HHS Public Access

Author manuscript

FEBS J. Author manuscript; available in PMC 2018 February 01.

Published in final edited form as:

FEBS J. 2017 February ; 284(3): 362–375. doi:10.1111/febs.13898.

Cell cycle proliferation decisions: the impact of single cell analyses

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Abstract

Cell proliferation is a fundamental requirement for organismal development and homeostasis. The mammalian cell division cycle is tightly-controlled to ensure complete and precise genome duplication and segregation of replicated chromosomes to daughter cells. The onset of DNA replication marks an irreversible commitment to cell division, and the accumulated efforts of many decades of molecular and cellular studies have probed this cellular decision, commonly called the restriction point. Despite a long-standing conceptual framework of the restriction point for progression through G1 phase into S phase or exit from G1 phase to quiescence (G0), recent technical advances in quantitative single cell analysis of mammalian cells have provided new insights. Significant intercellular heterogeneity revealed by single cell studies and the discovery of discrete subpopulations in proliferating cultures suggests the need for an even more nuanced understanding of cell proliferation decisions. In this review, we describe some of the recent developments in the cell cycle field made possible by quantitative single cell experimental approaches.

Graphical abstract

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Conflicts of interest: The authors have no conflicts of interest.

Author contributions

J.P.M. and J.G.C. co-wrote the manuscript and designed the figures.

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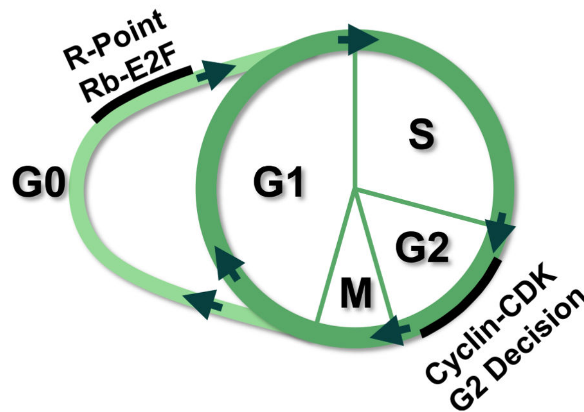

Proliferating cells decide to pursue a next cell division or exit to G0. Recent single cell studies provide new insights into when in the cell cycle cells commit to division. Actively proliferating cells have at least two decision points, the first in G2 phase and the second in G1, whereas cells entering the cycle from G0 (quiescence) decide in G1.

Keywords

cell cycle; restriction point; cell division; single cell; G1; G2; G0; quiescence; biosensor; review

Introduction

The cell cycle is a series of tightly-regulated molecular events controlling DNA replication and mitosis, producing two new daughter cells from a single parent cell. Every cell cycle, individual metazoan cells have the opportunity to adopt one of two mutually-exclusive proliferation-related fates: continued cell cycle progression or cell cycle exit. Cell cycle exit encompasses multiple distinct states including permanent arrests associated with terminal differentiation or senescence and reversible exit known as quiescence (G0). Settings where the importance of cell cycle entry is particularly apparent include hematopoiesis, replenishing the epithelial lining of the digestive tract, replacing shed skin cells, activation of adaptive immune responses, and wound healing. Tight control over cell cycle entry and exit is also critical for tumor prevention since most transformed cells do not appropriately respond to cues that trigger quiescence. It is thus important to understand the processes that determine cell cycle entry, cell cycle progression, and exit to quiescence. In this review, we highlight advances in understanding such proliferation decisions in mammalian cells and particularly the impact of recent improvements in single cell analysis methods.

Much of our understanding of the molecular determinants of cell cycle progression comes from analyses of cell populations. There are well-documented substantial changes in gene expression, protein abundance, protein post-translational modifications, and DNA synthesis as a population of cells moves from one cell cycle phase to the next, or when a population becomes quiescent. Commonly-used methods to monitor these changes include immunoblotting and quantitative real-time PCR for individual proteins or messenger RNAs

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or proteomics and transcriptomics for global analyses. The limits of detection for these methods have, until very recently, required pooled material from many thousands or millions of cells. A culture of genetically identical cells grown together are presumed to behave very similar to one another in the interpretation of such ensemble techniques, though this assumption is not necessarily valid.

Even when two cells begin a cell cycle phase at the same time - such as immediately after cytokinesis - marked variation among individual cells and heterogeneous responses to external and internal cell cycle cues lead to different rates of cell cycle progression [1]. Cell cycle heterogeneity arises from intrinsic and extrinsic variability in gene expression resulting in heterogeneity in mRNA and protein levels among individual cells [2–4]. Additional cell cycle variation could be a consequence of cell division itself. Although DNA is actively distributed equally to daughter cells, RNA, proteins, metabolites, and organelles may be randomly and unequally distributed at every cell division [5,6]. For example, Czerniak *et al.* used antibody staining to examine the distribution of several individual proto-oncoproteins that each have the potential to profoundly impact cell cycle progression, including the Ras GTPase and the c-Myc transcription factor. These authors found intercellular variability particularly in G1 phase cells, and most notably, differences in antigen concentrations in newly-born sister cells [7]. Once a cell acquires higher or lower concentrations of a key protein, the difference from the population average can remain for two or more cell cycles [8]. There may also be coexisting and interconverting subpopulations of cells with different molecular states that influence proliferative fate [9–11].

Each individual cell “decides” to proliferate or exit the cycle separately from the whole population, integrating numerous signals to time the transition from one phase of the cycle to the next [12]. The intercellular heterogeneity and generally asynchronous growth of proliferating cells creates challenges for precisely tracking their behavior. A popular method to overcome heterogeneity is artificially synchronizing cells with inhibitors in one cell cycle phase, releasing them from the block, and monitoring molecular markers over time. Although cell cycle synchronization creates generally homogenous populations, the further each time point is removed from the initial synchronizing block, the more heterogeneous the population becomes (Illustrated in Figure 1A). Furthermore, the arrests themselves can induce unique stress signaling pathways that aren’t normally engaged (or are engaged at much lower intensity) in normal cell cycles [13–15]. For example, nocodazole synchronization in mitosis can create genome instability after mitotic exit [16].

Moreover, a population of cells may harbor consistent or transiently interconverting distinct subpopulations with different proliferation dynamics. Methods that measure the aggregate concentration or activity in a population may mask subpopulations containing different levels of individual protein abundance or activity [17]. If a culture contains two very distinct populations in significant numbers (such as a mixture of proliferating cells and quiescent or senescent cells, ensemble molecular detection methods will report an intermediate level of activity that may not be a true reflection of either state (Figure 1B). Only experiments measuring cell cycle parameters from single cells can identify distinct subpopulations that are hidden by methods that average the whole population.

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Methods to assess proliferation in individual cells

Single cell analysis is not itself new in the cell proliferation field. Counting the individual mitotic cells per field in a biopsy tissue sample has been a long-standing practice in clinical settings as one prognostic indicator for cancer patients. Similarly, for decades patient samples have been routinely probed with antibodies to proteins that are only expressed in proliferating cells, and the expression intensity and number of expressing cells provide valuable diagnostic information [18–21]. With respect to understanding fundamental cell cycle regulation, early studies assessed DNA synthesis (using radioactive nucleotides or nucleotide analogs), protein abundance, localization, post-translational modifications, and cell division in single cells grown under a variety of culture conditions. In this way, the eukaryotic cell cycle was divided into discrete phases [22,23].

The development of flow cytometry that measures the intensity of fluorescent DNA or antibody stains revolutionized the cell cycle field. One of the many advantages over the earlier methods is the routine and rapid quantification of thousands of cells per sample and the relatively unbiased comparisons among different samples [24]. Moreover, multiple molecular parameters can be assessed simultaneously, and the number of distinct measurements is limited only by the spectral properties of the reagents and the fluorescence detectors. Since large numbers of cells are analyzed by flow cytometry simultaneously, researchers have opportunities to derive rates of cell cycle phase transitions and identify minor subpopulations of cells [25,26]. The opportunities for multiplex measurements have expanded significantly with the recent development of mass cytometry [27].

The advent of genetically-encoded fluorescent proteins expanded the capabilities of single cell analysis in many fields, including the cell cycle. An important recent contribution by Sakaue-Sawano *et al.* took advantage of existing knowledge about specific ubiquitin-mediated protein degradation events to create a pair of cell cycle biosensors they named FUCCI for “fluorescent ubiquitination-based cell cycle indicator.” [11]. They generated and co-expressed red and green fluorescent protein fusions that are degraded in S phase or G1 phase respectively. Using flow cytometry, fixed cell microscopy and time-lapse live cell imaging, these biosensors were utilized initially by Sakaue-Sawano and subsequently by many others in a variety of physiological, developmental, and pathological settings to visualize cell cycle phases [28–30]. New genome editing tools to efficiently incorporate fluorescent tags at endogenous genes will expand the proliferation reporters beyond the original FUCCI pair [31].

The high time resolution of fluorescent live cell imaging using fluorescent reporters is particularly powerful for determining the relationships among two or more events, even when they are closely-spaced in time [12,32]. Live cell microscopy reveals when a stereotypical order of events characterizes a given cell cycle phase [33]. For example, live imaging studies in both budding yeast and human cells identified sequences of protein degradation events that characterize passage through the individual subphases of mitosis or at the onset of S phase [34–36]. Furthermore, automated cell identification and tracking computer programs and scripts now make it possible to generate robust datasets of reporter signal intensities from many hundreds of cells without the potential biases of quantifying

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cells by hand [37,38]. While these software tools are rapidly advancing, each imaging data set usually requires a customized image analysis pipeline and parameter optimization.

Cell cycle proliferation and the restriction point phenomenon

The commitments to either cell cycle progression or quiescence are mutually exclusive. Moreover the collection of molecular states that differ between quiescent cells and cells committed to S phase entry is extensive and varied [39]. It's worth emphasizing here that quiescent cells are not characterized simply as the absence of proliferation-associated activities. Quiescence is actively maintained not only through continual transcriptional repression of genes required for DNA replication and mitosis, but also through active expression of anti-apoptotic, anti-senescence, and anti-differentiation genes [40–43].

The commitment to proliferation is a binary decision that is marked by the onset of DNA replication, an irreversible process. For this reason the transition from G1 to S phase is under tight and multifaceted controls; disruption in those controls drive many aspects of cancer cell phenotypes. Moreover, partial replication of chromosomal DNA is particularly dangerous, because the eventual dissolution of replication forks that do not ultimately meet one another during normal termination leads to double-stranded chromosome breaks [44]. Thus, cells that start replication must be fully prepared to finish replication in a time-frame compatible with the biochemical limitations of the replication program (number of initiation sites, replication fork stability and speed, etc.).

Cells commit to either quiescence (G0) or future S phase entry at the restriction point. The term "restriction point" or "R-point" was first proposed by Arthur Pardee in 1974 based on the timing of DNA synthesis in populations of hamster fibroblasts after a cell cycle block and release [45]. The restriction point was the cell cycle point past which cells were irreversibly committed to S phase entry, whether or not extracellular mitogens were removed [46]. It is sometimes described as the "point of no return" in the cell cycle with respect to S phase entry. Mammalian cells in culture require external mitogens for proliferation, and since the DNA content of quiescent cells is the same as G1 cells, and mitogen stimulation leads to DNA synthesis before mitosis, it seems obvious that cells exit to quiescence from G1. What has not always been obvious is precisely *when* during the cell cycle mitogens are sensed to execute the commitment to proliferation nor what the precise molecular nature of the decision itself is [47].

Pardee noted the limitations of measuring a cell population for cell cycle commitment and acknowledged the possibility that intercellular heterogeneity or the presence of fast-responding subpopulations would be missed in his study [45]. A subsequent single cell microscopy study in 1985 by Zetterberg and Larsson indicated that the only time mitogens are essential for the commitment to proliferation is in G1 phase [48]. In these pioneering experiments, the authors conducted time lapse microscopy of proliferating mouse fibroblasts that were transiently deprived of mitogens. They found that the only cells that responded to mitogen withdrawal (by markedly delaying the next cell division) were very "young" cells in the first few hours after mitosis. They also noted intercellular variability in G1 length. Their

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observations have guided discussions of cell cycle commitment as an exclusively G1 event for the past 30 years.

Cell cycle commitment during re-entry to G1 from quiescence

The mechanisms by which mitogens stimulate passage through the restriction point and cell cycle commitment initiate at the plasma membrane with mitogen receptors (Figure 2A). Of central relevance, external mitogens activate signal transduction activities including the Ras GTPase [49]. Ras activation stimulates a pathway culminating in activated gene expression through transcription factors such as c-Myc, serum response factor, and others [50–52]. Among the principle proliferation genes under control of these transcription factors are those encoding D-type cyclins (hereafter Cyclin D). Cyclin D protein complexes with either of two redundant cyclin-dependent protein kinases, Cdk4 or Cdk6, and cyclin binding activates Cdk4/6 to phosphorylate the Retinoblastoma protein (Rb) transcriptional regulator at one of many individual phosphorylation sites. Rb does not itself bind DNA but rather is recruited to specific genes through interaction with the family of DNA binding transcription factors known as the E2F family. E2F controls the expression of a suite of genes necessary for DNA replication, S phase progression, and mitosis. Rb binds E2F which results in active transcriptional repression of those E2F target genes. For many years, the prevailing paradigm – based largely on molecular assays of cell populations - has been that initial partial Rb phosphorylation by Cyclin D/Cdk4 (or Cyclin D/Cdk6) gradually releases E2F inhibition to activate transcription [49,50,53–55]. One of the E2F target genes that is induced is *cyclin E*, and the Cyclin E protein binds and activates the Cdk2 protein kinase which then participates in Rb phosphorylation in a positive feedback loop; this feedback loop is further reinforced by the fact that E2F1 is one of the E2F target genes. Full Rb hyperphosphorylation completely releases Rb from E2F relieving repression of E2F target genes and allowing for activated E2F-dependent transcriptional induction and S phase entry.

To ensure adequate preparation before S phase onset and a robust start and finish for DNA replication, the G1/S transition is a “bistable switch.” Bistability is a steady state distribution of two populations which may interconvert in a rapid, switch-like manner. It characterizes many cell cycle transitions so that progression from one phase to the next is rapid, complete, and unidirectional [56–58]. In the case of the G1/S transition, bistability applies to the activation of the cyclin dependent kinase (Cdk) Cyclin E/Cdk2 which triggers replication initiation [59]. A series of molecular feedback and feedforward relationships ensure that very little Cdk2 is active during early-to-mid G1 but in late G1, Cdk2 activity is sharply upregulated and stably maintained until later in S phase. These include: Cyclin E synthesis, degradation of a Cdk2 inhibitor protein ($p27^{KIP}$), Cdc6-dependent loading of the replicative DNA helicase to license replication origins, and Cyclin E/Cdk2 activation. Cdk2 activation is abrupt, but not at the exact same time in different cells. At any time in a population there may be subpopulations of cells with different discrete Cdk2 activity levels. The experimental challenges posed by this intercellular variability in the timing of rapid, irreversible, switch-like transitions from one phase to the next are greatly alleviated by single cell analyses.

Pardee proposed that cells exist in two states – either proliferating or quiescent – and that the G1 restriction point marked the shift from quiescence to proliferation. E2F activation

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emerged later as a popular marker of post-restriction point cells which have committed to division. The prevailing model of gradual and progressive Rb phosphorylation during G1 was not easily reconciled with an all-or-none proliferation decision however. Methods that measure entire populations of cells – such as DNA synthesis by general thymidine incorporation or immunoblotting cell lysates for Rb hyperphosphorylation, report only a global average of any subpopulations, making it difficult to precisely detect a switch between two states. To overcome this hurdle and focus specifically on E2F control, Yao *et al.* used single cell flow cytometry with fluorescent E2F or Cyclin D transcriptional reporters to correlate mitogen-induced gene expression with the switch triggering cell cycle commitment during re-entry from quiescence (G0) [60]. They synchronized cells in G0 by mitogen withdrawal then re-stimulated with serum. Transcriptional activity displayed an all or none bistable response resulting in two distinct cell populations. That is, each individual cell either remained quiescent with no reporter transcription, or displayed maximally-active expression. Significantly, there were no intermediate states and no partial commitment to the cell cycle. Sustained mitogen stimulation shifted the probability that any individual cell committed to S phase entry. This shifting probability was detectable as changes in the distribution of the population between the two states. Single cell flow cytometry clearly distinguished and quantified the two subpopulations of maximal or minimal responses.

The study by Yao *et al.* was also the first demonstration of a bistable commitment point directly related to E2F activity. A feature of this bistability was that cell cycle commitment measured by reporter activation required sustained and strong E2F stimulation, but once activated, was largely irreversible. Low levels of mitogens were insufficient to pass the restriction point and activate E2F. On the other hand, withdrawing mitogens after a cell passed the restriction point with high E2F transcriptional activity neither reduced E2F reporter activity nor prevented S phase entry. Thus, quiescent cells require a threshold of E2F activity to re-enter the cell cycle, and committed cells retain their high E2F activity even if the environment favoring proliferation changes.

One mechanism that maintains E2F activity once it crosses the commitment point is feedforward regulation of the *E2F1* gene itself. E2F1 transcription is stimulated not only by E2F but also by the c-Myc transcription factor [61] which is stabilized by mitogen signaling [62]. The dynamics of mitogen activated E2F1 expression were precisely monitored by Dong *et al.* with an updated fluorescent E2F1 transcriptional reporter for single cell live imaging to compare the contributions of Cyclin/Cdk and Myc to E2F-dependent cell cycle commitment [63]. Importantly, they tracked the behavior of individual cells over time, and correlated commitment to divide with each cell's history of reporter activity. At any time before irreversible commitment to the cell cycle, the E2F transcriptional activity in a cell predicted the probability for commitment to cell division. Greater levels of transcription before the restriction point correlated with higher probability to re-enter the cell cycle, passing the restriction point.

A recent study further challenges the notion that E2F is progressively activated by progressive Rb phosphorylation via Cyclin D in early-to-mid G1 and then via Cyclin E in late G1 [64]. A team of scientists from the Dowdy lab (Narasimha, Kaulich, Shaprio, *et al.*) used isoelectric focusing gels and a series of phospho-specific Rb antibodies to show that Rb

is exclusively monophosphorylated by Cyclin D throughout most of G1 phase (Figure 2B). Fully un-phosphorylated Rb was restricted to non-transformed cells under conditions of profound quiescence induced by mitogen deprivation or after terminal differentiation. Surprisingly, Rb was monophosphorylated at any one of the 14 individual Cdk sites by Cyclin D/Cdk4 (or Cyclin D/Cdk6), and the sites seemed to be functionally equivalent, though potential differences in their effects at different E2F-regulated genes remain to be investigated. Importantly, no intermediate Rb phospho-isoforms appeared at any time during G1. Moreover, a sharp cyclin E mRNA increase coincided with Cdk2 activation and abrupt switch-like Rb hyperphosphorylation at 10 sites. Each of the 14 different Cyclin D-induced monophosphorylated Rb isoforms remained bound to E2F, suggesting that some degree of active repression at E2F-regulated genes may be maintained throughout G1 even after Cyclin D/Cdk4-mediated Rb phosphorylation [64]. The apparent requirement for Cyclin E/Cdk2 to rapidly convert monophosphorylated Rb to fully hyperphosphorylated Rb suggests a strict division of labor between Cyclin D- and Cyclin E-activated kinases under normal conditions. The model proposed is that Cyclin D-mediated Rb phosphorylation is not the sole late G1 event that marks passage through the restriction point. Cyclin D may instead promote entry from quiescence into early G1 while other events are likely required to drive cells through G1 past the restriction point.

The prime candidate for the event needed for restriction point passage was long-held to be *cyclin E* transcription which is under Rb-E2F control. Cyclin E protein activates Cdk2 protein kinase which both hyperphosphorylates Rb and can trigger DNA replication initiation. Certainly, high Cdk2 activity corresponds with both Rb hyperphosphorylation and post-restriction point behavior (i.e. mitogen-independent cell cycle progression) [64]. On the other hand, multiple recent studies using time-lapse microscopy of single cells have found inconsistencies with the Cdk2 and Rb-centric model that have not yet been fully resolved. In one study pharmacological Cdk2 inhibition did not block fibroblast cell passage through the restriction point, and cyclin E accumulation appeared later than the calculated restriction point time of 3–4 hours post-mitosis [65]. Similarly Rb phosphorylation was not elevated until several hours *after* cells had already committed to mitogen-independent proliferation [66]. The Cdk2 inhibitor used in that study, roscovitine, inhibits enzymes in addition to Cdk2 however, though it is not yet clear how important other kinases non-specifically inhibited by roscovitine are for restriction point passage [67]. Highly selective Cdk2 inhibition in untransformed epithelial cells using chemical genetics suggested a positive role for Cdk2 in restriction point passage [68]. Thus, although a sharp increase in Cdk2 activity is required for Rb hyperphosphorylation and for origin firing in S phase (at least in cells passing through G1 from quiescence), it is not clear that Cyclin E/Cdk2 activity alone normally causes commitment to future S phase entry.

Classic studies with overproduced Rb mutant forms that block G1 progression, overproduced Cyclin that accelerates G1 progression, or genetic Cyclin and Cdk null alleles should be carefully interpreted in light of our current understanding about the redundancies among Rb and cyclin family members. The question remains if Rb hyperphosphorylation itself is the principal molecular determinant of restriction point passage or if it is a downstream consequence of restriction point passage or some combination of both models. Cell type and whether the cells are actively proliferating or first entering the cycle from

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quiescence may be important determinants of when and how the commitment is made (discussed below).

Regardless of whether Cdk2 causes restriction point passage or is a subsequent reflection of that commitment, the correlation between an increase in Cdk2 activity and commitment to S phase entry is widely accepted. The molecular mechanisms that drive the increase in Cyclin E/Cdk2 activity are all in feedback relationships with Cdk2 to reinforce bistability (Figure 2B). One of these relationships is Cdc6-dependent DNA replication origin licensing, and Cdc6 is itself stabilized by Cdk2 phosphorylation in late G1 [69–72]. A second major Cdk2 regulator is the p27^{KIP} protein which is both a direct inhibitor of Cdk2 kinase activity and also a substrate of Cyclin E/Cdk2. p27 is abundant in quiescent cells but is partially degraded shortly after cell cycle re-entry from G0 in early G1 which reduces, but does not eliminate p27 [73]. In late G1, the remaining p27 is eliminated by Cyclin E/Cdk2-induced degradation [73]. In another example of single cell biosensor use to track cell proliferation decisions, Oki *et al.* followed both the initial p27 drop from G0 to G1 and the subsequent elimination at G1/S by time-lapse fluorescence microscopy and flow cytometry of cells expressing a fluorescent p27 fusion [74]. Altogether, this collection of distinct mitogen-dependent molecular events cooperate in late G1 to activate Cyclin E/Cdk2, and the feedback mechanisms serve to maintain high activity through the G1/S transition [75].

Cell cycle decisions in actively-proliferating populations

The proliferation decision committing cells to complete the cell cycle or exit to quiescence has until recently, been thought to occur only in G1 [46]. However, single cell studies suggest the cell proliferation decision can occur during a window of G2 in the previous cell cycle in actively proliferating cells. Hitomi and Stacy first demonstrated that mitogen-induced Ras activity is required in G2 to complete the next cell cycle [76]. Spencer *et al.* subsequently explored this G2 mitogen requirement by removing mitogens from proliferating cultures during time lapse fluorescence microscopy [10]. They observed a large subpopulation of cell in mitosis or early G1 when the mitogens were withdrawn that were already committed to complete one more cell cycle.

A key component of this study was the use of a fluorescent fusion protein that responds to changes in Cdk2 activity, a Cdk2 "biosensor". At the start of G1 (i.e. just after mitosis), new cells were either already on a trajectory to increase biosensor activity or they started G1 with measurably lower activity that remained low for many hours. Cells born with the already increasing kinase activity contained hyperphosphorylated Rb and were apparently irreversibly committed to completing the next cell cycle, even if mitogens were withdrawn or mitogen signaling was inhibited in early G1. Mitogen withdrawal, mitogen pathway inhibition or high levels of a Cdk2 inhibitor during the preceding G2 phase reduced biosensor activity in the subsequently-born daughter cells and blocked progression into S phase in the following cell cycle (though cells completed the ongoing G2, M phase, and cytokinesis). Conversely, cells born with naturally low Cdk2 activity entered a prolonged G1/G0-like state and required sustained mitogen signaling through early G1 to commit to cell division (similar to the Zetterberg and Larsson restriction point study) before committing to G1 progression. Sister cells were born with the same biosensor activity and

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cell cycle commitment, supporting the idea of a decision occurring in the previous cell cycle. The low activity cells were interpreted as transiently exiting to quiescence and then re-entering the cell cycle at G1 whereas the high activity cells were pre-committed to finishing the next cell cycle.

Tracking cells from G2 through division and monitoring subpopulations in the next G1 was only possible by single cell analysis. Moreover live imaging of the fluorescent reporter in single cells revealed the two subpopulations of cells with different Cdk2 activity levels and subsequently different G1 phases. Interestingly, different cell lines had different relative amounts of the two subpopulations; transformed cell lines had more of the increasing Cdk2 cells and non-transformed more of the low Cdk2 activity cells. These differences generally correlated with the propensity of the different cells to exit the cell cycle, a characteristic that varies with cell cycle and especially transformation status.

A second study by Naetar *et al.* supports the role for a Cdk2-driven G2 decision window committing cells to the next cell cycle [77]. These authors withdrew mitogens from cells that they had pre-synchronized in G2 and used flow cytometry to measure DNA synthesis in the following cell cycle to test commitment to the next cell cycle. Removing serum mitogens during G2 caused cell cycle exit in the following G1 as expected. However, simultaneously inhibiting protein phosphatase 2A (PP2A) during the G2 mitogen withdrawal prevented cell cycle exit in the following G1. The cells committed irreversibly to completing the next cell division even though they entered G1 without mitogens present. Specific Cdk2 inhibition by chemical genetics or dominant-negative Ras expression coincident with PP2A inhibition during G2 mitogen withdrawal imposed the expected cell cycle exit in the following G1. It seems that G2 specific PP2A-dependent protein dephosphorylation of substrates phosphorylated by Cdk2 and Ras-dependent kinases is required to re-establish mitogen dependence in early G1 phase. The requirement for mitogen dependent Ras signaling in G2 for commitment to the next cycle is also consistent with the pioneering single cell studies by Hitomi and Stacy [76]. Thus, a cellular decision to proliferate or become quiescent can occur during the previous cell cycle in a window of G2 receptive to mitogens. The idea that the events in the previous cell cycle control the outcome of the next cycle is a relatively new concept in the field.

The notion that a G2 proliferation decision can produce distinct subpopulations of G1 cells with different molecular characteristics likely includes molecular distinctions beyond the activity of Cdk2 and its regulators. One possibility for an additional molecular distinction is the chromatin loading of replicative DNA helicases, known as Minichromosome maintenance complexes (MCM), to license DNA replication origins [78,79]. MCM loading is restricted to G1 and thought to be largely irreversible in that the only mechanism known for MCM unloading occurs during replication completion itself. [80–82]. Therefore, differences in G2 proliferation decisions may be reflected in the kinetics or patterns of G1 MCM loading. In support of this idea Håland *et al.,* using an innovative flow cytometry technique to measure protein chromatin association in single cells, compared the timing of MCM loading to the timing of Rb chromatin release (i.e. Rb hyperphosphorylation) [83]. Cells re-entering G1 from G0 only loaded MCM complexes after Rb chromatin release (MCM loading only after the restriction point). On the other hand, cells entering G1 from a

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preceding mitosis were distributed into two populations: cells with loaded MCM before Rb chromatin release and cells with loaded MCMs after Rb chromatin release (MCM loading both before and after the restriction point). The potential for cycling cells with two modes of MCM regulation in G1 is consistent with the idea of two populations after the G2 decision window.

A second study suggesting distinct G1 subpopulations focused on the inactivation of the APC^{Cdh1} ubiquitin ligase at the G1/S transition. Cappell *et al.* co-expressed the Cdk2 biosensor from Spencer *et al.* with a second biosensor derived from the FUCCI system [75]. Cells with increasing kinase activity in G1 not only spent little time in G1 but they also rapidly inactivated APC^{Cdh1}. Conversely, the subpopulation of cells with low kinase activity and an extended G1/G0 inactivated APC^{Cdh1} with delayed and variable timing. Interestingly, the G1 subpopulation of G2-committed cells could be further divided into multiple smaller populations with variable responses to DNA damage, oxidative and osmotic stresses. We expect future single cell live cell imaging studies will discover even more unique subpopulations in many cell cycle phases [24].

Multiple proliferation decision points – multiple mechanisms?

The suggestion that mitogen-dependent proliferation commitments can occur in G2 rather than G1 raises new questions about the molecular mechanisms that drive proliferation decisions. Cell cycle commitments during both the G2 decision window and the G1 restriction point require mitogen signaling through the Ras GTPase pathway [76,84–86], but what are the key molecular targets of this pathway in either setting? Is Cyclin D/Cdk4 activity important for cells to commit to proliferation in G2? While the details of the G2 decision window are incompletely understood, we suggest that: (1): The G1 restriction point and the G2 decision window operate by related but not identical molecular mechanisms through Cdk and Rb-E2F regulated networks. (2): The G1 restriction point is most important during the transition from G0 to cell cycle re-entry in G1, while the G2 decision window functions in continuously cycling cells. We also favor the idea that actively proliferating cells - especially non-transformed cells – can spontaneously and transiently divert to quiescence, and that their eventual progression through G0/G1 requires passage through the same G1 restriction point as mitogen-starved and re-stimulated cells (Figure 3).

In cells re-entering G1 from quiescence, mitogen-dependent Cyclin D expression supports an Rb-E2F molecular state that is required, but not sufficient, for subsequent G1 progression. Progression from this permissive state past the G1 restriction point requires sustained mitogen signaling and a collection of interconnected and reinforcing mechanisms that stimulate Cdk2 activation. E2F-stimulated Cyclin E expression is one of these mechanisms, but p27 degradation at the G0/G1 transition and again in late G1, and MCM loading (which supports Cdk2 activity by an as-yet unknown mechanism) are also important contributors to Cdk2 activation [87]. In addition, Cdk2 is cytoplasmic in at least some quiescent cells which prevents it from acquiring an essential activating phosphorylation by a nuclear kinase [72,88]. Mitogen-stimulated signaling causes Cdk2 to translocate to the nucleus for activation, but Cdk2 is constitutively nuclear and phosphorylated in actively

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proliferating cells. Thus at least one additional requirement to activate Cdk2 in cells entering G1 from G0 is already met by cells entering G1 from the preceding cell cycle.

The Myc transcription factor also has different roles at the G1 and G2 proliferation decisions. For passage through the G1 restriction point, Ras-driven Myc accumulation regulates and cooperates with E2F transcriptional activity to control a suite of proliferation-related genes [61,63,89]. For commitments made during G2 however, Myc is apparently important primarily for *cyclin E* transcription, possibly to prime G2 cells for high Cyclin E/Cdk2 activity after mitosis [77]. Neither Cyclin D nor other E2F target genes including *pcna*, *ccna2* (Cyclin A), and *e2f1* are transcribed in G2 under the same conditions [77]. This observation suggests the G2 decision window, unlike the G1 restriction point during cell cycle re-entry, does not heavily rely on Rb-E2F control as a primary mechanism. Moreover cells lacking all three Rb family members, (Rb, p107 and p130), "triple knockout" mouse embryonic fibroblasts, require mitogen signaling in G2, but not G1, for proliferation [90,91]. We suggest that E2F transcription is not required in G2 for commitment to the following cell cycle, but rather continues to promote cell cycle transcription in the following G1 in cells which are already committed to division.

The G2 decision window ultimately leads to increasing and self-reinforcing Cdk activity as the switch that determines proliferation or quiescence in the next cell cycle. Of note, Cdk2 activity itself peaks in S phase, but many Cdk2 functions can also be carried out by Cdk1, so the proliferation commitment may be set by a combination of Cdk2 and Cdk1 activity in G2 phase [92]. The precise downstream target(s) of Cdk mediating the G2 proliferation decision are not clear, but it does not appear to be E2F-stimulated transcription in G2 [77]. Spencer *et al.* provided evidence that an important determinant of high or low Cdk activity in G2 is the abundance of the CDK inhibitor, p21 (which is related to p27 but regulated by different mechanisms) [10]. Cells with high p21 and low Cdk activity in G2 entered a variable, but prolonged G1/G0-like state in the following cell cycle, and cycling cells lacking p21 altogether rarely (if ever) entered the low Cdk activity state in early G1 [10,93]. The relative contributions of CDK inhibitors, kinase activity, and Rb-E2F-controlled transcription to the ultimate commitment to proliferation are likely influenced not only by the differences between mitogen signaling for cell cycle re-entry from quiescence and mitogen-dependent cell cycle progression in proliferating cells but also by cell type and cell context. From a systems level perspective, both proliferation decisions require the same bistable switch focused on Cdk2 activity to commit to the cell cycle, even though the importance or timing of the individual molecular components are different. Recent computational modeling studies support the idea that actively proliferating G2 committed cells and cells re-entering from quiescence facing the G1 restriction point use a similar overarching network to commit to the next cell cycle [94,95].

G2-committed cells consistently pass quickly through a subsequent short G1 phase [10,77]. Interestingly, G1 length varies considerably between different human cell types in culture: human embryonic stem cells spend as little as 2–3 hours in G1 whereas many differentiated cell lines spend 12 hours or more in G1 [96]. It is intriguing to speculate that in a population of cells with very short G1 lengths, all the cells make the proliferation commitment in the G2 decision window and never face the G1 restriction point. Both cancer cells with short G1

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lengths and embryonic stem cells have high levels of CDK activity, which may guarantee consistent G2 commitment in nearly every cell cycle [96,97]. Mutations that cause high CDK activity might dictate G2 commitment to the next cell cycle, and consequently a short G1 phase.

Outlook

Technological advances in microscopy, flow cytometry, and fluorescent reporters have made it increasingly clear that individual cells make different proliferative decisions, even when they are genetically identical to one another and are cultured together. These intercellular differences are likely to be more pronounced in tissues where variations in cellular microenvironments affect local proliferation signals. For example, the intestinal epithelium comprises a broad range of proliferative stem cells and differentiated cells that respond to diverse spatial signaling cues [98]. Understanding the behavior of a cell population requires a nuanced appreciation that each cell responds to intrinsic or extrinsic cues by shifting its probability of entering or exiting the cell cycle. Thus, statistical models that take these probabilities into account will be particularly useful predictive tools [99]. Mathematical models of cell cycle transitions have been in development for several years, and the increasingly-sophisticated ability to acquire real-life parameters for molecular events using robust quantitative single cell approaches will both test and refine these models [57,100–102].

One long-term goal held by many in the field is deep mechanistic understanding of proliferation decisions and the factors that affect the probabilities of progressing from G1 to S phase or exiting from G1 to quiescence. An important contributor to the achievement of that goal will be combinations of assays that reveal the relationships among molecular events and cell behaviors. For example, simultaneously tracking two or more biosensors in the same cell is the best way to determine the order of two events and if one is likely the cause of the other [31,36,75]. Furthermore combining different types of single cell assays, such as live cell imaging of fluorescent biosensors with single-cell transcriptomics or genomics, ties the recent molecular behavior of one cell with its later fate [103–106]. We anticipate that ongoing technological and conceptual breakthroughs will ultimately yield practical benefits such as precision cancer diagnoses and treatments, interventions into degenerative diseases and aging, and cell-based therapies.

Acknowledgments

We thank Dr. Michael Emanuele, Dr. Jeremy Purvis, and members of the Cook lab for helpful feedback and discussion of the manuscript. This work was supported by a National Science Foundation graduate research fellowship (DGE-1144081) to J.P.M. and by funding to J.G.C. from the National Institutes of Health NIGMS (R01GM102413) and the W.M. Keck Foundation.

Abbreviations

CDK	Cyclin dependent kinase
FUCCI	fluorescent ubiquitination-based cell cycle indicator

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PP2A	protein phosphatase 2A
MCM	Minichromosome maintenance complexes
Rb	Retinoblastoma

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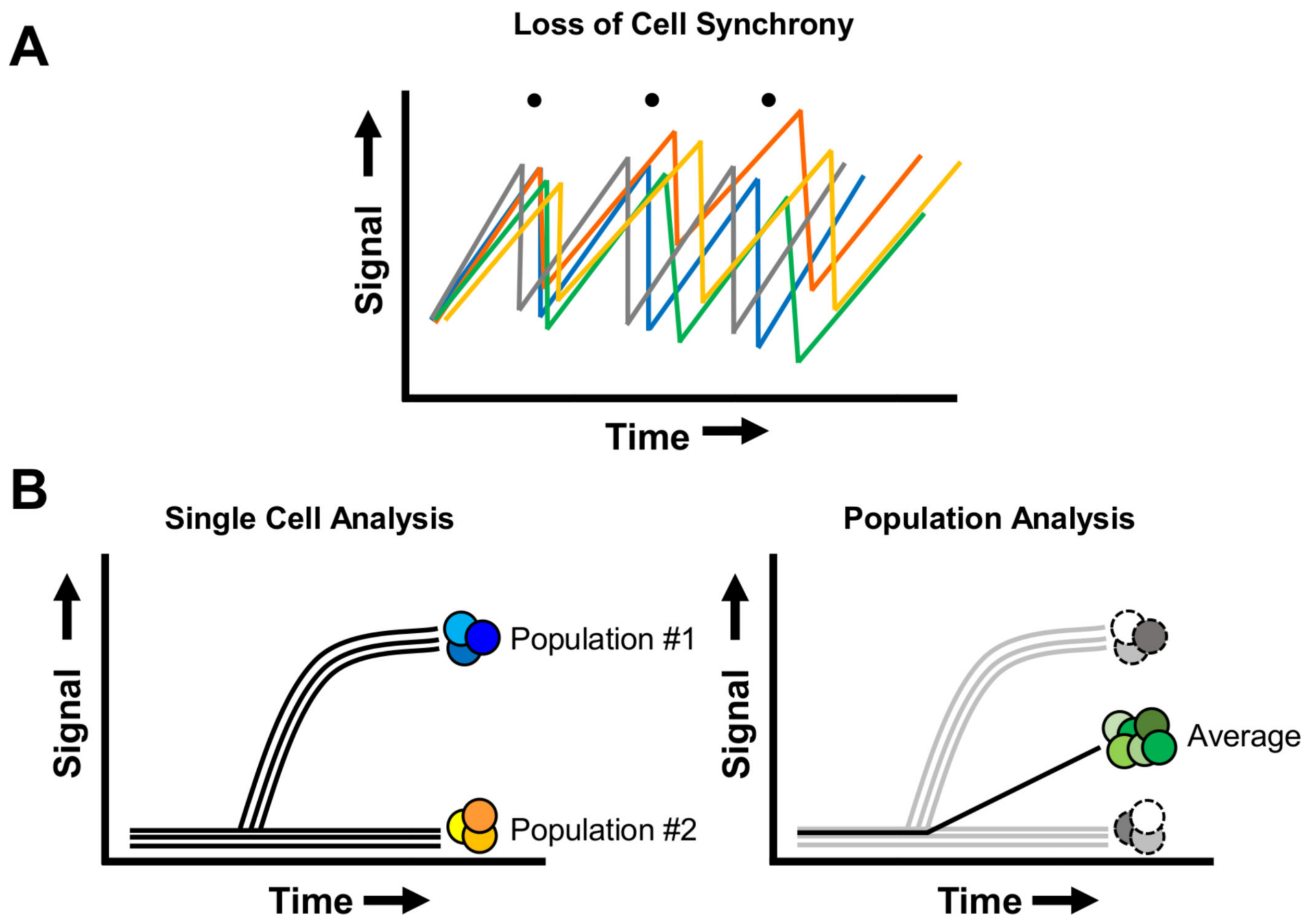

Figure 1. Advantages of single cell analysis

(A) Hypothetical molecular signal in individual cells of an artificially-synchronized population. Black dots represent cell divisions. Cells are synchronized in the first cell cycle, but within two to three cell cycles the population is completely asynchronous. (B, Left) Single cell analysis identifies and tracks a representative molecular signal in coexisting cell populations where different subpopulations adopt different cell cycle fates. (B, Right) Ensemble analysis of the same coexisting populations reports only the average signal that may not represent either subpopulation's cell cycle fate.

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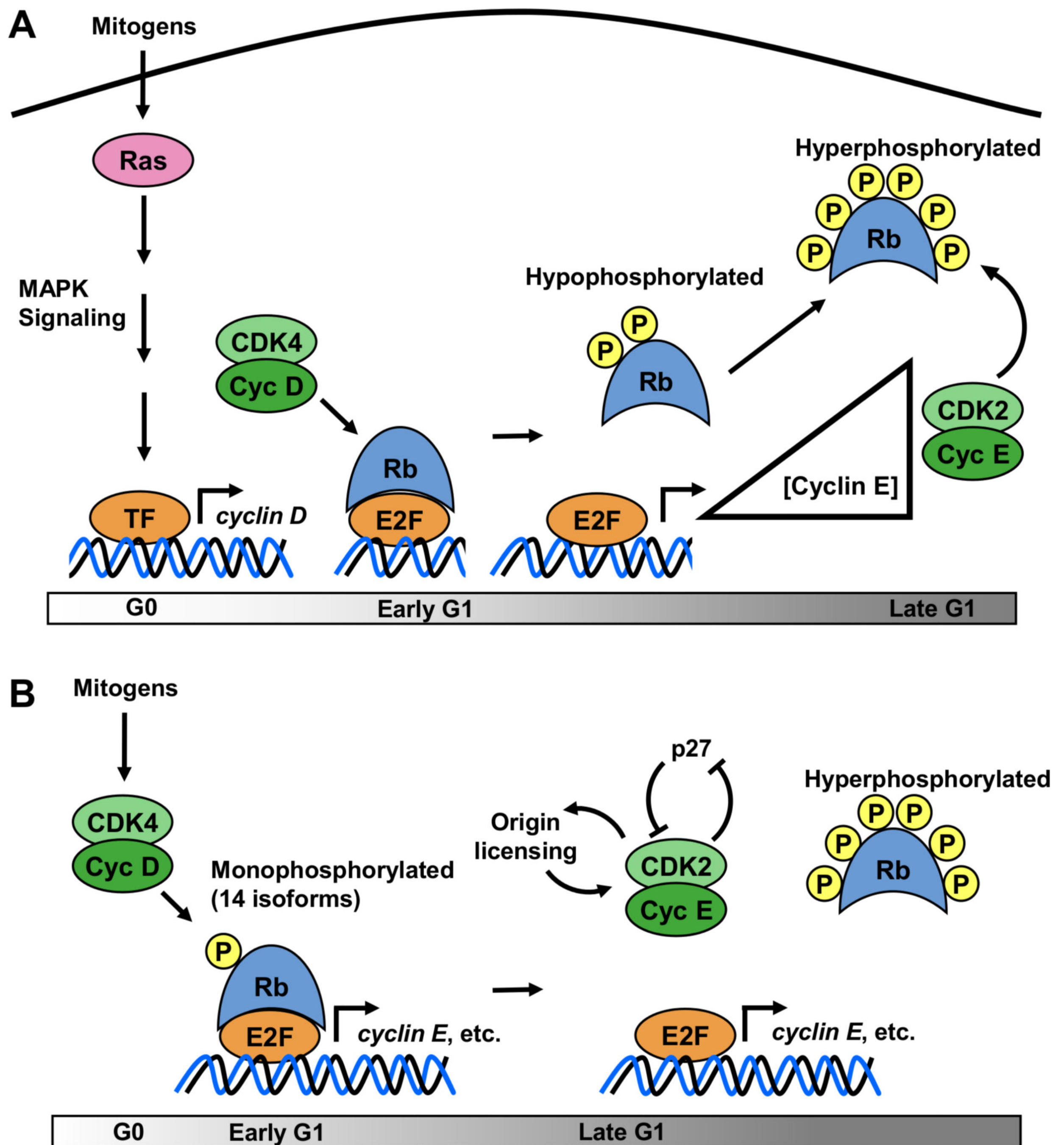

Figure 2. The restriction point during re-entry to G1 from quiescence

(A) The prevailing paradigm of the restriction point. Mitogens activate Ras signaling, downstream MAP Kinases, and ultimately Cyclin D transcription. Cyclin D complexes with CDK4 and CDK6 to partially phosphorylate Rb, and this hypophosphorylation causes release from E2F and partially activates Cyclin E expression. Cyclin E complexes with CDK2 to hyperphosphorylate Rb, creating a positive feedback loop where progressively increasing Cyclin E levels increase Rb phosphorylation leading to increased Cyclin E transcription and S phase commitment. (B) An alternate model for the restriction point.

Cyclin D-CDK complexes only monophosphorylate Rb on 14 unique sites. The monophosphorylated Rb remains E2F-bound, but at least some aspects of Rb-mediated gene repression are relieved moving cells from G0 to G1. Multiple sustained mitogen-dependent inputs (p27 degradation, origin licensing, etc.) activate Cyclin E-CDK2 complexes which rapidly hyperphosphorylate Rb in a switch-like fashion to drive commitment to S phase.

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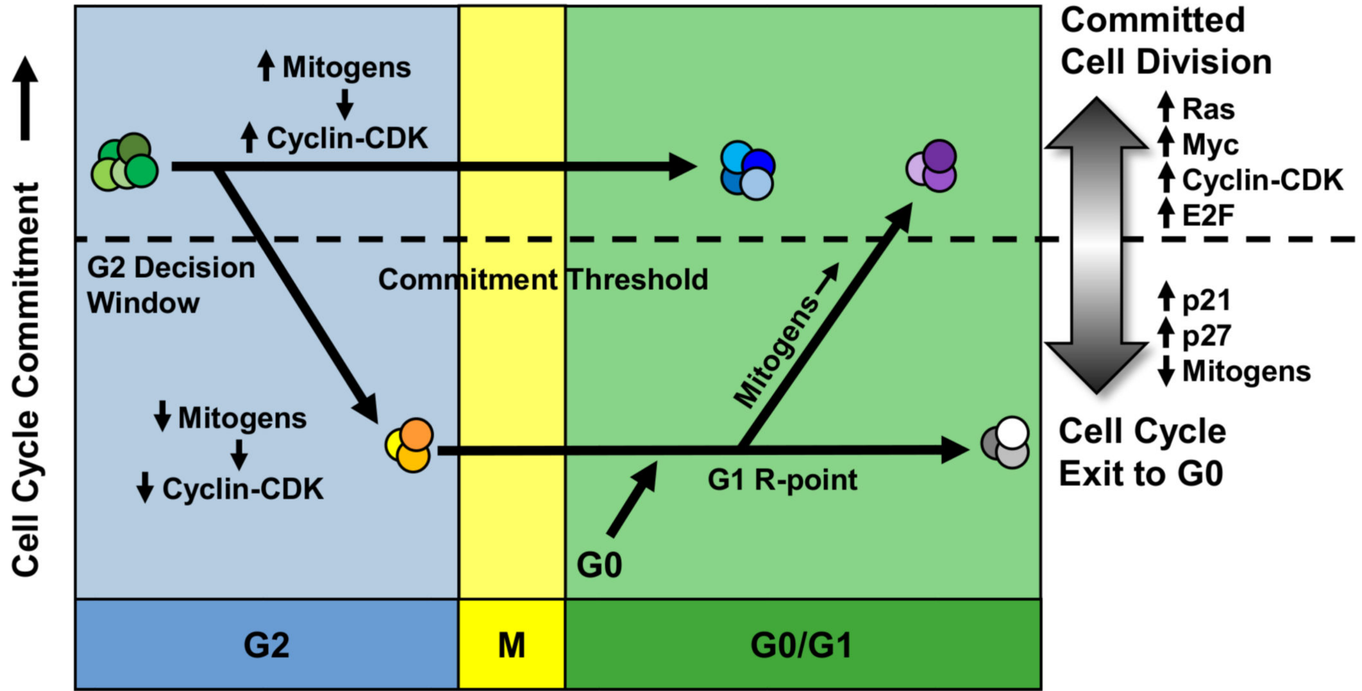

Figure 3. Multiple proliferation decisions in actively dividing cells

Actively dividing cells have two distinct proliferation decision points. The first is a window during G2, in which cells with CDK activity above a critical threshold pre-commit to completing the next cell cycle with a short G1. A subpopulation of G2 cells with reduced mitogen signaling and/or low Cyclin-CDK activity do not commit to the next cell cycle. These cells have a long, variable length subsequent G1/G0-like phase and are presented with a second proliferation decision at the G1 restriction point. Sustained mitogen signaling promotes increased CDK activity above the commitment threshold which is self-sustaining through multiple feedback and feedforward relationships. In the absence of sustained signaling and increasing CDK activity, cells exit to G0. The probability of cells committing to the cell cycle or exiting to G0 is the cumulative probability of multiple molecular events.