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REVIEW ARTICLE

Restriction point regulation at the crossroads between quiescence and cell proliferation

Betheney R. Pennycook[1,2] and Alexis R. Barr[1,2]

1 Institute of Clinical Sciences, Faculty of Medicine, Imperial College London, London, UK
2 MRC London Institute of Medical Sciences, Imperial College London, London, UK

Correspondence
A. R. Barr, Institute of Clinical Sciences, Faculty of Medicine, Imperial College London, Du Cane Road, London W12 0NN, UK
Tel: +44 02083838235
E-mail: abarr@ic.ac.uk

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The coordination of cell proliferation with reversible cell cycle exit into quiescence is crucial for the development of multicellular organisms and for tissue homeostasis in the adult. The decision between quiescence and proliferation occurs at the restriction point, which is widely thought to be located in the G1 phase of the cell cycle, when cells integrate accumulated extracellular and intracellular signals to drive this binary cellular decision. On the molecular level, decision-making is exerted through the activation of cyclin-dependent kinases (CDKs). CDKs phosphorylate the retinoblastoma (Rb) transcriptional repressor to regulate the expression of cell cycle genes. Recently, the classical view of restriction point regulation has been challenged. Here, we review the latest findings on the activation of CDKs, Rb phosphorylation and the nature and position of the restriction point within the cell cycle.

Keywords: cell cycle; cyclin; cyclin-dependent kinase; proliferation; quiescence; restriction point; single-cell imaging

Tight control of cell proliferation is vital for normal development and tissue homeostasis. Loss of cell cycle control can lead to proliferative diseases, including cancer and fibrosis [1,2]. Cell proliferation is controlled by regulating the entry into, and passage through, the cell cycle. The cell cycle can be defined as consisting of four consecutive phases: G1 (gap 1), S (DNA replication), G2 (gap 2) and M (mitosis – cell and nuclear division). Whilst initially viewed as 'gap' phases, we now know that G1 and G2 are periods of active signal integration and protein synthesis and can instead be thought of as pre- and postreplication states [3]. G0, or quiescence, exists outside of the proliferative cycle and is an enigmatic state, or better a collection of states [4] that can be broadly defined by their lack of proliferation but maintenance of proliferative potential. For example, stem cells spend the majority of their time in G0 and only re-enter proliferative cell cycles on receiving appropriate input from growth factors. Growth factors are necessary for the transition from G0 to G1 and the point at which S-phase entry becomes independent of growth factor stimulation is known as the restriction point. Therefore, passage through the restriction point has been viewed as the point of no return into the cell cycle.

The first description of the restriction point emerged from experiments that showed that nontransformed cells exposed to different suboptimal environmental conditions (e.g. low serum, isoleucine withdrawal) arrested at the same position in the cell cycle – at a unique 'restriction point' located in G1 [5]. Later work mapped the position of this restriction point, in mouse 3T3 cells, to 2–3 h prior to S-phase entry [6]. Furthermore, in an early example of using single-cell time-lapse imaging to study the kinetics of restriction point passage, Zetterberg and Larsson [7] reported that, in response to transient serum deprivation in cycling 3T3 cells, the time taken to reach the restriction point was

Abbreviations
CAK, CDK activating kinase; CDK, cyclin-dependent kinase; CKI, CDK inhibitor; Rb, retinoblastoma.

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relatively invariant at 3–4 h, whilst the time between restriction point passage and S-phase entry was highly variable.

One of the obvious questions emerging from this work was what is the molecular mechanism underpinning restriction point passage? Addition of the protein synthesis inhibitor cycloheximide could delay S-phase entry in cells that had been stimulated to enter the cell cycle [8,9]. This work suggested that a labile protein, now understood to be one of the D-type cyclins, the accumulation of which is sensitive to growth factors, must reach a critical threshold for the cell to pass the restriction point [10]. Transformed cells may accumulate a more stable form of this protein that could compromise the restriction point and therefore growth regulation [11].

In this review, we will discuss our current understanding of the molecular events underpinning restriction point control. We focus on the control of the restriction point in somatic cells but direct interested readers to the review by Padgett and Santos [12], in this same series, for an in-depth discussion of how this control differs in embryonic cells. We will review recent results, mainly gathered from quantitative single-cell time-lapse imaging experiments, that challenge the position of the restriction point and question the 'traditional' view of the restriction point as a point of no return for commitment to DNA replication.

Activating CDK4 and CDK6

Progression into and through the cell cycle is driven by the activity of cyclin-dependent kinases (CDKs). The earliest CDKs to be activated in the cell cycle are CDK4 and CDK6. This section will address how these kinases are activated prior to restriction point passage. Extracellular and intracellular processes that influence restriction point passage all ultimately impact CDK activity by controlling the expression, stability and/or activity of one or more CDK regulatory processes (Fig. 1A). Below, we consider each of these regulatory processes and their impact on CDK4/6 activity. CDK4 and CDK6 are activated by the same cyclins, and the relative importance of CDK4 versus CDK6 for proliferation has some degree of cell type and tissue specificity [13–15]. For brevity, we consider the two kinases together.

Cyclin binding

CDK4 and CDK6 require binding to one of three D-type cyclins (D1, D2 or D3) to become active. Transcription of *CCND* (the gene encoding D-type cyclins)

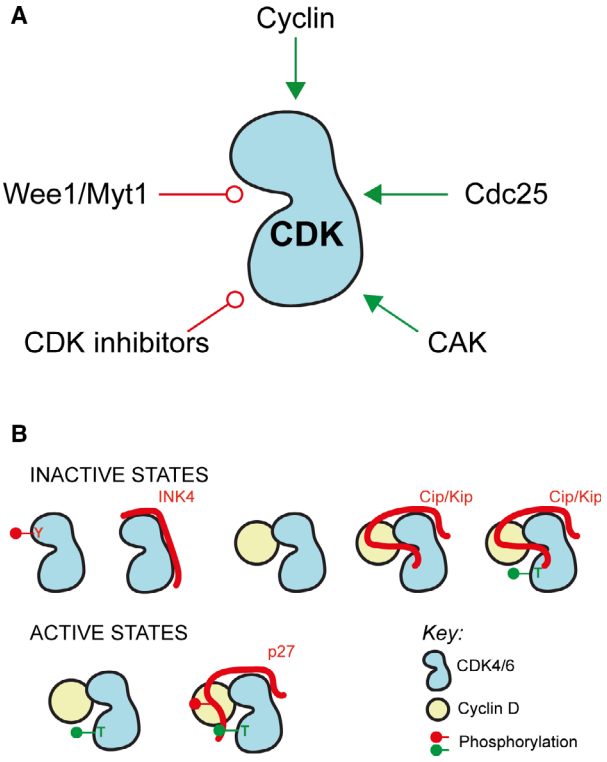

Fig. 1. (A) CDK regulatory mechanisms. Green arrows represent CDK activating mechanisms, and red lines represent CDK inhibitory mechanisms. The contribution of each of these mechanisms to CDK4/CDK6 regulation is described in the text. (B) A number of different CyclinD:CDK4/6 complexes have been characterised *in vitro* and in cells, with different degrees of kinase activity. Red represents phosphorylation on tyrosine residues, and green represents activating T-loop phosphorylation. The coordination of inhibitory tyrosine phosphorylation on CDK4 and CDK6 with Cyclin or CKI binding is not well understood and is not included in the figure.

can be driven by many factors, including mitogenic growth factors, cytokines, the extracellular matrix, Wnt and Notch signalling, as well as tissue-specific signals [16–18]. The best understood of these is regulation by mitogenic growth factors. Growth factor stimulation drives activation of the Ras-Raf-MEK-ERK signalling pathway, which ultimately stabilises the AP-1 family of dimeric transcription factors, including c-Fos and c-Jun [19,20]. Fos and Jun can drive the expression of Cyclin D, which, in turn, binds to CDK4 and CDK6. Overexpression of Cyclin D1 in fibroblasts is sufficient to shorten G1 and partially relieves cells of their growth factor dependency [21–23]. However, ectopically expressed Cyclin D and CDK4 do not readily associate in quiescent cells and a mitogen-dependent step is required for their stable association [24–26]. Moreover, unlike Cyclin:CDK1/2 complexes,

binding of D-type cyclins to CDK4 does not induce the structural changes within the kinase subunit that are associated with CDK activation, for example remodelling the ATP binding site or exposure of the T-loop [27–30]. Together, these data indicate that additional steps are required to activate CyclinD:CDK4/6 complexes.

T-loop phosphorylation

Activation of CyclinD:CDK4/6 requires phosphorylation on the T-loop of the CDK subunit by CDK activating kinase (CAK). CAK consists of three subunits – CDK7, Cyclin H and the assembly factor MAT1 (ménage à trois [31,32]). Phosphorylation of the T-loop displaces it from the from the CDK active site and exposes a Cyclin:CDK:substrate binding interface [33]. CAK phosphorylates T172 in CDK4 and T177 in CDK6, and both residues are more efficiently phosphorylated in CyclinD:CDK dimers than in CDK4/6 monomers [24,34,35]. Binding of the Cip/Kip CDK inhibitor (CKI) $p27^{Kip1}$ to CyclinD:CDK4 also exposes the activation segment of CDK4, promoting CAK phosphorylation. However, this complex remains inactive until p27 is phosphorylated or removed (see below [36,37]). This suggests that trimer complex formation of CyclinD:CDK4/6:p27 is upstream of phosphorylation by CAK and is one example of how CKIs may promote activation of CDK4/6.

Early work on CAK regulation suggested that CAK expression and activity were constitutive, even in quiescent cells [25]. However, recent work using the a human cell line expressing an analogue-sensitive CDK7 (*Cdk7as/as*) suggests that T-loop phosphorylation, and thus the activity of CAK itself, increases during the G0-G1 transition [38], although another group was unable to confirm this [39]. Experimental evidence from the *Cdk7as/as* cell line [38] and lambda phosphatase treatment of *in vitro* complexes [30] suggest that T-loop phosphorylation of CDK4/6 is labile. This implies that maintenance of CDK4/6 kinase activity would require constant CAK activity in cells, which would make T-loop phosphorylation an exquisite control point. Whether this would be mediated by changes in CAK activity, as mentioned above, or by regulation of T-loop phosphorylation by a distinct (as yet unknown) kinase or phosphatase [35] remains an open question.

CKIs

Two classes of cyclin-dependent kinase inhibitors (CKIs) exist in mammalian cells – the Cip/Kip and INK4 families. The Cip/Kip family consists of three proteins, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. These proteins are intrinsically disordered and fold upon binding to the Cyclin:CDK dimer [40]. Cip/Kip proteins are capable of inhibiting all Cyclin:CDK complexes. By contrast, the INK4 family has four members: $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$, which are specific inhibitors for CDK4 and CDK6. Unlike the Cip/Kip proteins, INK4 proteins inhibit kinase activity by binding to monomeric CDK4 or CDK6 and preventing Cyclin D binding [41].

In the case of Cip/Kip interactions with CDK4/6, the inhibitor label may be misleading. Evidence suggests that Cip/Kip proteins stabilise CyclinD:CDK4/6 complex formation and that p27 could activate CDK4 [36,42–46]. Cip/Kips may also facilitate the localisation of Cyclin D1 and Cdk4 to the nucleus, although this function is apparently not essential [43,44,47]. In the absence of Cip/Kip proteins, Cyclin D protein levels are decreased, suggesting that interaction with CDK4 and the Cip/Kips stabilises Cyclin D protein (p27: [43], p21: [48], p57: [42]). Binding of p21 and p27 to CyclinD:CDK4 also imposes a conformational constraint on the dimer complex and can act as a physical bridge between the two proteins [36]. Recent structural studies have shed light on a conundrum that has confused cell cycle enthusiasts for some time, that CyclinD:CDK4 bound to p27 appears to exist in both active and inactive conformations, and the switch to an active conformation can be driven by tyrosine phosphorylation of p27 [49]. Phosphorylation of p27 on Y74 induces conformational changes in CDK4 that promote ATP coordination and CDK4 activity [36]. Although CDK4 activity is boosted by p27 Y74 phosphorylation, the presence of tyrosine phosphorylated p27 alters the substrate specificity of the trimer compared to the active CyclinD:CDK4 dimer. The CyclinD:CDK4:PY74-p27 trimer preferentially inhibits the phosphorylation of CDK4 substrates that contain a docking site, for example the transcriptional repressor retinoblastoma (Rb), but does little to change the activity towards those substrates that do not, for example p107 [36]. This suggests that changes in both p27 levels and phosphorylation status as cells enter the cell cycle could impart some control over the order in which CDK4 substrates are phosphorylated. Another intriguing observation from the same work is that tyrosine phosphorylation regulation was specific to p27 and not p21, which lacks a similar phosphorylation site. This reveals the unique roles of p27 and p21. In the case of p27, tyrosine phosphorylation provides an additional route to link extracellular growth factor signalling, *via* tyrosine kinase activation, to restriction

point control. However, p21 is expressed in response to DNA damage, downstream of p53 [50,51]. Refractoriness to tyrosine phosphorylation would thus allow p21 to prevent CDK4 activation and cell cycle entry in the presence of ongoing DNA damage. Thus, the Cip/Kips appear to be ambivalent about CyclinD:CDK4/6 as they can activate or inhibit kinase activity, and their role is dependent on the cellular context.

Inhibitory phosphorylation

Inhibitory phosphorylation of T14 and Y15 in CDK1 by Myt1 and Wee1 kinases, respectively, prevents premature entry of cells into mitosis. CDK1-mediated activation of Cdc25 phosphatases (Cdc25A, B and C) and simultaneous inhibition of Wee1 kinase is required to dephosphorylate T14 and Y15 and initiate rapid mitotic entry [52,53]. CDK4 and CDK6 can be phosphorylated at analogous Y17 and Y24 sites, respectively, but the function of these residues in controlling CDK4 and 6 activity and in restriction point passage is poorly understood.

Phosphorylation of Y17 in CDK4 has been detected in cells induced into quiescence by serum starvation. Upon re-entry into the cell cycle, Y17 phosphorylation decreases around the same time that CDK4 is activated [54]. TGF-β treatment of cells leads to a reduction in Cdc25A expression and induces cells to arrest with increased tyrosine phosphorylation of CDK4 and CDK6 [55]. Furthermore, Cdc25A is no longer required for cell cycle re-entry from quiescence in cells expressing the nonphosphorylatable mutant, $CDK4^{Y17F}$, implying that a key role of Cdc25A in cell cycle entry is dephosphorylation of this residue [56]. More recent data suggest that inhibitory tyrosine phosphorylation of CDK4 and CDK6 may also prevent their interaction with Cyclin D [57]. Together, these data suggest that tyrosine phosphorylation does play a key role in the regulation of restriction point passage by controlling CDK4 and CDK6 activity, although precise molecular details, including the roles and regulation of Wee1 (and potentially Myt1) kinase, are still lacking.

It appears that multiple states of CDK4 and CDK6 can exist in cells at any one time (Fig. 1B). The presence of multiple states of CDK4 and CDK6 in cells has likely contributed to difficulties in reaching a consensus concerning how these CDKs are activated to regulate restriction point passage. However, maintaining different pools of CDK4/6 or CyclinD:CDK4/6 complexes would allow cells to tune their CDK4/6 activity to different conditions and to respond rapidly to changing conditions [49].

Rb phosphorylation: a shifting paradigm

Passing the restriction point is coincident with hyperphosphorylation of the product of the retinoblastoma gene, Rb [58] and activation of CyclinE:CDK2 [59,60]. Rb is a member of the pocket protein family, which also includes p107 and p130 [61,62]. Pocket proteins bind to and inhibit E2F transcription factors, a family of proteins that drive the gene expression programme required for entry into the cell cycle [63]. For full activation of E2F transcriptional activity, Rb must be hyperphosphorylated to prevent its binding to E2Fs. Our understanding of how the cell achieves this has recently changed and below we will discuss the 'classical' and 'new' models of Rb phosphorylation.

'Classical' model: progressive hypophosphorylation of Rb

Until 2014, our understanding of Rb phosphorylation during restriction point passage was that as CyclinD:CDK4/6 activity increased during early G1, CDK4/6 could progressively phosphorylate unphosphorylated Rb protein on any one of 14 potential CDK sites – an event termed hypophosphorylation [64,65]. Hypophosphorylation of Rb was understood to release a fraction of Rb from inhibiting E2F transcription factors, allowing E2F-mediated transcription to initiate. Targets of activator E2Fs include the *CCNE1* and *CCNE2* genes, encoding cyclins E1 and E2 [66,67], and E2F itself [68]. This initiates two positive feedback loops that promote E2F activity. The former is that E2F drives its own transcription. The latter is that newly synthesised Cyclin E protein binds to and activates CDK2, which can drive the hyperphosphorylation, and consequent inactivation, of Rb (Fig. 2A). This promotes the release of more E2F from Rb and more *CCNE1/2* transcription, initiating a second positive feedback loop to drive restriction point passage [69] and the increase in transcription necessary for S-phase entry.

'New' model: mono-phosphorylation of Rb

Although aspects of the old model had previously been called into question [70–72], the most convincing evidence contradicting the existing model was published in 2014 [73]. Using two-dimensional isoelectric focussing (2D-IEF) to separate phospho-isoforms of Rb, the authors were only able to identify unphosphorylated, monophosphorylated and hyperphosphorylated forms of Rb. No hypophosphorylated Rb form was found to exist in cells. A timecourse of serum-starved cells,

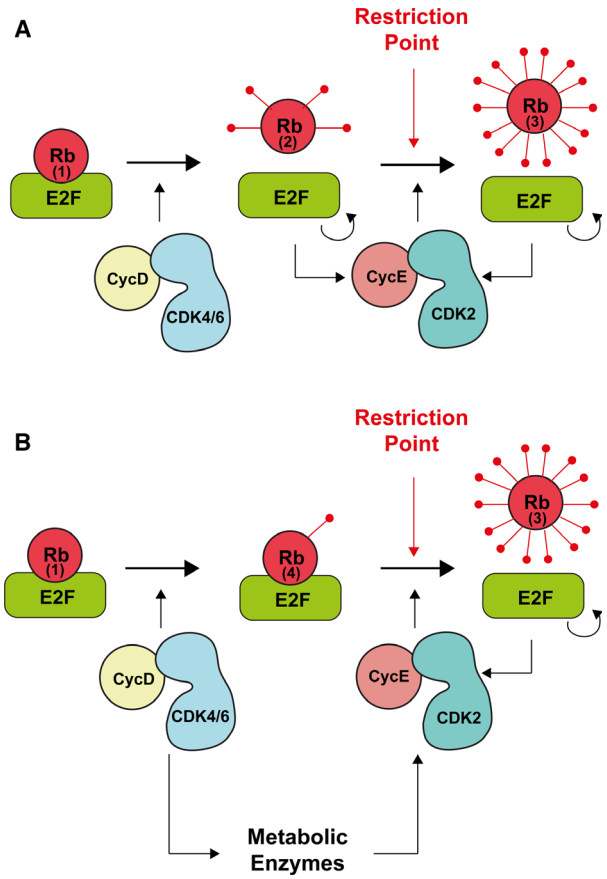

Fig. 2. 'Classical' (A) and 'new' (B) models of restriction point control by Rb hyperphosphorylation. In cells entering the cell cycle from quiescence, Rb is unphosphorylated (state (1)). (A) In the old model of restriction point regulation, CyclinD:CDK4/6 was proposed to progressively, but incompletely, phosphorylate Rb (hypophosphorylation – state (2)), releasing its inhibition of E2F. E2Fs initiate *CCNE* transcription and Cyclin E binds to and activates CDK2. Active CyclinE:CDK2 complexes further phosphorylate Rb, initiating a positive feedback loop driving more E2F release, *CCNE* transcription, CyclinE:CDK2 activity and eventual hyperphosphorylation (state (3)) and inactivation of Rb. (B) More recent data suggest that CyclinD:CDK4/6 complexes serve to mono-phosphorylate Rb (state (4)), on any one of 14 CDK sites, and simultaneously initiate metabolic changes in the cell that drive CyclinE:CDK2 activation. If this is the case, it is then CyclinE:CDK2 activity alone that drives the hyperphosphorylation of monophosphorylated Rb and restriction point passage. In both models, once Cyclin E is expressed, the positive feedback loop between CyclinE:CDK2 activity and E2F activation is the same.

stimulated to re-enter the cell cycle, revealed a rapid shift from a monophosphorylated Rb to hyperphosphorylated Rb at 14 h postserum stimulation, coincident with CyclinE:CDK2 activation. This led to a new model being proposed where CyclinD:CDK4/6 complexes can *only* mono-phosphorylate Rb protein, on any one of 14 CDK sites (Fig. 2B). Monophosphorylated Rb can still bind to E2F and inhibit its activity. A separate, as yet uncharacterised event, then drives triggers *CCNE* transcription to activate CDK2 activity and drive the conversion of monophosphorylated Rb to inactive, hyperphosphorylated Rb. Only then would E2F transcription factors be released and activated, and cells would pass the restriction point.

At least three unanswered questions emerge from this model. The first is how does CyclinD:CDK4/6 only mono-phosphorylate Rb, and how is phosphorylation at other sites prevented? This becomes particularly difficult to understand when we consider that in this model CDK4/6 can phosphorylate any of the 14 CDK sites, but only one. It is possible that monophosphorylation of Rb induces some structural changes in Rb protein [74–76], that could inhibit further phosphorylation by CDK4/6. Or perhaps CyclinD:CDK4/6 (or CyclinD:CDK4/6:pY-p27) has such a weak affinity for Rb that mono-phosphorylation is the most likely outcome? The second question is how is CyclinE:CDK2 activated if E2F is not activated by monophosphorylated Rb? There are suggestions that CyclinE:CDK2 activation may be downstream of CDK4/6 phosphorylation of metabolic enzymes [73,77] or regulated by E2F-independent *CCNE* transcription factors [78–80]. The third is, in the case of Rb mono-phosphorylation by CyclinD:CDK4, how do Cyclin D1 levels then relate to G1 progression? CCND1 is overexpressed in many cancers [81] and overexpression of D-type cyclins accelerates G0-S progression [22], yet too much Cyclin D protein may also prevent or delay normal S-phase entry and progression [82,83]. Perhaps the answer is that timely degradation of Cyclin D1 may be involved in restriction point passage [84]. These will be significant and exciting questions to address in future work.

The Rb phosphorylation code

The observation that Rb can be monophosphorylated at any one of 14 CDK sites raises the intriguing possibility that these 14 isoforms of Rb may have independent functions. Indeed, an Rb phosphorylation 'code' has been mapped using quantitative proteomics to look at the interactions of Rb species phosphorylated at each of the different sites [85]. For example, independent mono-phosphorylation events can regulate processes involved in either mitochondrial oxidative phosphorylation or chromatin remodelling. How the abundance of different monophosphorylated isoforms of Rb are generated by CyclinD:CDK4/6 complexes

and how the activities of multiple monophosphorylated species are coordinated remain unknown.

The position of the restriction point in the cell cycle

In addition to challenging the canonical model of restriction point passage, recent work has called into question the position and nature of a commitment point in the cell cycle. As previously mentioned, the 'point of no return' for commitment to DNA replication has been defined, at a molecular level, as the point at which Rb is hyperphosphorylated, E2F-dependent transcription is activated and CDK2 activity increases, and has historically been placed in G1 [7,69,86–88]. However, recent work, in particular using single-cell imaging, has started to alter how we think about commitment to proliferation.

Timing of cell cycle decision-making

For cells entering the cell cycle from quiescence, signalling during G0/G1 results in commitment to a new round of division. However, for cycling cells it is clear that signalling during the previous cell cycle can be important for restriction point passage [89–94]. Early studies suggested that commitment decisions are solely made during G1, indicating that cells in a population begin the cell cycle all equally likely to divide [5,7,58]. However, this model was challenged by the finding that, in asynchronously cycling cell cultures, Ras activity during G2 in the mother cell is necessary to promote cell cycle commitment in daughter cells, in part through promoting Cyclin D synthesis [90,91,95]. Recent single-cell work has strengthened the mother cell G2 model of mitogen sensing, demonstrating an early divergence in CDK2 activity in daughter cells [93,96]. This is reflected in Rb hyperphosphorylation, which can be detected as cells exit mitosis, suggesting that cells are born committed to a new cell cycle [97]. However, how CDK2 activity remains high after mitosis, when both Cyclin A and E are degraded, is unclear. One possibility is that in committed cells the major Rb phosphatase, PP1, is unable to dephosphorylate Rb, which remains hyperphosphorylated. Committed cells also degrade Cyclin D1 protein early after mitosis [84] and Cyclin D1 may not be needed if Rb remains mono- or hyperphosphorylated from the previous cycle. As Cyclin D protein has a short half-life, any inheritance of previous mitogen exposure is likely mediated through CCND1 mRNA, which has a longer lifetime [94]. As Cyclin D protein levels are highly dependent on the global protein translation rate, which responds to mitogenic signalling through MAPK, the protein translation rate may also enable a form of cellular mitogenic memory [92]. In addition, endogenous DNA damage in mother cells can also influence proliferation–quiescence decisions in daughter cell cycles [98,99]. This can be mediated through the inheritance of DNA damage by daughter cells, which results in increased p21 protein in the daughter cell and cells entering quiescence [98–100]. It is likely that the cell bases the decision to divide not on signal strength *per se*, but on signalling dynamics over time [87,101].

In cells returning to cycle from quiescence, it has been proposed that the threshold of growth factor stimulation required for restriction point passage can be modulated by cells [102]. This results in different 'depths' of quiescence from which cells require different threshold of serum stimulation, in terms of time or strength of stimulation, to return to the cell cycle [102–105].

What is commitment?

The restriction point is defined as the 'point of no return' for commitment to DNA replication. By its very definition, once the restriction point has been passed, cell cycle progression becomes independent of growth stimuli. However, the restriction point is only the first commitment point, sensitive to mitogens, and it should be noted that completion of the cell cycle can still be halted by downstream checkpoints, such as in response to DNA damage. Whilst the original definition of the restriction point is unchanged, its molecular makeup remains unclear. Below, we outline the evidence for the molecular events that could underpin this irreversibility.

Rb-E2F

The inactivation of pocket proteins and thus the activation of E2F-dependent transcription has a central role in the canonical restriction point model [58,63,87]. Early evidence strongly implicated the inactivation of Rb in the promotion of proliferation. Unphosphorylated Rb prevents S-phase progression and promotes cell cycle exit and differentiation, whilst conditions that result in its phosphorylation increase proliferation [58]. Whilst the pocket proteins are rarely mutated in cancer, Rb is the target of viral oncoproteins [106], and the inactivation of the upstream Rb-E2F pathway is a common feature of cancer [81,107,108]. The activation of E2F-dependent transcription alone has been shown to be sufficient to induce S-phase entry in quiescent cells [109,110].

In the classical model of the restriction point, Rb phosphorylation is initiated by CyclinD:CDK4/6 activity during G1 [111]. The hyperphosphorylation and inhibition of Rb allows the activation of E2F-dependent gene expression, which positively feeds back on itself through E2F and Cyclin E expression. A third feedback loop reinforcing commitment involves another E2F target, early mitotic inhibitor-1 (Emi1). Emi1 is a competitive inhibitor of the E3 ubiquitin ligase APC/C^{Cdh1}, and APC/C^{Cdh1} must be inactivated to allow S-phase entry [112,113]. APC/C^{Cdh1} activity is high in early G1, but Emi1 binding triggers its irreversible inactivation [114,115], which occurs following restriction point passage [59]. Inactivation of APC/C^{Cdh1} allows for an increase in the protein levels of its substrates, including Skp2 and Cyclin A, also E2F targets [116–120]. A time lag between the activation of E2F-dependent transcription and APC/C^{Cdh1} inactivation means that the levels of these proteins are initially low following restriction point passage. CDK2 complexes phosphorylate Cdh1, preventing binding to APC/C in a negative feedback loop [119,121]. Skp2 is an F-box protein, which is rate limiting for SCF^{Skp2} E3 ubiquitin ligase activity [122]. SCF^{Skp2} has been shown to target both E2F1 and p27 for degradation [123]. This means that Skp2 forms part of both a negative feedback loop for E2F activity and a positive feedback loop for CDK activity [124]. Thus, transcriptional changes at the restriction point are coupled to changes in protein degradation to give sustainable changes in protein levels.

Together, these feedback loops reinforce an initial increase in CDK activity and Rb hyperphosphorylation and inactivation. This enables CDK activity to be sustained independently of decreases in growth factor signalling, conferring bistability on the restriction point [69,88,125]. This property of the restriction point ensures irreversibility and means that this first step in cell cycle commitment is switch-like and that progression between phases of synthesis and division is unidirectional [88,125,126].

CDK2 activity

Ultimately, at the restriction point the combination of multiple different graded signals must be converted to a binary cellular output encoded through CDK activity. Recent single-cell analyses using a live-cell fluorescent sensor for CDK2 activity during G1 have extended the classical restriction point model and enabled a quantitative definition of the restriction point [60,93]. The activity of this sensor is able to predict restriction point passage with a high accuracy, defined by the response of cells to serum withdrawal [60]. A threshold of CDK2 activity may, therefore, be the defining feature of restriction point passage [127]. The activity threshold is reached several hours before full Rb-E2F pathway activation and is therefore predicted to reflect the onset of E2F activation rather than the peak [128]. The quantification of CDK2 activity has enabled a more precise definition of the restriction point and the timing of molecular events associated with it. The CDK2 threshold for restriction point passage seems to depend on cell type and the kind of proliferative inputs to which a cell is exposed [60].

APC/C^{Cdh1} inactivation

In the classical model of cell cycle commitment, the sensing of proliferative signals during G1 culminates in the hyperphosphorylation (or lack thereof) of Rb. This has been thought to be an irreversible step due to the bistability of the Rb-E2F network [69]. CDK activity inhibits a major Rb phosphatase PP1, therefore, Rb phosphorylation is difficult to reverse in healthy cells until cell cycle exit brings a decrease in CDK activity [86,129]. In stress conditions, PP2A may also dephosphorylate Rb [130].

Rb inactivation is separated in time, and is proposed to be independent from, the inactivation of APC/C^{Cdh1} [59,88]. Whilst the restriction point has classically been defined by cellular mitogen sensing, it is also possible that cells are able to respond to stress after Rb is hyperphosphorylated [59,131]. In this model, the time between Rb hyperphosphorylation and APC/C^{Cdh1} inactivation represents a window during which stress, such as DNA damage, is still able to prevent progression into S-phase. During this time, it is reported that, contrary to CDK4/6 only being able to mono-phosphorylate Rb [73], CDK4/6 activity is still required for Rb hyperphosphorylation, despite the detectable activation of CDK2, until APC/C^{Cdh1} is inactivated [132]. This suggests that both the restriction point and the inactivation of the APC/C^{Cdh1} contribute towards the irreversible transition between G1 and S-phase. Indeed, the activity of Emi1, which inhibits APC/C^{Cdh1}, is essential for the maintenance of an irreversible transition to S-phase [124,133]. In this model, the restriction point is not a discrete point during the cell cycle but rather a window during which cells are still sensitive to mitogen removal due to a dependence on CyclinD:CDK4/6 activity until APC/C^{Cdh1} inactivation. However, the physiological conditions under which this response would be required, that is how often cells are exposed to a high level of stress between

the onset of Rb hyperphosphorylation and APC/C^{Cdh1} inactivation, are unclear. The mechanism of Rb dephosphorylation after stress induction during G1 is also uncertain. Further, it is unclear if these cells retreat to a true quiescent state or whether they could still be said to be in a distinct postrestriction point state but with dephosphorylated Rb. Recovery from arrest appears to be faster in these stressed cells returning to cycle than from a postmitotic quiescence [59].

This window of reversibility between the restriction point and S-phase entry may not be closed by APC/C^{Cdh1} inactivation, but by the rapid degradation of p21 at S-phase entry by $CRL4^{Cdt2}$, which generates a bistable switch and prevents p21 upregulation in S-phase, that could otherwise promote premature S-phase exit [126]. APC/C^{Cdh1} inactivation and p21 degradation overlap in time and so the defining event is difficult to identify. However, interfering with p21 degradation by Cdt2 depletion allows cells to prematurely exit S-phase, even in the presence of intact APC/C^{Cdh1} control, suggesting that it is p21 degradation at S-phase entry that closes the window of reversibility. Moreover, even though APC/C^{Cdh1} is still active, cells become less sensitive to DNA damage the closer they are to S-phase entry which correlates with the time taken to induce p21 expression to a threshold capable of inhibiting Cdks [126].

Cell growth/size

In addition to mitogens, cells must be able to respond to other growth inputs to make appropriate proliferation decisions. An essential part of cell cycle control is to balance growth and division. Indeed, a negative correlation between cell size at birth and G1 length has been long established [134,135]. This indicates a size-dependent component to cell cycle commitment. We will briefly summarise work-relating cell size and cell cycle commitment here, but direct the interested reader to reviews of the current literature on growth and size control [136,137].

There is evidence that growth information can be integrated by Rb phosphorylation, as Rb inhibition has been shown to result in smaller cell size [138–140] and its overexpression, a larger cell size [141]. Recent work has proposed a link between CDK4/6 activity and mammalian target of rapamycin complex 1 (mTORC1), a key regulator of cell growth [142]. The mTOR protein kinase is a key part of a signalling network integrating environmental inputs such as amino acid and oxygen levels, with mTOR activity promoting protein, lipid and nucleotide synthesis and inhibiting autophagy [143]. In addition to Rb phosphorylation, CDK4/6 may also promote mTor activity [142]. In this way, CDK4/6 could couple the cell cycle machinery and cell growth. mTor was proposed to form part of a two-step model of cell cycle commitment, involving both a growth and nutrient sensing step [144,145]. This arose from data demonstrating the necessity for multiple factors, targeting Myc, Rb, p53 and mTor, in cellular transformation. This suggested that Rb inactivation alone is insufficient for cell cycle entry, but that other steps, mTor signalling and downregulation of p53 for example, are also important for commitment. However, other work has suggested that, although it regulates cell size, mTor is not involved in coordinating cell size and G1 length [146]. An inhibitor screen revealed that compounds targeting the PI3K/Akt/mTOR pathway altered both G1 length and cell size. In contrast, compounds targeting the p38 MAPK pathway perturbed the coordination between cell size and G1 length, indicating this pathway is important for cellular size control [137].

Whilst it is clear that cell size and growth are important inputs in a proliferation–quiescence decision, how cells measure these properties, and how this is integrated with restriction point control, is unknown. In yeast, cell growth leads to dilution of the Whi5, a transcriptional inhibitor similar in function to Rb, promoting the passage of START [147]. A similar inhibitor dilution model has recently been proposed for Rb in mammalian cells [148].

The point of no return

The exact position and nature of a commitment point in the cell cycle are still unclear. However, it is important to remember that studies investigating cell cycle control mostly use immortalised cell lines cultured in rich growth media. Whilst this has enabled much progress to be made in understanding the capabilities of cell cycle regulation, it remains to be seen which of these mechanisms are physiologically relevant given that most cells are quiescent *in vivo* [149]. Indeed, recent work has indicated stark differences in restriction point control between commonly grown cell lines and primary cells [60].

Together, recent single-cell work challenges the definition of the restriction point as the 'point of no return' for cells, suggesting that progression through to S-phase requires multiple inputs. It reveals an important caveat of the initial definition of the restriction point as the point beyond which cells are insensitive to the removal of mitogens. Whilst an important step in cell cycle commitment, this does not account

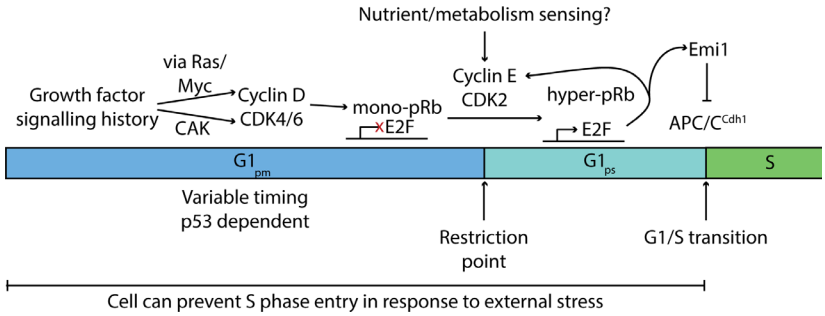

Fig. 3. A two-step model for cell cycle commitment. During G1, two bistable switches regulate the proliferation–quiescence decision. The activation of the first switch, the Rb-E2F pathway, is dependent on the integration of mitogenic signals both during G0/G1, and the previous G2 in cycling cells, *via* CyclinD:CDK4/6 activity. Rb-E2F switch activation is also dependent on the integration of DNA damage signals by p21. Other inputs, currently unknown but potentially linked to growth or metabolic signalling, increase Cyclin E transcription. Following the activation of the Rb-E2F pathway, CDK2 activity increases, and this is the molecular definition for restriction point passage. A second bistable switch is mediated by p21 degradation at S-phase entry, which marks the end of a period of stress sensitivity in G1, that is able to reverse the commitment decision made at the restriction point. p21 degradation is mediated by SCF^{Skp2} in late G1 and early S-phase, and by $CRL4^{Cdt2}$ in S-phase, which targets substrates through binding to PCNA. p21 can also inhibit $CRL4^{Cdt2}$ through inhibiting DNA replication. This coincides with APC/C^{Cdh1} inactivation by Emi1, which switches from a substrate to an inhibitor of APC/C^{Cdh1}.

for the cellular response to other inputs such as stress or metabolism, and in so doing possibly oversimplifies the process of commitment. How then, do we define commitment? It seems clear that CDK2 activity is highly predictive in determining restriction point passage as assessed by its original definition [7,60]. But how we define this 'decision' point molecularly, especially when molecular changes may not be as binary as once assumed, remains unclear.

Since there are different types of quiescence [4], it is likely that there are multiple ways of passing the restriction point, in that many potential inputs are able to increase CyclinD:CDK4/6 activity. Whilst there are many possible routes to restriction point passage, cells likely 'forget' their history after cell cycle commitment, meaning that there can be many paths to passing the restriction point but following passage, all cells are equally committed [150].

We think that a useful model for thinking about commitment to a new cell cycle involves two bistable switches. One is the restriction point, the transition of which is specific to extracellular mitogenic signals. The other is the G1/S transition (Fig. 3) [126]. The time between these two points represents a period of reversibility and further signal integration, which becomes important during stress conditions ($G1_{ps}$ in Fig. 3). Given the importance of restriction point control, and its dysregulation, in understanding development, tissue homeostasis and tumorigenesis, key questions still surround the molecular mechanisms of cell cycle entry in these systems. CDK4/6 inhibitors, such as Palbociclib, Abemaciclib and Ribociclib, are being used to treat ER+ breast cancers, and being trialled in other tumour types [151], generating renewed interest in how cells commit to proliferation. With the development of new single-cell tools to quantify and probe these mechanisms, in addition to improved imaging technologies, including light-sheet imaging and label-free microscopy, we anticipate exciting discoveries in these areas in the next few years.

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