs.

and sufficient for cell-cycle reentry and DNA synthesis, regardless of the status of the Raf/MEK/ERK pathway (reviewed in Ref. 26). Recent reports have pointed to the significance of the ribosomal protein S6, a component of the 40S ribosome, in coordinating cell growth with division^{27–29} and the PI3K/S6K pathway could be an important signaling pathway in this coordination. Figure 2 shows an arrow from Raf to Cdc25A to signify the physical association between these two proteins and the activation of Cdc25A by Raf thereby enhancing the mitogenic signal from the MAP kinase pathway.³⁰ Note that *p27* is downregulated by mechanisms involving both PI3K and ERK, which is necessary for cell cycle reentry because quiescent cells generally have high levels of *p27*. For in-depth discussions of the pathways shown in Fig. 2, the reader is referred to Refs. 22 and 26.

B. Signals regulating cyclin E/CDK2 activity

The relationship between *Myc* and the cell cycle has been reviewed^{10,31} and experimental evidence suggests that *Myc* drives the cell cycle primarily through the activation of cyclin E/CDK2. Figure 3 shows the expression of *Myc* immediately after activation of membrane receptors which leads to activation of tyrosine kinases such as *Src* and *JAK*. *Myc*'s transcriptional targets include cyclin D2,^{32,33} CDK4,^{23,32} Cdc25A,³⁴ E2F-2,³⁵ and cyclin E.³⁶ It has also been shown in some cells that *Myc* suppresses the transcription of cyclin D1 and *gas1* (growth-arrest specific gene 1) (see Ref. 1 for review).

Figure 3 suggests at least three ways that *Myc* could regulate the activity of CDK2: One is via induction of cyclin E expression, another is via induction of Cdc25A expression, and, third, via induction of cyclin D2 which has been implicated in downregulating *p27* and *p21* by a sequestration mechanism.^{33,37} Furthermore, expression of *p21* has been observed to be repressed by *Myc*.³² It is, therefore, not sur-

prising that Myc could bypass the effects of the tumor-suppressor proteins *p16* and *pRB*.¹⁰ Indeed, Santoni-Rugiu *et al.*¹¹ report evidence for the involvement of Myc in a *G1/S*-promoting mechanism that is parallel to the *pRB/E2F* pathway; that is, either E2F alone or Myc alone could induce DNA synthesis. However, both E2F and Myc were shown to be required for an orderly completion of cell cycles. Santoni-Rugiu *et al.*¹¹ further demonstrated that the effect of Myc is associated with Cdc25A and activation of cyclin E/CDK2.

C. Adapters, scaffolds, and signaling modules

Despite the tangled web of signaling pathways, the cell has developed means to analyze, route, and convey signals to their specific targets. For a perspective on the main issues in intracellular signaling, the reader is referred to Weng *et al.*³⁸ Here, we briefly discuss the role of adapter and scaffolding proteins that mediate the formation of macromolecular assemblies or signaling modules. Adapter proteins contain domains that mediate protein–protein interactions; an example is the Grb2 protein which connects an activated membrane receptor and, for example, the SOS protein, a guanine-exchange factor required for the activation of Ras. Scaffold proteins are thought to prevent or minimize crosstalks between related pathways as well as increase the responsiveness of the signaling module to incoming signals. An example of a scaffolding protein is MP1 which binds ERK1 and MEK1, components of the MAP kinase cascade.³⁹ Indeed, this macromolecular organization appears to extend to a higher level of subcellular organization because of evidence that the entire signaling complex, from receptor through Ras to ERK, is located in membrane structures called “caveolae.”⁴⁰ Thus, important kinetic principles in signal transduction may be associated with macromolecular organization beyond the individual activities of the components of a signaling pathway.⁴¹

An interesting quantitative modeling of the involvement of scaffold proteins in MAPK signaling has been carried out by Levchenko *et al.*⁴² Their results suggest that there exists a certain scaffold concentration that generates an optimal signal amplitude, and that this optimal scaffold concentration does not depend on the binding constants of kinase-scaffold interactions but only on the kinase concentrations and the character of kinase–kinase interactions. Thus, the authors conclude that scaffolds contribute to channeling of cascade reactions through scaffold-specific regulation of the reactions and not by sequestering the cascade components from the cytosol.

D. Resolving the complex network of signaling pathways

Structural analyses of the cytoplasmic regions of some membrane receptors have shown that pathways generating contradictory signals may emanate simultaneously from the same receptor (see Ref. 43, for examples). Furthermore, the character and duration of a given signal may evoke completely different cellular responses; for example, although the stimulation of the MAPK cascade usually drives prolif-

eration, sustained vigorous activation of this pathway can induce cell cycle arrest, possibly by upregulation of *p21* as shown schematically in Fig. 2.⁴⁴ More specifically, activation of Raf, a member of the MAPK cascade, can trigger different responses such as proliferation, growth arrest, apoptosis, or differentiation.⁴⁵ There is evidence that the state of the *p53* tumor suppressor protein determines whether a Raf signal stimulates or inhibits proliferation;¹ thus, according to the “orchestrating model” of Hirano,⁴³ *p53* could be the “orchestrator” of the conflicting signals from Raf. Crosstalks among signaling pathways also provide ways of enhancing one pathway over another; a good example shown in Fig. 2 is the inhibition of the Raf/MEK/ERK pathway by the PI3K/PKB pathway through the phosphorylation of Raf by Akt.⁴⁵ This particular crosstalk has been interpreted as the cause for the shift in cellular response of a human breast cancer cell line from cell cycle arrest to proliferation. In addition to crosstalks in signaling networks, several feedback loops have been discovered that can self-regulate Ras/Raf signaling; examples include ERK likely phosphorylating SOS thereby releasing SOS from Grb2 and stopping signals from Ras,¹ and ERK inducing the expression of phosphatase MKP-1 thereby dephosphorylating and deactivating ERK.²⁴

How can we understand and control the complex signaling pathways that determine the fate of a cell? The behavior of computer-based neural networks has been offered as a paradigm.⁴⁶ As more details of particular signaling pathways are elucidated, it may be possible to carry out detailed computer simulations of their operation. For example, Kholodenko *et al.*⁴⁷ have performed both computational modeling and experimental analysis of short-term signaling from the epidermal growth factor receptor of isolated hepatocytes; their kinetic model is able to show under what conditions the activation patterns of signaling proteins are transient or sustained which is crucial in determining the cellular response.

IV. INITIATION OF DNA REPLICATION

Significant advances have been made recently towards understanding the process of initiating DNA replication; most of the knowledge gathered so far comes from studies with budding yeast (*Saccharomyces cerevisiae*), but the emerging picture seems to be conserved from yeast to human.^{48,49} This picture requires the assembly and activation of pre-replication complexes (pre-RCs) onto replication origins on DNA prior to recruitment of DNA polymerases. The pre-RC is composed of the following proteins (see Fig. 1): the origin-recognition complex (ORC), Cdc6, and complexes of MCM proteins (MCM2–7). ORC recruits Cdc6 which, in turn, loads the MCMs onto the chromatin. Some of the MCMs have been shown to possess helicase (DNA-unwinding) activity (see Ref. 48 for review). The replication-competent state of the chromatin is characterized by the presence of chromatin-bound MCMs while the replication-incompetent state is maintained by preventing MCM binding; thus, the MCMs are referred to as “licensing factors” for DNA replication. The CDKs of the cell cycle machinery are then expected to enter into the picture by render-

ing the chromatin replication-competent in *S* phase and, just as important, ensuring that DNA is replicated only once per cell cycle.

A tentative model has been proposed by Fujita⁴⁹ to explain the processes involved in the assembly and activation of pre-RCs. This model assumes that in *G1*, ORC, and Cdc6 are associated with the nuclear matrix and MCM complexes are loaded onto chromatin regions that are not associated with the nuclear matrix. Upon *S*-phase entry, members of the MCM complexes are activated through phosphorylation by kinases such as Cdc7 and CDK2. According to the Fujita model, the MCMs are dissociated from chromatin once replication starts and reloading could be prevented by some mechanism involving CDK2. Recently, Petersen *et al.*⁵⁰ provide evidence that phosphorylation of Cdc6 by cyclin A/CDK2 leads to the export of Cdc6 out of the nucleus, hence preventing the reloading of MCMs. In *G2/M*, CDK2, and Cdc2 could hyperphosphorylate Cdc6 and the MCMs to reinforce the replication-incompetent state of the chromatin. Finally, during exit from mitosis, Cdc2 kinase activity is suppressed and the replication-competent chromatin could again be formed.

Leone *et al.*¹⁸ provide evidence of a collaboration between cyclin E/CDK2 and members of the family of E2F transcription factors in the induction of *S* phase. These authors sketched a model wherein the accumulation of members of the pre-RCs depends on E2F and that either the assembly of the components of the pre-RCs or the activation of an assembled pre-RC is facilitated by phosphorylation carried out by cyclin E/CDK2. Arata *et al.*⁵¹ have investigated the molecular mechanism of E2F-mediated initiation of chromosomal DNA replication in mouse fibroblasts and their results indicate a cascade of processes starting from E2F activation to the induction of the expression of pre-RC components such as the MCMs, Cdc6, and another replication protein, Cdc45. Cdc45 is thought to be a scaffold protein that binds the MCM helicase and the replicative polymerases together (reviewed in Ref. 48). The results of Arata *et al.*⁵¹ suggest that CDK2 activity was required only for chromatin binding of Cdc45 and, therefore, E2F and cyclin E/CDK2 must cooperate to induce chromosomal DNA replication.

The details of the ‘‘firing’’ of replication origins are still sketchy. However, Masai *et al.*⁵² have proposed a model wherein the Cdc7 kinase acts as a switch for DNA replication. Cdc7 kinase activity is cell cycle-dependent, primarily through the availability of its binding partner Dbf4 (called ASK in human cells) which is expressed maximally at the *G1/S* transition. According to Masai *et al.*,⁵² the following hypothetical sequence of events occurs prior to DNA unwinding: Cdc7/Dbf4 phosphorylates MCM2, the inhibitory MCM2 and MCM3/5 subunits then dissociate from the MCM complex (note that hyperphosphorylation of MCM2 has been detected during *S* phase in yeast and mammalian cells), and finally the helicase activity of the MCM4/6/7 subassembly unwinds the DNA duplex. Indeed, experiments *in vitro* have shown that MCM2 inhibit the helicase activity of MCM4/6/7 complex (reviewed in Ref. 52).

V. PUTTING THE PIECES TOGETHER

A rigorous biochemical definition of the quiescent or *G0* state for somatic mammalian cells is difficult to formulate because of widely varying attributes that depend on cell type or lineage (see Ref. 53 for a short review). However, common characteristics shared among quiescent cells have been identified. *G0* cells generally exhibit a low level or an absence of transcription of immediate early (IE) genes. The fact that the mRNAs of many of these IE genes are quite unstable would explain why sufficiently strong and sustained growth-factor stimulation is needed to reenter the cell cycle. This low-level transcription in *G0* is consistent with, and perhaps due to, the finding that there is a predominance of hypophosphorylated *pRB* family members especially *p130*, the predominant repressor of transcription in the quiescent state.^{54,55} The active (hypophosphorylated) state of *pRB* family members suppresses DNA replication in at least two ways: first, as we have mentioned in Sec. II, *pRB* represses transcription of *S*-phase genes by binding and deactivating E2F, and, second, the recruitment of a histone deacetylase by *pRB* induces a relatively closed chromatin structure that suppresses the transcription as well as the replication machinery.

After mitogenic stimulation, the immediate induction of IE genes encoding transcription factors would stimulate the expression of *G1*- and *S*-phase genes and, perhaps equally important, they contribute to chromatin remodeling to counteract the effects of active *pRB*, by recruiting co-activators possessing histone acetyltransferase (HAT) activity (e.g., CBP/*p300*) or by recruiting chromatin remodeling factors to replication origins that would likely facilitate initiation of DNA replication. We have also mentioned above the possible role of the IE gene *c-myc* in the global influence on transcription through chromatin remodeling.¹³ Future studies in this exciting area of research are expected to enhance our understanding of the cell cycle.

To further counteract the transcription-repressive effect of active *pRB*, activation of CDK4 is required so that CDK4 can initiate the deactivation of *pRB* via phosphorylation. CDK4 expression has recently been shown to be induced by *c-myc*.²³ In the quiescent state, however, CDK4 is found in a tyrosine-phosphorylated and inactive state (at least in rat fibroblasts),⁵⁶ mitogen-stimulated coincidental expression of cyclin D1 and Cdc25A phosphatase (which removes the inhibitory tyrosine phosphorylation of CDK4) would then lead to the activation of CDK4.

In addition to *pRB* as a major obstacle to cell cycle entry, *p27* is usually present at high levels in quiescent cells and would be expected to inhibit the CDKs. We have seen in Sec. III (Fig. 2) that *p27* is a target of the Ras effector pathways and is rapidly downregulated once the autocatalytic activation of cyclin E/*cdk2* sets into motion as described in Sec. II. Once CDK4 or CDK6 is sufficiently activated, our recent modeling of the restriction point suggests a minimum duration of mitogenic stimulation to achieve a threshold of cyclin E/CDK2 kinase activity.⁴ Our computer simulations demonstrated that a sharp switching behavior could be generated by the presence of a positive feedback loop between cyclin E/CDK2 and Cdc25A along with a mutually negative

interaction between cyclin E/CDK2 and *p27*. This model is supported by recent experimental evidence.^{20,21} Recently, it has also been shown⁵⁷ that *Cdc25A* is a target of a *p53*-independent *G1* DNA damage checkpoint pathway; according to our model, this is expected because *Cdc25A* is part of the core mechanism of the R-point switch.

To further our understanding of the coupling between the *G1/S* cell cycle machinery and the initiation of DNA replication, more details must be known about the molecular mechanism of the collaboration between cyclin E/CDK2 and E2F in the assembly and activation of pre-RCs. The cell cycle-dependent kinase activity of *Cdc7* and its possible role as the switch for the initiation of replication, as proposed by Masai *et al.*,⁵² must be taken into consideration in future studies of the mechanism of *S*-phase entry. However, it must be emphasized that, as observed in normal animal cells, it is the R point that represents the checkpoint beyond which the cells are observed to be committed to DNA replication; whether or not the assembly of pre-RCs is part of the control network of the R point, and how replication seems to follow automatically after crossing the R-point, are important questions that need to be further investigated.

VI. CONCLUDING REMARKS

Most existing mathematical models of the eukaryotic cell cycle employ continuous deterministic differential equations to describe the dynamics of CDK activation and deactivation under spatially homogeneous conditions.⁵⁸ Future models, however, must take into consideration the regulation of the cell cycle by compartmentalization or subcellular localization of the proteins involved. For example, an interesting observation⁵⁹ on quiescent cells implicates compartmentalization of CDK2 in the control of cell cycle reentry; it was shown that there is an abundance of cyclin E, *p21* and *p27* but very little CDK2 in *G0* nuclei. Upon serum stimulation, it was observed that there is a rapid increase in the amount of cyclin E bound to CDK2 as a result of the serum-stimulated entry of CDK2 into the nucleus. Another important example of how protein relocalization controls entry into the cell cycle is provided by ERK which enters the nucleus as a result of phosphorylation via the MAP kinase cascade (reviewed in Ref. 3).

Beyond establishing the players and the topology of the network of pathways, a quantitative modeling of the cell cycle requires a proper and careful consideration of the kinetics of the following dynamical elements: Protein–protein and protein–nucleic acid interactions leading to formation of signaling modules and various complexes involved in transcription and replication; post-translational modifications such as phosphorylation and dephosphorylation that affect the conformations or activities of enzymes; and other epigenetic mechanisms such as histone acetylation and DNA methylation that affect chromatin structure and ultimately exert some global influence on transcription and replication. Undoubtedly, mathematical and computer-based analyses will help us sort out the increasingly complex picture of the cell cycle machinery.

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APPENDIX: LIST OF ABBREVIATIONS

CAK	cdk-activating kinase
CDK	cyclin-dependent kinase
CKI	cdk inhibitor
ERK	extracellular signal-regulated kinase
GPCR	G protein-coupled receptor
GSK3 β	glycogen synthase kinase 3 β
HAT	histone acetyltransferase
HDAC	histone deacetylase
IE	immediate-early
MAPK	mitogen-activated protein kinase
MCM	minichromosome maintenance
MEK	MAPK/ERK kinase
mRNA	messenger ribonucleic acid
ORC	origin-recognition complex
PI3K	phosphatidylinositol 3-kinase
PKB	protein kinase B
pRB	retinoblastoma protein
pre-RC	pre-replication complex
R-point	restriction point
RTK	receptor tyrosine kinase
S6K	ribosomal S6 protein kinase

¹D. T. Denhardt, "Signal transduction pathways and regulation of the mammalian cell cycle: Cell-type dependent integration of external signals," in *The Molecular Basis of Cell Cycle and Growth Control*, edited by G. S. Stein, R. Baserga, A. Giordano, and D. T. Denhardt (Wiley-Liss, New York, 1999), pp. 225–304.

²F. B. Engel, L. Hauck, M. C. Cardoso, H. Leonhardt, R. Dietz, and R. von Harsdorf, "A mammalian myocardial cell-free system to study cell cycle reentry in terminally differentiated cardiomyocytes," *Circ. Res.* **85**, 294–301 (1999).

³J. Pines, "Four-dimensional control of the cell cycle," *Nature Cell Biol.* **1**, E73–E79 (1999).

⁴B. D. Aguda and Y. Tang, "The kinetic origins of the restriction point in the mammalian cell cycle," *Cell Prolif.* **32**, 321–335 (1999).

⁵K. Helin, "Regulation of cell proliferation by the E2F transcription factors," *Curr. Opin. Gen. Dev.* **8**, 28–35 (1998).

⁶H. Muller and K. Helin, "The E2F transcription factors: Key regulators of cell proliferation," *Biochim. Biophys. Acta* **1470**, M1–M12 (2000).

⁷L. Magnaghi-Jaulin, R. Groisman, I. Naguibneva, P. Robin, S. Lorain, J. Le Villain, F. Troalen, D. Trouche, and A. Harel-Bellan, "Retinoblastoma protein represses transcription by recruiting a histone deacetylase," *Nature (London)* **391**, 601–605 (1998).

⁸R. X. Luo, A. Postigo, and D. C. Dean, "Rb interacts with histone deacetylase to repress transcription," *Cell* **92**, 463–473 (1998).

⁹A. Brehm and T. Kouzarides, "Retinoblastoma protein meets chromatin," *Trends Biochem. Sci.* **24**, 142–145 (1999).

¹⁰B. Amati, K. Alevizopoulos, and J. Vlach, "Myc and the cell cycle," *Frontiers in Bioscience* **3**, d250–268 (1998).

¹¹E. Santoni-Rugiu, J. Falck, N. Mailand, J. Bartek, and J. Lukas, "Involvement of Myc activity in a *G1/S*-promoting mechanism parallel to the pRb/E2F pathway," *Molecular and Cellular Biology* **20**, 3497–3509 (2000).

¹²T. Moroy, "Restriction point control in human cancer," *Tumordiagn. u. Ther.* **19**, 93–100 (1998).

¹³M. D. Cole and S. B. McMahon, "The Myc oncoprotein: A critical evaluation of transactivation and target gene regulation," *Oncogene* **18**, 2916–2924 (1999).

¹⁴S. Ait-Si-Ali, A. Polesskaya, S. Filleur, R. Ferreira, A. Duquet, P. Robin, A. Vervish, D. Troucher, F. Cabon, and A. Harel-Bellan, "CBP/p300

- histone acetyl-transferase activity is important for the *G1/S* transition," *Oncogene* **19**, 2430–2437 (2000).
- ¹⁵ Y. Murakami and Y. Ito, "Transcription factors in DNA replication," *Frontiers in Bioscience* **4**, d824–833 (1999).
 - ¹⁶ P. L. Puri, T. K. Maclachlan, M. Levrero, and A. Giordano, "The intrinsic cell cycle: From yeast to mammals," in *The Molecular Basis of Cell Cycle and Growth Control*, edited by G. S. Stein, R. Baserga, A. Giordano, and D. T. Denhardt (Wiley-Liss, New York, 1999), pp. 15–79.
 - ¹⁷ A. B. Pardee, "A restriction point control of normal animal cell proliferation," *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1286–1290 (1974).
 - ¹⁸ G. Leone, J. DeGregori, L. Jakoi, J. G. Cook, and J. R. Nevins, "Collaborative role of E2F transcriptional activity and *G1* cyclin-dependent kinase activity in the induction of *S* phase," *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6626–6631 (1999).
 - ¹⁹ V. Sexl, J. A. Diehl, C. J. Sherr, R. Ashmun, D. Beach, and M. F. Roussel, "A rate-limiting function of *cdc25A* for *S* phase entry inversely correlate with tyrosine dephosphorylation of *Cdk2*," *Oncogene* **18**, 573–582 (1999).
 - ²⁰ N. Takuwa and Y. Takawa, "Ras activity late in *G1* phase required for *p27Kip1* downregulation, passage through the restriction point, and entry into *S* phase in growth factor-stimulated NIH 3T3 fibroblasts," *Mol. Cell. Biol.* **17**, 5348–5358 (1997).
 - ²¹ I. Blomberg and I. Hoffmann, "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.* **19**, 6183–6194 (1999).
 - ²² C. Marshall, "How do small GTPase signal transduction pathways regulate cell cycle entry?" *Curr. Opin. Cell Biol.* **11**, 732–736 (1999).
 - ²³ H. Hermeking, C. Rago, M. Schuhmacher, Q. Li, J. F. Barrett, A. J. Obaya, B. C. O'Connell, M. K. Mateyak, W. Tam, F. Kohlhuber, C. V. Dang, J. M. Sedivy, D. Eick, B. Vogelstein, and K. W. Kinzler, "Identification of *CDK4* as a target of *c-Myc*," *Proc. Natl. Acad. Sci. U.S.A.* **97**, 2229–2234 (2000).
 - ²⁴ V. R. Iyer, M. B. Eisen, D. T. Ross, G. Schuler, T. Moore, J. C. F. Lee, J. M. Trent, L. M. Staudt, J. J. Hudson, M. S. Boguski, D. Lashkari, D. Shalon, D. Botstein, and P. O. Brown, "The transcriptional program in the response of human fibroblasts to serum," *Science* **283**, 83–87 (1999).
 - ²⁵ M. J. Robinson and M. H. Cobb, "Mitogen-activated protein kinase pathways," *Curr. Opin. Cell Biol.* **9**, 180–186 (1997).
 - ²⁶ S. Frame and A. Balmain, "Integration of positive and negative growth signals during ras pathway activation in vivo," *Curr. Opin. Gen. Dev.* **10**, 106–113 (2000).
 - ²⁷ J. Montagne, M. J. Stewart, H. Stocker, E. Hafen, S. C. Kozma, and G. Thomas, "Drosophila *S6* kinase: A regulator of cell size," *Science* **285**, 2126–2129 (1999).
 - ²⁸ S. Volarevic, M. J. Stewart, B. Ledermann, F. Zilberman, L. Terracciano, E. Montini, M. Grompe, S. C. Kozma, and G. Thomas, "Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein *S6*," *Science* **288**, 2045–2047 (2000).
 - ²⁹ G. Thomas, "An encore for ribosome biogenesis in the control of cell proliferation," *Nature Cell Biol.* **2**, E71–E72 (2000).
 - ³⁰ K. Galaktionov, C. Jessus, and D. Beach, "Raf1 interaction with *Cdc25* phosphatase ties mitogenic signal transduction to cell cycle activation," *Genes Dev.* **9**, 1046–1058 (1995).
 - ³¹ A. J. Obaya, M. K. Mateyak, and J. M. Sedivy, "Mysterious liaisons: The relationship between *c-Myc* and the cell cycle," *Oncogene* **18**, 2934–2941 (1999).
 - ³² H. A. Collier, C. Grandori, P. Tamayo, T. Colbert, E. S. Lander, R. N. Eisenman, and T. R. Golub, "Expression analysis with oligonucleotide microarrays reveals that *myc* regulates genes involved in growth, cell cycle, signaling, and adhesion," *Proc. Natl. Acad. Sci. U.S.A.* **97**, 3260–3265 (2000).
 - ³³ C. Bouchard, K. Thieke, A. Maier, R. Saffrich, J. Hanley-Hyde, W. Anzorge, S. Reed, P. Sicinski, J. Bartek, and M. Eilers, "Direct induction of cyclin D2 by *Myc* contributes to cell cycle progression and sequestration of *p27*," *EMBO J.* **18**, 5321–5333 (1999).
 - ³⁴ K. Galaktionov, X. Chen, and D. Beach, "Cdc25 cell-cycle phosphatase as a target of *c-myc*," *Nature (London)* **382**, 511–517 (1996).
 - ³⁵ R. Sears, K. Ohtani, and J. R. Nevins, "Identification of positively and negatively acting elements regulating expression of the *E2F2* gene in response to cell growth signals," *Mol. Cell. Biol.* **17**, 5227–5235 (1997).
 - ³⁶ I. Perez-Roger, D. L. C. Solomon, A. Sewing, and H. Land, "Myc activation of cyclin E/CDK2 kinase involves induction of cyclin E gene transcription and inhibition of *p27Kip1* binding to newly formed complexes," *Oncogene* **14**, 2373–2381 (1997).
 - ³⁷ I. Perez-Roger, S.-H. Kim, B. Griffiths, A. Sewing, and H. Land, "Cyclin D1 and D2 mediate *Myc*-induced proliferation via sequestration of *p27Kip1* and *p21Cip1*," *EMBO J.* **19**, 5310–5320 (1999).
 - ³⁸ G. Weng, U. S. Bhalla, and R. Iyengar, "Complexity in biological signaling systems," *Science* **284**, 92–96 (1999).
 - ³⁹ E. A. Elion, "Routing MAP kinase cascades," *Science* **281**, 1625–1626 (1998).
 - ⁴⁰ P. Liu, Y.-S. Ying, and R. G. W. Anderson, "Platelet-derived growth factor activates mitogen-activated protein kinase in isolated caveolae," *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13666–13670 (1997).
 - ⁴¹ C. S. Zuker and R. Ranganathan, "The path to specificity," *Science* **283**, 650–651 (1999).
 - ⁴² A. Levchenko, J. Bruck, and P. W. Sternberg, "Scaffold proteins may biphasically affect the levels of mitogen-activated protein kinase signaling and reduce its threshold properties," *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5818–5823 (2000).
 - ⁴³ T. Hirano, "Molecular basis underlying functional pleiotropy of cytokines and growth factors," *Biochem. Biophys. Res. Commun.* **260**, 303–308 (1999).
 - ⁴⁴ D. Woods, D. Parry, H. Cherwinski, E. Bosch, E. Lees, and M. McMahon, "Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by *p21Cip1*," *Mol. Cell. Biol.* **17**, 5598–5611 (1997).
 - ⁴⁵ S. Zimmermann and K. Moelling, "Phosphorylation and regulation of Raf by Akt (protein kinase B)," *Science* **286**, 1741–1744 (1999).
 - ⁴⁶ D. Bray, "Intracellular signaling as a parallel distributed process," *J. Theor. Biol.* **143**, 215–231 (1990).
 - ⁴⁷ B. N. Kholodenko, O. V. Demin, G. Moehren, and J. B. Hoek, "Quantification of short term signaling by the epidermal growth factor receptor," *J. Biol. Chem.* **42**, 30169–30181 (1999).
 - ⁴⁸ J. Leatherwood, "Emerging mechanisms of eukaryotic DNA replication initiation," *Curr. Opin. Cell Biol.* **10**, 742–748 (1998).
 - ⁴⁹ M. Fujita, "Cell cycle regulation of DNA replication initiation proteins in mammalian cells," *Frontiers in Bioscience* **4**, d816–823 (1999).
 - ⁵⁰ B. O. Petersen, J. Lukas, C. S. Sorensen, J. Bartek, and K. Helin, "Phosphorylation of mammalian *CDC6* by cyclin A/CDK2 regulates its subcellular localization," *EMBO J.* **18**, 396–410 (1999).
 - ⁵¹ Y. Arata, M. Fujita, K. Ohtani, S. Kijima, and J. Kato, "Cdk2-dependent and -independent pathways in E2F-mediated *S* phase induction," *J. Biol. Chem.* **275**, 6337–6345 (2000).
 - ⁵² H. Masai, N. Sato, T. Takeda, and K. Arai, "Cdc7 kinase complex as a molecular switch for DNA replication," *Frontiers in Bioscience* **4**, d834–840 (1999).
 - ⁵³ A. Zetterberg and O. Larsson, "Cell cycle progression and cell growth in mammalian cells: Kinetic aspects of transition events," in *Cell Cycle Control*, edited by C. Hutchison and D. M. Glover (IRL, Oxford, 1995), pp. 206–227.
 - ⁵⁴ K. Ohtani, "Implication of transcription factor E2F in regulation of DNA replication," *Frontiers in Bioscience* **4**, d793–804 (1999).
 - ⁵⁵ E. J. Smith, G. Leone, J. DeGregori, L. Jakoi, and J. R. Nevins, "The accumulation of an E2F-*p130* transcriptional repressor distinguishes a *G0* cell state from a *G1* cell state," *Mol. Cell. Biol.* **16**, 6965–6976 (1996).
 - ⁵⁶ S. Jinno, S.-C. Hung, and H. Okayama, "Cell cycle start from quiescence controlled by tyrosine phosphorylation of *Cdk4*," *Oncogene* **18**, 565–571 (1999).
 - ⁵⁷ N. Mailand, J. Falck, C. Lukas, R. G. Syljuasen, M. Welcker, J. Bartek, and J. Lukas, "Rapid destruction of human *Cdc25A* in response to DNA damage," *Science* **288**, 1425–1429 (2000).
 - ⁵⁸ B. Novak and J. J. Tyson, "Numerical analysis of a comprehensive model of *M*-phase control in *Xenopus* oocyte extracts and intact embryos," *J. Cell. Sci.* **106**, 1153–1168 (1993); M. N. Obeyesekere, E. S. Knudsen, J. Y. J. Wang, and S. O. Zimmerman, "A mathematical model of the regulation of the *G1* phase of RB+/+ and RB-/- mouse embryonic fibroblasts and an osteosarcoma cell line," *Cell Prolif.* **30**, 171–194 (1997); K. W. Kohn, "Functional capabilities of molecular network components controlling the mammalian *G1/S* cell cycle phase transition," *Oncogene* **16**, 1065–1075 (1998).
 - ⁵⁹ W. A. Bresnahan, I. Boldogh, T. Ma, T. Albrecht, and E. A. Thompson, "Cyclin E/Cdk2 activity is controlled by different mechanisms in the *G0* and *G1* phases of the cell cycle," *Cell Growth & Differentiation* **7**, 1283–1290 (1996).