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Integrating old and new paradigms for G1/S control

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Summary

The Cdk-Rb-E2F pathway integrates external and internal signals to control progression at the G1/S transition of the mammalian cell cycle. Alterations in this pathway are found in the vast majority of human cancers and specific Cdk4/6 inhibitors are approved or in clinical trials for the treatment of diverse cancers. In the long-standing paradigm for G1/S control, Cdks inactivate Rb through phosphorylation, which releases E2F transcription factors to drive cell-cycle progression from G1 to S. However, recent observations in the laboratory and clinic challenge central tenets of the current paradigm and demonstrate that our understanding of the Rb pathway and G1/S control is still incomplete. Here, we integrate these recent findings with the previous paradigm to synthesize a current molecular and cellular view of the mammalian G1/S transition. A more complete and accurate understanding of G1/S control will ultimately lead to improved therapeutic strategies targeting the cell cycle in cancer.

Introduction

The cellular decision to transition from G1 to S phase of the cell cycle is critical to normal development and is necessarily misregulated in cancer. During G1, cells decide to either enter the cell cycle, initiate DNA replication, and divide, or to exit the cell cycle and enter quiescence, senescence or differentiation. The G1/S transition is controlled by Cyclin-dependent kinases (Cdks), the retinoblastoma tumor suppressor (Rb), and E2F transcription factors (Figure 1A). These components are typically assembled into a linear pathway, in which the upstream Cdk inhibits Rb to drive E2F-dependent transcription and cell-cycle progression. Abrogation of the G1/S control point is a hallmark of cancer and typically occurs through loss of Rb or through hyper-activation of Cdks (Burkhart and Sage, 2008; Kent and Leone, 2019; Malumbres and Barbacid, 2009; Otto and Sicinski, 2017). Importantly, the linear understanding of how the G1/S transition is controlled by the Cdk-Rb-E2F pathway has led to the development of Cdk4 and Cdk6 (Cdk4/6) kinase inhibitors for cancer therapy (O'Leary et al., 2016; Otto and Sicinski, 2017; Sherr et al., 2016). These Cdk4/6 ATP-competitive inhibitors, which were developed to arrest proliferating cells by

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inhibiting Rb phosphorylation and inactivation, are the first clinically approved drugs targeting the G1/S transition.

Despite the progress over the past several decades, important questions remain. While the canonical G1/S pathway is understood as linear (Figure 1A), regulation is still quite complex. There are multiple homologous proteins for each pathway component, each protein is subject to post-translational modifications, and there are critical positive and negative feedback loops that connect the different pathway components (Figure 1B) (for past reviews see (Bertoli et al., 2013; Burkhart and Sage, 2008; Dick and Rubin, 2013; Kent and Leone, 2019; Sherr and Roberts, 1999). As a consequence, the field still struggles to define the critical molecular interactions and activities leading to Rb inactivation and the timing of those mechanisms relative to passage through G1 and into S phase. Moreover, it remains unclear how upstream growth signals feed into the pathway to coordinate cell growth with division, one of the earliest identified functions of G1/S regulation. Finally, there is a need to understand the variable responses to Cdk4/6 inhibitors observed both in the partial arrest of cells cultured *in vitro* and in the diverse outcomes of patients in clinical trials. According to the textbook linear model, inhibition of the upstream Cdk complexes should arrest cell division. That it often does not implies that there is a lot we do not know about the G1/S transition. Here, we review recent progress in understanding these important aspects of G1/S control in mammalian cells.

Mechanisms and consequences of Rb inactivation

The most critical event that marks the transition from G1 to S is the activation of a transcription program regulated by E2F transcription factors (Kent and Leone, 2019). Toward the end of G1, activating members of the E2F family stimulate expression of a set of hundreds of genes that promote DNA replication and enforce irreversible progression into the cell cycle (Bertoli et al., 2013; DeGregori et al., 1995). This E2F activity driving entry into S phase is restricted by Rb family proteins, which form repressive complexes with E2F proteins on the promoters of the S phase genes. Repression is relieved by Cdk phosphorylation of Rb proteins, which takes place on up to 15 sites and drives structural changes in Rb that lead to E2F dissociation (Dick and Rubin, 2013). Accordingly, a hallmark of the G1/S transition is the hyperphosphorylation of Rb that necessarily precedes DNA synthesis.

The requirement of Rb inactivation for G1/S progression has motivated studies to define the mechanisms by which Cdk complexes phosphorylate Rb and by other means regulate the G1/S transition. In the prevailing model, Cdk4/6-CycD is first to phosphorylate Rb. Then, later in G1, Cdk2-CycE completes phosphorylation to release and fully activate E2F (Figure 2, Model I). This model is supported by the timing of cyclin expression through G1, some reported evidence for specificity of the kinases for non-overlapping phosphorylation sites in Rb, and experiments inhibiting Cdk4/6 or Cdk2 activity (Harbour et al., 1999; Lundberg and Weinberg, 1998). The understanding that Rb phosphorylation is ordered in this manner also fits with models for how Cdk4/6 and then Cdk2 activity gradually increase throughout G1. Two mechanisms explain how Cdk4/6-CycD itself induces the increase in Cdk2-CycE activity. First, the gene encoding CycE is an E2F target that could be expressed if Cdk4/6

phosphorylation of Rb partially inactivated it (DeGregori et al., 1995). Second, evidence suggests that increasing the abundance of Cdk4/6-CycD complexes leads to Cdk2 activation by sequestering the Cdk inhibitors p21 and p27 away from Cdk2 (Sherr and Roberts, 1999). Somewhat paradoxically, p21 and p27 are generally considered cell-cycle inhibitors, but also play a role in Cdk4/6 assembly and activation (Guiley et al., 2019; Sherr and Roberts, 1999).

New models for cell-cycle progression at the G1/S transition

The canonical model, in which both Cdk4/6 and Cdk2 activity contribute to Rb phosphorylation and inactivation during G1, has been challenged by a number of recent studies that support two distinct alternative models (Figure 2). By careful quantification of Rb phosphorylation patterns in synchronized cells, it was found that Cdk4/6-CycD activity only induces Rb monophosphorylation, whereas Rb hyperphosphorylation is co-incident with the onset of Cdk2 activity (Narasimha et al., 2014). E2F-dependent gene expression remained low while Rb was monophosphorylated by Cdk4/6-CycD, leading to the conclusion that only Cdk2 directly inactivates Rb (Figure 2, Model II). These observations also change our conception of Rb phosphorylation during G1. Rather than existing in a continuum of phosphorylation states, which results from progressive and successive phosphorylation by the G1 Cdks, Rb is present in discrete states. Rb is either unphosphorylated in cells that exit the cell cycle and lack CycD, monophosphorylated when active in G1 in the presence of CycD, or hyperphosphorylated once Cdk2 is activated (Figure 2B, Model II). Through use of phosphospecific antibodies, monophosphorylation was observed at each of the Rb Cdk consensus sites that were examined, suggesting that there are multiple monophosphorylated forms of Rb. The possibility of different functions for the different monophosphorylated states was further explored by identifying Rb complexes in G1 that contain a particular monophosphorylated species (Sanidas et al., 2019). Specific phosphorylation events assembled Rb with factors, for example the NuRD chromatin remodeling complex, which may induce unique transcriptional outputs. This mass spectrometry-based proteomics study also found that CycD association with several of the monophosphorylated forms of Rb was poor compared to association with unphosphorylated Rb, which could account for how Cdk4/6-CycD activity toward Rb is limited to a single phosphorylation event. If CycD no longer interacts with monophosphorylated Rb, then it could not be responsible for hyperphosphorylation. The mechanism by which Cdk4/6 monophosphorylates various Cdk sites in Rb needs further exploration. Moreover, additional corroborating research is needed to support the conclusion that Cdk4/6-CycD strictly activates Rb and to reconcile this model with other observations, several of which are described below, implicating Cdk4/6-CycD as an Rb-inactivating kinase.

That Cdk4/6-CycD monophosphorylates Rb and that this monophosphorylated Rb inhibits E2F raises the question of whether or not Cdk4/6-CycD inactivates Rb, a central tenet of the current paradigm. If Cdk4/6 activity does not inactivate Rb, then why is Cdk4/6 activity important for cell-cycle progression and proliferation? If Cdk2 is the inactivating kinase, why do Cdk4/6 specific inhibitors like palbociclib result in loss of hyperphosphorylated Rb and cell-cycle arrest in G1? These questions are further motivated by recent evidence affirming that Rb-directed Cdk4/6-CycD activity is critical for Rb hyperphosphorylation and G1/S progression. It was found that mutation of a Cdk4/6-specific docking site in Rb results

in impaired Rb hyperphosphorylation, increased G1 arrest, and enhanced Rb tumor suppressor activity *in vivo* (Topacio et al., 2019). There are several potential explanations for how Cdk4/6 activity leads to Cdk2 activity, and inversely how Cdk4/6 inhibition may indirectly lead to Cdk2 inhibition. In such mechanisms, Cdk4/6 is necessary for the ultimate inactivation of Rb, even if Cdk4/6 activity alone does not directly hyperphosphorylate Rb (Figure 2, Model II). One possibility is that Cdk4/6 monophosphorylation is required for priming Rb for Cdk2 hyperphosphorylation. Such priming mechanisms for Cdk hyperphosphorylation have been observed in other cell-cycle substrates in yeast (Koivomagi et al., 2013), though they have not yet been implicated in the mechanism of Rb hyperphosphorylation. A second possibility is that additional Cdk4/6 targets currently being identified, such as enzymes controlling metabolism (Caillot et al., 2020; Wang et al., 2017), can explain the role of Cdk4/6 in promoting cell-cycle progression and proliferation. In this model, Cdk2 activation would result from Cdk4/6 targeting these other substrates. Importantly, both of these mechanisms are not mutually exclusive and are consistent with the observation that upon expression of CycE, Cdk4/6-CycD is dispensable for G1/S progression (Keenan et al., 2004).

A third possibility for how Cdk4/6 impacts G1/S progression without hyperphosphorylating Rb invokes the idea that Cdk4/6 regulates Cdk2 through their interactions with the network of Cdk protein inhibitors. For example, increasing CycD levels through G1 could increase Cdk2 activity to inactivate Rb by sequestering p21 and p27. Recent studies of the effects of palbociclib support the importance of these connections between the G1 Cdks. For example, it was found that palbociclib does not directly inhibit cellular Cdk4/6 activity toward Rb, but may indirectly inhibit Cdk2 by increasing the abundance of p21 in Cdk2-CycE complexes (Guiley et al., 2019). Other evidence demonstrates that palbociclib still arrests the cell cycle even when Cdk4 with impaired kinase activity is expressed (Persky et al., 2020; Schade et al., 2019). These results highlight the importance of non-catalytic Cdk4-CycD functions and suggest that small molecule Cdk4/6 inhibitors may both directly inhibit Cdk4/6 and also free p21 and p27 to inhibit Cdk2.

In contrast to the body of work suggesting mechanisms through which Cdk4/6-CycD and Cdk2-CycE cooperate in Rb phosphorylation and the G1/S transition, some other recent studies have challenged the conclusion that Cdk2-CycE plays any significant role in inactivating Rb during G1 (Figure 2, Model III) (Chung et al., 2019; Yang et al., 2020). Rather, it is proposed that Cdk4/6-CycD is exclusively responsible for hyperphosphorylating Rb to activate E2F. These studies, which primarily used fluorescent reporters of putative Cdk4/6 and Cdk2 activity in live cells, corroborated the canonical model that Cdk4/6 activity precedes Cdk2 activity and that Cdk2 activity precedes the onset of S phase (Yang et al., 2020). However, upon manipulating activities, primarily by using chemical inhibitors, it was concluded that Cdk4/6 and not Cdk2 is necessary and sufficient for Rb phosphorylation during G1 (Chung et al., 2019; Yang et al., 2020). Then, only once cells were in S phase, Cdk2 function was required to maintain Rb phosphorylation and positive feedback-driven E2F expression.

The observation that Cdk2 is dispensable for Rb hyperphosphorylation contrasts with other experiments demonstrating that acute Cdk2 inhibition results in G1 accumulation (Merrick

et al., 2011; Narasimha et al., 2014). This discrepancy may be due to how observations were made, for example using different markers of S phase entry and different approaches to manipulating Cdk activities. There are also limitations of the Cdk activity sensors that need be considered when interpreting those data. In particular, the engineered live-cell reporters likely detect a combination of Cdk activities due to the cross binding of cyclins to non-canonical Cdk partners and the presence of multiple cyclin docking sites on the sensors (Jirawatnotai et al., 2011; Schwarz et al., 2018; Topacio et al., 2019). In addition, the chemical Cdk inhibitors used in these experiments may lack specificity and have off-target effects that have not been properly considered or discovered.

Yet, even if interpreted cautiously, the experiments analyzing Cdk reporters in single cells have reopened important questions regarding the timing of cell-cycle events through the G1/S transition and highlight the acute need for studies that integrate or explain the seemingly contradictory results leading to the different models for Rb inactivation. Considering the complexity of the Cdk network, the precise manner in which perturbations are made and subtle differences in cellular model system may impact outcomes. For example, requirements for traversing G1-S are known to be different in cells re-entering the cell cycle from quiescence compared to continuously cycling cells (Matson and Cook, 2017).

Revisiting the restriction point

The study of the G1/S transition and the Rb pathway has been motivated by a desire to understand commitment to cell division, a process that is necessarily misregulated in cancer. Classically, the restriction point in mammalian cells has been defined as the irreversible point of commitment to division whose traversal requires growth factor signaling (Johnson and Skotheim, 2013; Pardee et al., 1978; Pennycook and Barr, 2020). Beyond the restriction point, cells progress through to division even if growth factors, typically serum, are removed from the media. The restriction point was originally determined to occur in late G1 just prior to the initiation of DNA replication (Pardee et al., 1978). This classical picture fits well with the consensus molecular model for the G1/S transition (Figure 2, Model I) (Bertoli et al., 2013). Once initially activated by Cdk4/6-CycD, the CycE-Rb-E2F positive feedback loop can maintain its activity without CycD, which explains how cells become insensitive to decreases in upstream growth factor signaling in the classic restriction point analyses (Barr et al., 2016).

While the CycE-Rb-E2F feedback loop is an elegant molecular mechanism that explains the physiology of an irreversible G1/S transition, this model has now been challenged by recent single-cell analyses revealing a greater complexity in how cells commit to cell division. Primary fibroblast cell lines exhibit the classical G1 restriction point, but many commonly cultured immortal cell lines do not (Martinsson et al., 2005; Naetar et al., 2014; Schwarz et al., 2018; Spencer et al., 2013). Many cells are already committed to division and insensitive to growth factor removal before completing mitosis in the previous cell cycle (Spencer et al., 2013). These pre-committed cells are born with initially high and rising Cdk activity and phosphorylated Rb, showing that signaling events regulating the G1/S transition take place in the previous cell cycle and are remembered in the subsequent generation (Moser et al.,

2018). One contribution to this cellular memory of the previous cell cycle is DNA damage, which, if present, leads to reduced Cdk activity and a longer G1 in the next cell cycle (Arora et al., 2017; Barr et al., 2017; Yang et al., 2020; Yang et al., 2017). In addition, global protein synthesis in the previous cell cycle is required for a rapid G1 in the subsequent cell cycle through its effect on CycD synthesis and possibly through its effect on cell growth and thereby on the size of newborn cells (Min et al., 2020). Taken together, these studies show that signaling events from the previous cell cycle can be integrated to control CycD expression and cell-cycle commitment in the following cell cycle. That signaling history and not just current activity impacts the decision to divide was anticipated by a series of yeast studies looking at how the pheromone-activated MAPK pathway integrates its activity over time to restrict cell division (Doncic et al., 2015; Doncic and Skotheim, 2013). It is likely that such integrated signaling responses may be a common feature of MAPK pathways across eukaryotes to promote accurate cellular decision making.

While cells clearly can integrate signals and pre-commit to division, it is not clear when they do so. Certainly, we anticipate the response to growth factor dynamics to be both context-dependent and cell type specific. Inhibiting PP2A phosphatase signaling results in commitment to cell division in the preceding cell cycle, but such signaling is likely present in wild-type cells (Naetar et al., 2014). In general, active dephosphorylation during mitosis would be expected to reset the cell cycle so that there is little memory of past signaling and the cell cycle would have the modular structure some have observed (Araujo et al., 2016; Chao et al., 2019). One possibility to explain the pre-commitment phenomenon in immortal cell lines is that culturing cells selects for alterations upregulating the Cdk4 activity that has been associated with the pre-commitment phenomenon. Indeed, likely due to its crucial role in regulating cell proliferation, mutations weakening or advancing the restriction point to the previous cell cycle have been associated with cancer-derived cell lines (Pardee et al., 1978; Sherr and DePinho, 2000; Zetterberg et al., 1995). Rapid evolution of lab cultures has revealed that lineages within a yeast population rapidly generated mutations that were then selected for increased proliferation (Levy et al., 2015). While it is easy to imagine something similar happening during the long-term culture of human cell lines, an analogous evolutionary study is needed to test this hypothesis. In addition to the possibility of lab generated mutations affecting the restriction point, we do not know the *in vivo* context in which these signaling pathways operate that can affect the signaling dynamics. For example, the pulsatile and wave-like dynamics of MAPK signaling observed in mouse epidermis (Hino et al., 2020; Hiratsuka et al., 2015) highlights the importance of understanding the dynamics of growth factor signaling *in vivo* across diverse cell types. A better understanding of signaling dynamics *in vivo* could situate the restriction point studies of cultured cells in their physiologically relevant context. One possible outcome is that the location of the restriction point is developmentally regulated to take place in the prior cell cycle or in G1 phase depending on cell type.

APC^{Cdh1} and p21/p27 inactivation and the irreversible commitment to S phase

While Rb inactivation is required for the G1/S transition, cells that have hyperphosphorylated Rb can still arrest before S phase entry in response to a stress that activates p53, such as DNA damage (Cappell et al., 2016; Yang et al., 2020). This observation suggests the presence of additional processes that trigger irreversible entry into S phase, and recent evidence implicates inactivation of the anaphase promoting complex (APC) and degradation of the Cdk inhibitors p21 and p27 (p21/p27) as such mechanisms (Barr et al., 2016; Cappell et al., 2016; Cappell et al., 2018; Heldt et al., 2018) (Figure 3). The APC is a ubiquitin ligase that, with the adaptor subunit Cdh1, promotes degradation of cell-cycle substrates during G1 (Schrock et al., 2020). Several of these substrates, including the Cdk2-activator CycA and the ubiquitin ligase adaptor Skp2, play important regulatory roles during S phase. The development of a live-cell reporter for APC^{Cdh1} activity led to the observation that Cdh1 inactivation follows Rb inactivation but precedes DNA replication (Cappell et al., 2016). Notably, Cdh1 inactivation could not be reversed through Cdk inhibition or cellular stress including DNA damage. Two mechanisms have been described for APC^{Cdh1} inactivation, including phosphorylation by Cdk2 (Keck et al., 2007; Lukas et al., 1999), which directly inhibits the ubiquitylation reaction, and binding of the inhibitor protein Emi1 (Figure 3) (Cappell et al., 2018; Miller et al., 2006). The gene encoding Emi1 is a transcriptional target of E2F, which places Rb inactivation upstream of Cdh1 inactivation. Emi1 is an APC^{Cdh1} substrate that becomes an inhibitor when its protein concentration increases and Cdh1 is phosphorylated. This dual function of Emi1 has been invoked as a mechanism for how Cdh1 inactivation acts as an irreversible switch (Cappell et al., 2018).

Another important event at the G1/S transition is the degradation of the p21/p27 inhibitors, which is required for activation of Cdk2 in S phase (Barr et al., 2016; Heldt et al., 2018). Skp2, a substrate adaptor of the cullin ubiquitin ligase that targets p21/p27 for degradation, is itself a Cdh1 target (Schrock et al., 2020), and therefore p21/p27 inactivation likely occurs downstream of Cdh1 inactivation (Figure 3). Other parallels between Cdh1 and p21/p27 inactivation are apparent. p21/p27 degradation is stimulated by Cdk2 activity (Sheaff et al., 1997), which provides positive feedback as p21/p27 inhibits Cdk2 (Figure 3). p21 is a p53 target that is induced upon DNA damage, and so p21 degradation, like Cdh1 inactivation, also commits the cell to S phase despite subsequent stress. The degradation of p21/p27 at the onset of S phase may have implications for why Cdk4/6 inhibitors are limited in their efficacy to G1. Once levels of p21/p27 decrease, there is no pathway to indirectly inhibiting Cdk2 activity through reshuffling of the trimer Cdk complexes. As these additional mechanisms of Cdh1 and p21/p27 inactivation implicate, the critical determinant of S phase progression is the complete activation of Cdk2 (Figure 3).

Coupling cell growth to cell-cycle progression in G1/S

An important function of the G1/S transition in animal cells is to link cell growth to division (Ginzberg et al., 2015; Zatulovskiy and Skotheim, 2020). This connection manifests as an

inverse correlation between how big a cell is when it is born and how long it spends in the G1 phase of the cell cycle. The inverse correlation allows cells that are initially born smaller to catch up in size before entering the cell division cycle. Recent single-cell studies confirmed that cell size at cell birth negatively correlates with the time cells spend in the G1 phase of the cell cycle, although the quantitative relationship between cell size, growth, and G1/S progression depended on the particular cell line (Cadart et al., 2018; Ginzberg et al., 2018; Varsano et al., 2017). Importantly, the G1/S transition of epidermal stem cells *in vivo* was more size-dependent than the G1/S transition of cultured cells (Xie and Skotheim, 2020), supporting the role of the G1/S transition in coupling cell growth to division

One mechanism human cells use to link cell growth to the G1/S transition is intimately related to the size-dependent scaling of cellular biosynthesis that generally maintains cellular components at constant concentrations (Schmoller and Skotheim, 2015). Differential patterns of biosynthetic scaling enable cells to measure their size through size-dependent concentration changes of particular proteins and to use these concentration changes to regulate G1/S in a size-dependent manner. This possibility was first discovered in budding yeast, where the synthesis of cell-cycle activators is roughly proportional to cell size and thereby follows the scaling pattern typical of most proteins, but the synthesis of the cell-cycle inhibitor Whi5 is independent of cell size (Schmoller and Skotheim, 2015). This means Whi5 concentration is lower in larger cells, which reduces its inhibitory effect to trigger earlier cell-cycle entry compared to smaller cells. Strikingly, the same phenomenon of dilution during G1 was discovered in human cells for Rb, which is the functional ortholog of Whi5 (Zatulovskiy et al., 2020) (Figure 4). Thus, it is possible that in both yeast and human cells, growth triggers cell division by diluting a protein that inhibits cell cycle-dependent transcription, while activators remain at constant concentration.

In addition to Rb dilution, cell size likely impacts the G1/S transition through the activity of the p38 stress activated kinase. In the p38 model, small cell size activates p38 to restrict the G1/S transition until cells grow sufficiently large (Liu et al., 2018). However, the molecular mechanism through which cell size regulates p38 activity, and how such a size-activated p38 functions to inhibit the G1/S transition is unknown. Moreover, the effect on cell-cycle progression of p38 activity is context-dependent and may also be isoform-dependent. While p38γ was recently shown to directly phosphorylate Rb to drive G1/S progression in hepatocytes *in vivo* (Tomás-Loba et al., 2019), p38β and p38δ also contributed to the size-dependent G1/S control in cultured cells (Liu et al., 2018). In general, the p38 family has been linked to inhibition of the G1/S and G2/M transitions through activation of p53 and p21 ((Campbell et al., 2011) and reviewed in (Thornton and Rincon, 2009)). It will be interesting to learn the mechanism through which small cell size activates p38, and how size-dependent p38 activation regulates the G1/S transition.

Thus, while cell size and growth have long been linked to the G1/S transition, only now are we beginning to uncover the underlying molecular mechanisms. The recent mechanistic p38 and Rb studies mark a turning point in the field, and the next step is to understand if and how these mechanisms operate *in vivo*. Importantly, Rb dilution and p38 activation are not mutually exclusive mechanisms, and it is likely that there are additional mechanisms linking cell growth to division at the G1/S transition depending on cell type. In addition, these

mechanisms may work hand in hand with the classic view that growth factors activate CycD expression. For example, both Cdk activity, which weakens Rb-E2F affinity, and Rb concentrations can modulate the capacity of Rb to bind and inhibit E2F. Finally, a full accounting of how growth regulates the G1/S transition will also likely require a more detailed understanding of the mTOR signaling network and its mechanistic links to G1 control (Liu and Sabatini, 2020). We anticipate the nexus of cell size, growth, and the G1/S transition will be a very active area of research in the coming years.

Response of tumors to Cdk4/6 inhibitors and mechanisms of resistance

The Cdk4/6 inhibitor palbociclib was approved in 2016 for the treatment of estrogen receptor-positive breast cancer and was followed by the similar molecules abemaciclib and ribociclib (Finn et al., 2016; Im et al., 2019; Johnston et al., 2019; Sherr et al., 2016). Positive clinical trial results for pallbociclib (PALOMA 3), ribociclib (MONALEESA-7), and other inhibitors have validated Cdk4/6 as therapeutic targets in breast cancer (Im et al., 2019; Sledge et al., 2019; Spring et al., 2020; Turner et al., 2018), and it is likely that Cdk4/6 inhibitors will demonstrate efficacy in other cancer types currently being tested (clinicaltrials.gov). However, a number of questions remain, including why only some patients benefit from treatment with these drugs (Spring et al., 2020). Our lack of an answer arises from our current inability to predict short- and long-term response to Cdk4/6 inhibitors. As an example, there was a puzzling lack of response in the PALOMA-3 trial in a majority of breast tumors that continue to express Rb (Turner et al., 2018), even though Rb is thought to be the main target of Cdk4/6 in the control of cell-cycle progression. Why don’t Cdk4/6 inhibitors inhibit cancer more effectively in patients and what are the mechanisms that dictate the initial response to these molecules and that mediate resistance to their inhibitory effects?

A growing number of studies have been using tumor samples from breast cancer patients to identify pathways that mediate the intrinsic or selected resistance to Cdk4/6 inhibitors. These analyses identified expected alterations in the core Rb pathway and also in other pathways linked to the Rb pathway and cell-cycle control, including RAS/AKT/PTEN/PI3K/MTOR signaling (Costa et al., 2020; Wander et al., 2020), Hippo signaling (Li et al., 2018), p53 signaling (Wander et al., 2020), and molecules involved in the G2/M transition of the cell cycle (Wander et al., 2020). These studies in patients samples have been corroborated by studies in pre-clinical models (Álvarez-Fernández and Malumbres, 2020).

While the overall picture drawn from these studies support an on-target activity of the Cdk4/6 inhibitors, a number of new questions have arisen. First, because Rb is a key target of Cdk4/6 and Rb inactivation promotes tumorigenesis in multiple contexts, loss of Rb would be expected to be a frequent event in tumors that become resistant to Cdk4/6 inhibitors. However, the events leading to the functional inactivation of Rb (*e.g.*, inactivating mutations in the *RB1* gene) are relatively rare in clinical samples from tumors resistant to Cdk4/6 inhibitors (Condorelli et al., 2018; Costa et al., 2020; Li et al., 2018; Wander et al., 2020). This rarity raises the question of whether Rb is really the key target of Cdk4/6 in cell-cycle control or whether loss of Rb is actually detrimental to cancer cells - both these ideas challenge the current Rb pathway paradigm.

A second set of observations from clinical samples have shown that elevated levels of Cdk4 (Finn et al., 2020) or Cdk6 (Li et al., 2018) are also associated with increased resistance to Cdk4/6 inhibitors. One interpretation of these data is that, surprisingly, the levels of Cdk4/6 inhibitors that reach cancer cells are not enough to inhibit these kinases even with a few-fold increase in the levels of the drug target. However, data indicating that increased levels of D-type cyclins is linked with increased *sensitivity* of some cancer cells to Cdk4/6 inhibition (Gong et al., 2017) are at odds with the simplest hypothesis that high levels of CycD-Cdk4/6 complexes drive resistance to treatment in tumors. More active Cdk4/6-CycD complexes could mean that cancer cells have become more dependent on the kinase activity of Cdk4/6. Alternatively, increased expression of particular kinase subunits could lead to changes in protein complexes that render them less amenable to inhibition by small molecule inhibitors. It is also possible that CycD proteins have Cdk4/6-independent effects (Bienvenu et al., 2001; Jirawatnotai et al., 2011; Neuman et al., 1997), including reshuffling of protein partners that also impinge upon Cdk2 activity (Guiley et al., 2019). In support of increased Cdk2 activity as a mechanism of resistance, high CycE levels are associated with decreased response to palbociclib in breast tumors (Turner et al., 2019; Wander et al., 2020). But if increased Cdk2 activity is a major mechanism of resistance to Cdk4/6 inhibitors, why are Rb loss of function events not more frequent? One possible, but unattractive, answer is that Rb is not the key target of Cdk4/6 and Cdk2 in cancer.

Collectively, these analyses underscore our incomplete understanding of how cancer cells respond to the inhibition of Cdk4 and Cdk6 and, more generally, our incomplete understanding of the Rb pathway in G1/S control.

Concluding remarks

Decades of research have identified most, if not all the major regulators of the G1/S transition in mammalian cells. This work has led to the successful development of Cdk4/6 inhibitors now used in the clinic, and Cdk2 inhibitors are currently being tested in cancer patients (clinicaltrials.gov). However, the results of clinical studies with Cdk4/6 inhibitors show that we only partly understand the mechanisms underlying a durable response to these inhibitors. Recent laboratory studies, including the analysis of the G1/S transition at the single cell level, further reveal our incomplete grasp of how the decision to replicate DNA and progress towards mitosis is made at the molecular level.

Some of these gaps in our understanding will be filled by the analysis of cancer specimens from patients treated with Cdk4/6 and Cdk2 inhibitors. The identification of mechanisms of resistance to these inhibitors will lead to new clinical trials with combination therapies and will instruct new studies to better inform how different signaling pathways regulate cell-cycle progression in G1. An important aspect of these clinical studies will be to distinguish how various treatment strategies directly regulate the cell cycle of cancer cells relative to other cells in the tumor microenvironment, including immune cells, which have already been shown to be directly and indirectly impacted by treatment with Cdk4/6 inhibitors (Zhang et al., 2018).

It is likely that our understanding of the restriction point and G1/S regulators will further change as more investigators examine the mechanisms regulating cell-cycle progression in multiple contexts, including *in vivo* (Cuitino et al., 2019; Sakaue-Sawano et al., 2017; Yano and Hoffman, 2018). In most organs and tissues, cell density, cell-cell interactions, and metabolic activities are different than in cell culture. We anticipate that these differences between cells in culture and *in vivo* likely influence how the decision to progress from G1 to S is made. For example, the complete loss of Rb family proteins blocks the ability of these cells in culture to arrest in G1 in all contexts examined, but the same Rb family deletion in the mouse liver is not sufficient to block cell-cycle arrest (Ehmer et al., 2014). Furthermore, the definition of the restriction point and the mechanisms of cell-cycle progression in G1/S may differ between quiescent stem cells re-entering the cell cycle from G0 and stem/progenitor cells that cycle frequently and transition from M to G1 directly. It is also possible that differences exist between human cells and mouse cells. The identification of key regulators of G1/S in different *in vivo* contexts may considerably improve our understanding of cell-cycle progression and give us insight into the plasticity of this molecular network.

We find it striking that even with recent advances in genetic engineering, single cell analyses, and structural studies, so little has been done to investigate the molecular mechanisms underlying how key players in the extended Rb pathway function. For example, post-translational modifications of Rb pathway components (phosphorylation and others) have only been superficially investigated and similarities and differences between family members at every level in the pathway are still poorly understood. The mechanisms underlying protein-protein interactions and the dynamic nature of protein complexes in the pathway are also still mostly unclear. It is likely that further research in the next decade will not only refine our understanding of the molecular mechanisms of the G1/S transition but will also uncover new paradigms.

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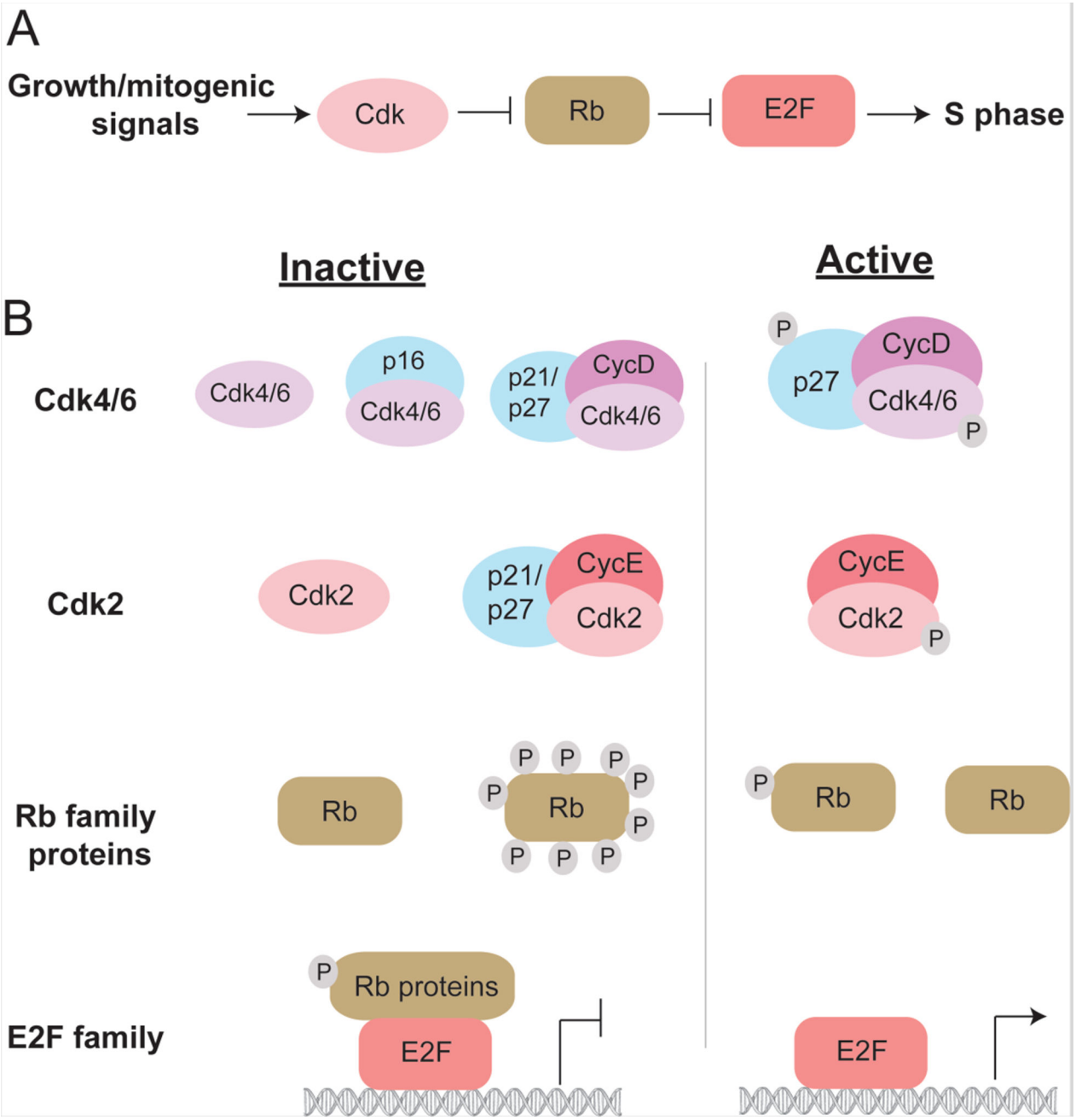

Figure 1: Components of the Cdk-Rb-E2F pathway controlling the G1/S transition.

(A) Simplified model for pathway. (B) Inactive and active states of the key players in the Rb pathway. Cdk4 and Cdk6 (Cdk4/6) have relatively high sequence homology among Cdks. They are inactive as monomers, bound to p16 family proteins, or bound by unphosphorylated p21 and p27 (p21/p27) proteins. Cdk4/6 are activated by association with Cyclin D (CycD) family proteins, but full activity also requires a phosphorylated from of p27 in the complex and phosphorylation on the kinase activation loop. Cdk2 is inactive as a monomer or in complex with p21/p27 family proteins, and it is activated by CycE binding in G1 (or CycA later in the cell cycle) and activation loop phosphorylation. Rb is considered

active when hypo-or monophosphorylated; in this state it binds and inhibits E2F. Hyperphosphorylation of Rb leads to its inactivation, dissociation from E2F and subsequent E2F activation. While Rb binds E2F when unphosphorylated during quiescence, it may also be considered inactive in that it cannot perform monophosphorylation-dependent functions.

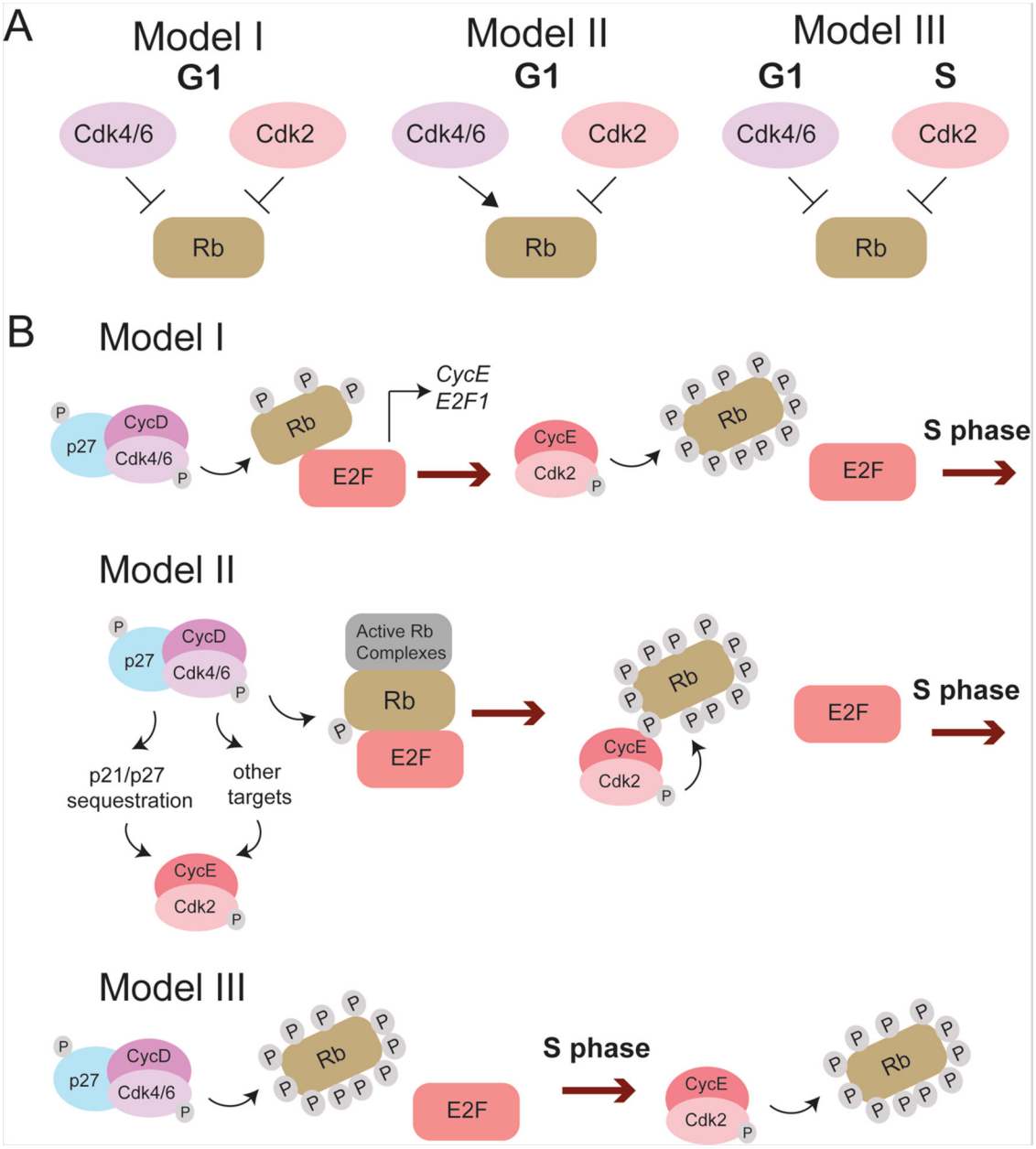

Figure 2: Proposed models for Rb inactivation during G1.

(A) The three models differ with respect to whether Cdk4/6 and Cdk2 activate (arrow) or inhibit (cross) Rb and in the timing of their activity relative to the G1/S transition. (B) Roles of Cdk complexes in modulating Rb function. In the canonical Model I, Cdk4/6 partially phosphorylates Rb, resulting in some E2F activity. Transcription of genes such as CycE then activates Cdk2 for full Rb hyperphosphorylation and inactivation for S phase. In Model II, Cdk4/6 monophosphorylates Rb, and the different active Rb species inhibit E2F and form functional protein complexes in G1. Cdk4/6 induces Cdk2 activity through several possible mechanisms, including sequestration of p21/p27 inhibitors and phosphorylation of other targets that may indirectly activate Cdk2. Active Cdk2 then hyperphosphorylates Rb, leading to Rb inactivation and S phase entry. In Model III, Cdk4/6 hyperphosphorylates Rb in G1,

which is sufficient for Rb inactivation and S phase entry. Cdk2 activity is required to maintain Rb hyperphosphorylation during S phase.

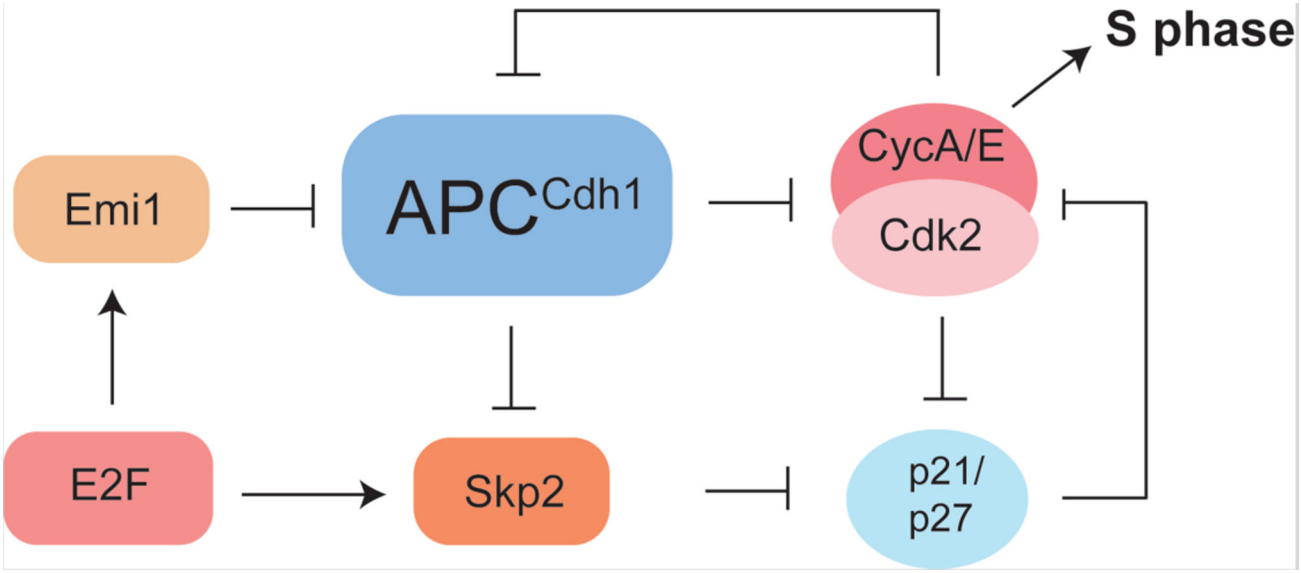

Figure 3: APC^{Cdh1} and p21/p27 inactivation are required for S phase entry.

S phase activity of Cdk2 is inhibited by p21/p27 and APC^{Cdh1}, which is a ubiquitin ligase that stimulates CycA degradation. APC^{Cdh1} is inactivated by Cdk2 phosphorylation and the protein Emi1. p21/p27 degradation is induced by Cdk2 phosphorylation and the ubiquitin ligase Skp2, which is in turn degraded by APC^{Cdh1}. Both Emi1 and Skp2 are transcriptional targets of E2F, which places these inactivation events downstream of Rb inactivation. These two connected double negative feedback loops involved in Cdk2 activation are thought to render S phase entry irreversible.

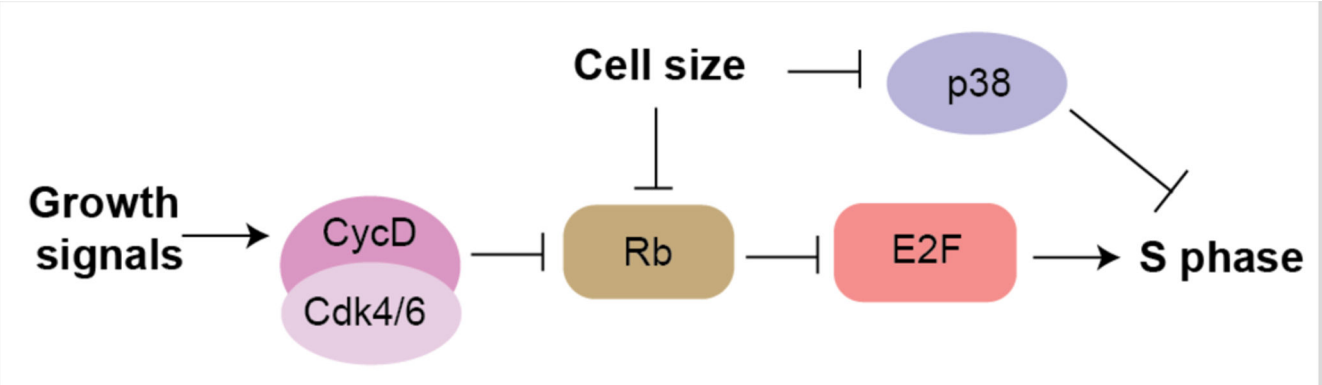

Figure 4: Cell growth and size signals drive the G1/S transition.

Growth factors trigger the synthesis of CycD and Cdk4/6-dependent activity. Cell growth in G1 dilutes Rb and leads to a decrease in activity of the p38 stress-activated kinase to trigger cell-cycle progression in larger cells.