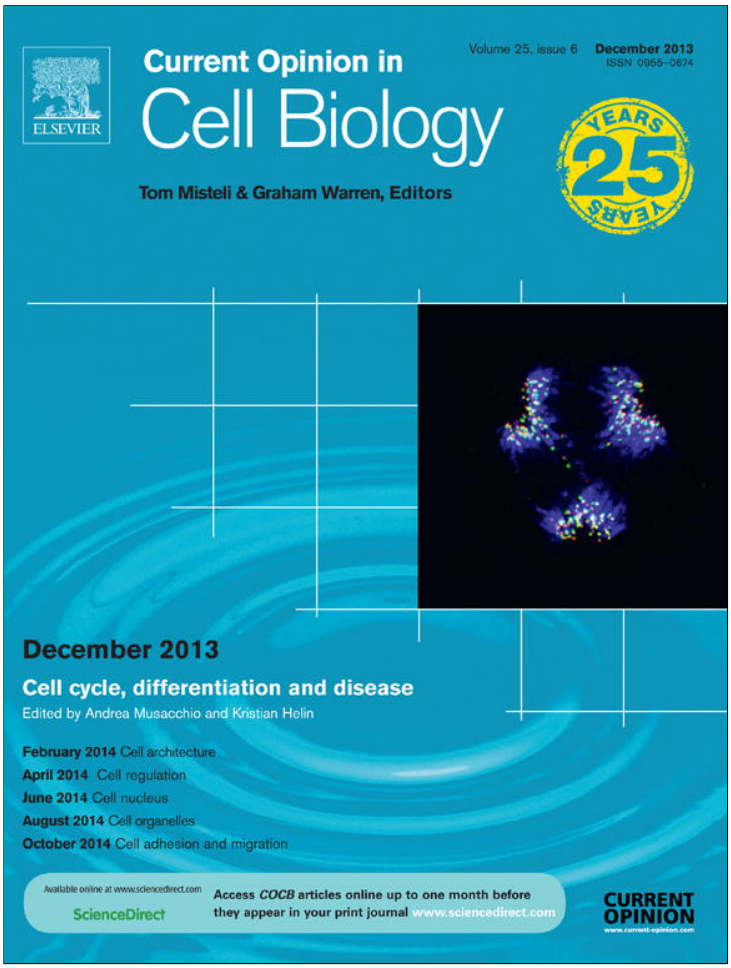

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Start and the restriction point

Amy Johnson and Jan M Skotheim

Commitment to division requires that cells sense, interpret, and respond appropriately to multiple signals. In most eukaryotes, cells commit to division in G1 before DNA replication. Beyond a point, known as *Start* in yeast and the restriction point in mammals, cells will proceed through the cell cycle despite changes in upstream signals. In metazoans, misregulated G1 control can lead to developmental problems or disease, so it is important to understand how cells decipher the myriad external and internal signals that contribute to the fundamental all-or-none decision to divide. Extensive study of G1 control in the budding yeast *Saccharomyces cerevisiae* and mammalian culture systems has revealed highly similar networks regulating commitment. However, protein sequences of functional orthologs often indicate a total lack of conservation suggesting significant evolution of G1 control. Here, we review recent studies defining the conserved and diverged features of G1 control and highlight systems-level aspects that may be common to other biological regulatory networks.

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Although both *Start* and the restriction point govern passage into S phase, their physiological input signals are quite different. A pre-*Start* cell exposed to mating pheromone immediately arrests in G1 while a post-*Start* cell proceeds once more through the cell cycle [1–3]. Similarly, beyond the mammalian restriction point, cells complete division irrespective of changes to some growth factor-dependent signals [4,5]. Thus, *Start* and the restriction point have very distinct physiologies which might place different requirements on their regulatory networks [6••].

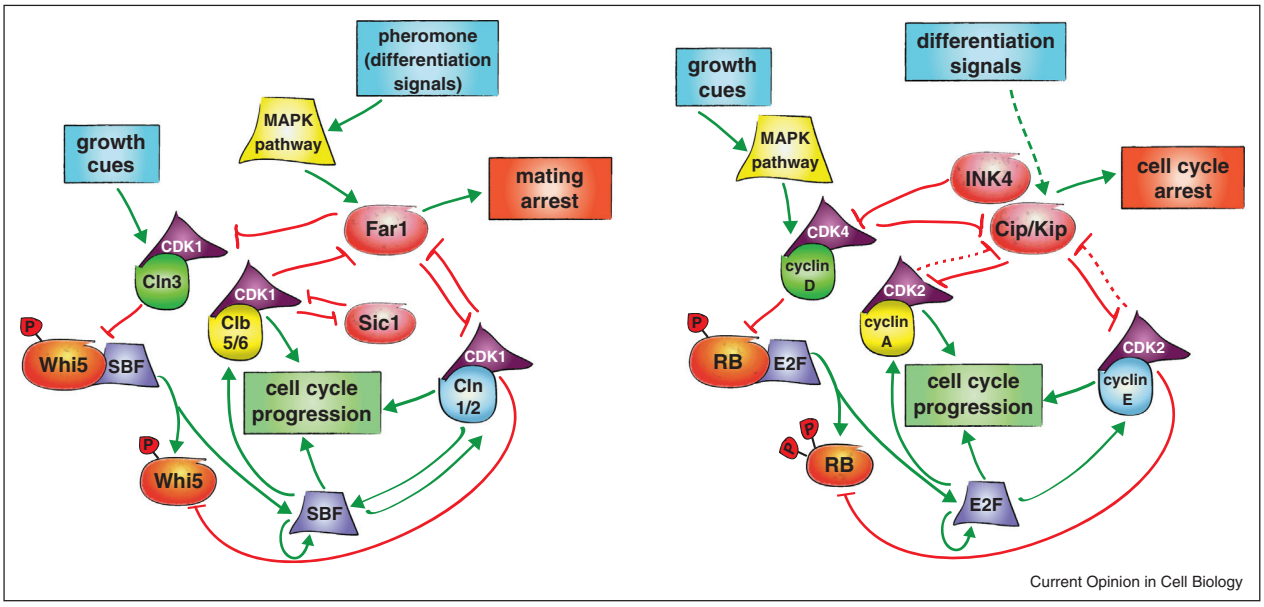
Conservation and evolution of G1 control networks

The core G1 control network appears highly similar in budding yeast and mammals (Figure 1) [7]. In both cases, signaling leads to an increase in cyclin-dependent kinase (CDK) activity via cyclin synthesis, which is largely responsible for promoting progression into S phase [8,9]. Before CDK activation, budding yeast spends variable amounts of time in G1, with smaller cells generally taking longer to reach *Start* [10]. This size-dependent progression functions primarily in daughter cells and requires that growth be coupled to the cell cycle. One likely coupling mechanism would rely on increasing levels of a cell cycle-regulating 'sizer' protein whose rate of synthesis is proportional to the overall protein production rate [11]. A good sizer candidate in budding yeast is the G1-S activator *CLN3*, which drives progression through *Start* in a dosage-dependent manner [12,13]. The levels of Cln3 are sensitive to both cellular growth rate and metabolic state [14,15]. Cln3 binds and activates CDK1, the sole yeast cyclin-dependent kinase required for cell cycle progression [16]. Cln3-CDK1 phosphorylates and initiates the inactivation of the transcriptional inhibitor Whi5, promoting its disassociation from the transcription factor SBF (Swi4/Swi6). This results in weak transcriptional activation of two downstream G1 cyclins, *CLN1* and *CLN2* [17,18]. Cln1 and Cln2 promote further inactivation of Whi5 and simultaneous activation of SBF and MBF (Mbp1/Swi6), which drive the cell cycle-dependent expression of over 200 genes including the S-phase cyclins that initiate DNA synthesis [19,20,21•,22•]. The SBF component Swi4 is also an SBF target, suggesting an additional positive feedback loop [23]. While an exact mechanism has yet to be elucidated, rising Cln3 levels may relay synthesis rate information to the G1 control network, with downstream positive feedback setting the threshold for cell cycle commitment.

The core G1 signaling network is similar in mammals, where growth factor stimulation leads to an increase in cyclin D, the upstream activator of G1 progression [24]. Cyclin D, functionally analogous to the yeast Cln3 protein, activates CDKs 4 and 6 to phosphorylate and initiate inactivation of the pocket proteins p107, p130, and the retinoblastoma (Rb) protein [25]. Inactivation of Rb leads to partial activation of the transcription factors E2F1-3, which then activate the transcription of downstream cyclins E and A that likely complete Rb inactivation and initiate DNA replication [25,26]. In both networks, activating signals proceed through an upstream cyclin that initiates a positive feedback loop of downstream cyclins.

While the core cell cycle control networks are broadly similar in both mammals and yeast, many functionally analogous proteins share no sequence

Figure 1



Despite a lack of sequence homology, G1 control networks are similar in both yeast (left panel) and mammals (right panel). Proteins and signals with similar functions are similarly shaped/colored. Upstream growth cues activate G1 cyclins, which drive progression into S phase via the activation of a positive feedback loop. Differentiation signals, including the pheromone-activated MAPK pathway in yeast, activate proteins that inhibit cyclin-CDK activity, leading to the increased stability of a low CDK activity cellular state.

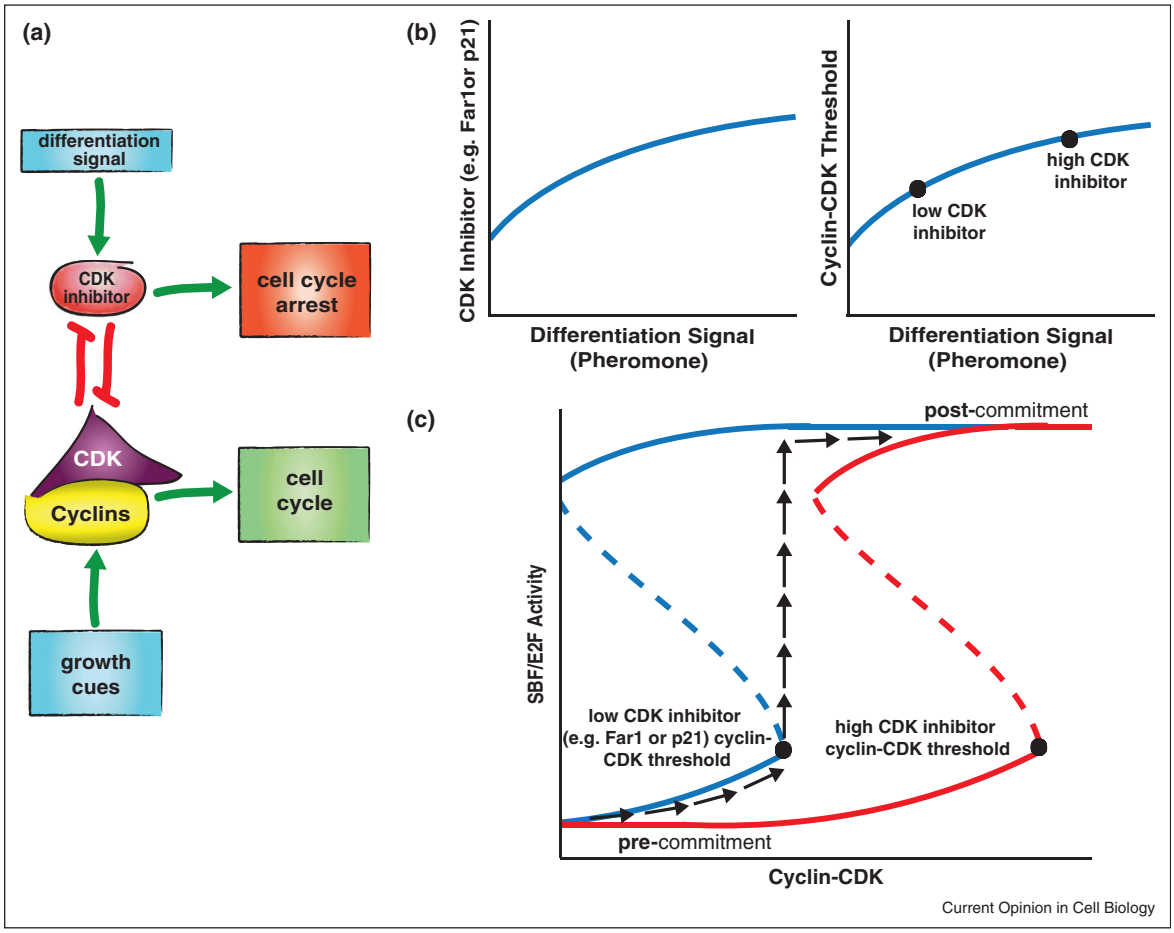
homology. For example, despite a lack of sequence similarity, Whi5 and Rb act as inhibitors of cell cycle progression through recruitment of histone deacetylases and are directly inhibited by CDK activity [13,27]. Similarly, SBF shares no sequence homology with E2F while performing an analogous function. In addition, known homologs such as CDK1 appear to have taken on a new role in G1 control in yeast while performing mitotic functions in mammals, which use CDKs 2, 4, and 6 to regulate cell cycle entry [28]. While network structure and the physiological function of integrating multiple signals into a decision to divide are conserved, there has been significant evolution of G1 control and direct inferences from one organism to another should be carefully considered. Nonetheless, the high degree of network similarity suggests that similar molecular mechanisms might be used to generate appropriate input–output relationships governing physiological function.

In both mammals and yeast, input signals of diverse origin increase CDK activity to reach a threshold level driving commitment. In this model, when the transcriptional inhibitor Whi5 or Rb is sufficiently inactivated, an all-or-none response occurs that prevents reversion to a precommitted state [29–31]. Such an all-or-none response, where a higher level of activation is required to initiate commitment than to maintain the downstream state, suggests that the underlying regulatory system undergoes a saddle-node bifurcation (Figure 2) [32]. During such a transition, the system is initially bistable, but the low-CDK state is destabilized by an increasing input signal leading to rapid cell cycle entry [30]. Loss of bistability via saddle-node bifurcation is encountered at other irreversible cellular transitions, such as oocyte maturation in *Xenopus laevis*, where progesterone treatment of cells above a threshold promotes meiosis [33]. The transition from interphase to meiosis is established through ultrasensitivity of the MAP kinase cascade within a positive feedback loop that drives maturation [34]. Thus, cells may employ bistable regulatory networks based on positive feedback to generate all-or-none threshold responses [35–37].

Redefining commitment with single-cell analysis

Our general framework for viewing commitment is that input signals raise cyclin-CDK levels until they traverse a threshold beyond which positive feedback becomes self-sustaining. However, additional pathways can act to modulate this threshold (Figure 1) [38]. In *Xenopus*, the meiotic progesterone threshold can be tuned by GSK-3β activity [34]. Threshold tuning has also been observed in *Xenopus* mitosis, where inhibitory phosphorylation of CDK1 must be overcome by Cdc25 phosphatase activity to permit mitotic entry. DNA checkpoint signaling that

Figure 2

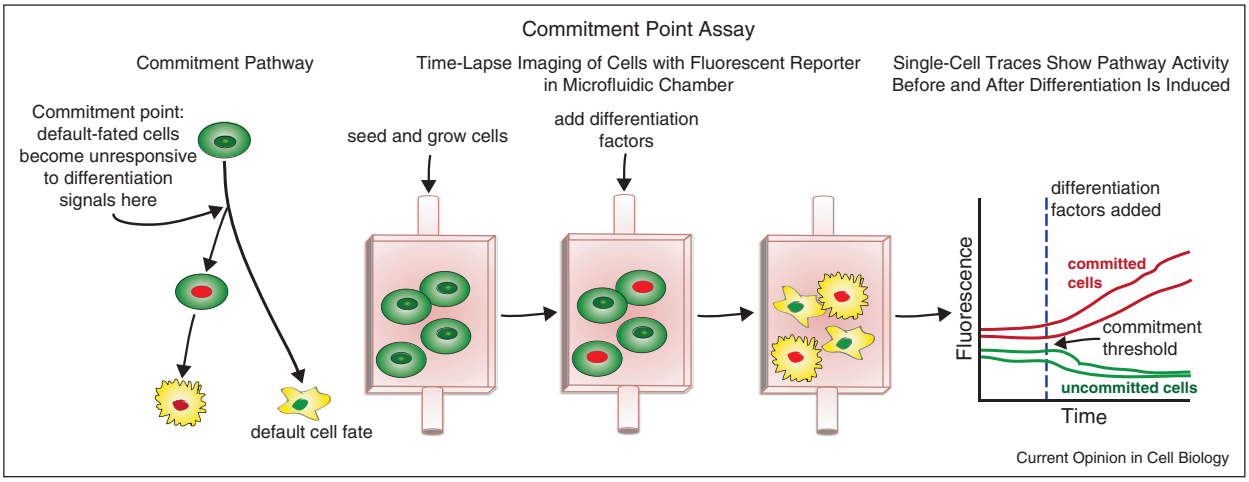


(a) Simplified scheme of the decision-making network regulating *commitment to cell division*. **(b)** Increased duration of a differentiation signal such as α-factor leads to the accumulation of CDK inhibitors such as Far1, which increases the amount of cyclin-CDK required to drive cells into the division cycle. In yeast, increasing Far1 reflects the corresponding temporal integration of pheromone pathway activity. **(c)** Cell cycle commitment is a bistable process. Once a threshold level of cyclin-CDK is reached, cells rapidly transition to the committed state. The black arrows illustrate one potential path. The dotted line represents an unstable intermediate state. For example, in yeast, increased Far1 concentrations favor the low SBF activity state and shift the curve toward the right indicating that higher cyclin-CDK concentrations are required to commit a cell to division.

opposes Cdc25 activity raises the threshold concentration of cyclin B required to progress into mitosis [39]. Thus, traversing a tunable threshold is a common theme among switch-like transitions.

The measurement of commitment thresholds has been greatly improved by recent technical advances. More accurate measurements are providing mechanistic insight into how these thresholds are tuned. While population-based studies have poor threshold resolution due to imperfect cell cycle synchrony, the combination of time-lapse imaging with microfluidic devices, which precisely control the extracellular environment, has become a powerful tool for quantitatively measuring commitment thresholds [40]. This combination of microfluidics and imaging has been successfully applied to yeast *Start*. Traversal of *Start* is driven by CDK activity, which can be measured in live cells via the CDK-dependent nuclear export of the transcriptional inhibitor Whi5 [29,41]. *Start* corresponds precisely to the abrupt activation of the G1 cyclin-positive feedback loop, which occurs when about 50% of a cell's Whi5 has left the nucleus [42•]. There is surprisingly little cell-to-cell variation in the threshold, as the percentage of nuclear Whi5 at the time of pheromone addition predicts cell fate with 97% accuracy. Interestingly, the threshold level of Whi5 is tuned by the addition of mating pheromone, and it was found that the threshold for exiting a pheromone-arrested state is higher (~65% of nuclear Whi5 must be exported). This study illustrates the potential for using microfluidic platforms to accurately determine thresholds in a variety of physiological contexts [43].

Figure 3



General experimental scheme for using time-lapse imaging, fluorescent reporters, and microfluidics to analyze commitment within a predictive framework. For pathways that employ irreversible transitions, following a cell before and after the transition allows the determination of molecular events committing a cell to a downstream fate if a threshold can be determined using a fluorescent reporter (see text).

Time-lapse imaging with fluorescent reporters has not yet been applied to the restriction point where there are many positive feedback loops and other signaling events that could be causal for commitment [6••,44•,45]. In mammalian cells, low levels of E2F1-3 activate expression of E2F1, forming a positive feedback loop that inactivates its key negative regulator Rb [30,46]. The E2F target cyclin E binds CDK2 to further phosphorylate and complete inactivation of Rb [26,47•], producing a second positive feedback loop. A third positive feedback loop involves the E2F target Skp2, the F-box protein specifically targeting the CDK inhibitor p27 for degradation [48–50]. While these positive feedback loops may contribute to the commitment process, recent studies using single-cell time-lapse imaging of cycling normal human fibroblasts concluded that the mammalian restriction point occurs 4–6 hours before significantly detectable Rb phosphorylation, suggesting that upstream events could define commitment [24,45,51,52]. Moreover, studies in transformed cells reveal a lack of proper restriction point control in response to serum removal [51,53,54]. Much like in yeast, single-cell time-lapse imaging experiments using live cell reporters in primary mammalian cells may precisely pinpoint the molecular events of the restriction point and hopefully resolve the controversy surrounding commitment timing.

Commitment regulation may be modified in distinct cell types and we currently lack understanding of restriction point control within a growing animal tissue. For example, stem cell division control may be distinct because these cells do not rely on a MAPK-dependent mitogenic pathway to enter the cell cycle, and they exhibit a short G1 phase before differentiation (Figure 3) [55–58]. We know what markers are expressed in differentiated cells, but we do not yet understand both the causal molecular mechanism and when exactly the decision to differentiate is made [59••]. By reconstructing the physiological environment of stem cells using microfluidics and studying activation timing of the molecular pathways underlying differentiation, one might be able to generate a predictive framework for how these cells make decisions.

Prediction is essential to analyzing cell cycle commitment because it provides a quantitative measure of how well a cellular decision is understood. In this context, commitment points can be analyzed by exposing cells to an abrupt step change in their extracellular environment and then observing the outcome of a binary cellular decision, such as differentiation into two distinct cell types or the distinction between cells committed to division and those responsive to growth factor signaling (Figure 3). It is important to only use information gathered before the step change to infer the cellular state because allowing the use of subsequent downstream information would produce trivial predictions: for example, the expression of any of 1000 differentiation-regulated genes predicts cell fate with 100% accuracy. However, expression of these proteins will not be highly predictive of the decision because they occur downstream of the commitment point. Nevertheless, such trivial predictions are the current state-of-the-art in the stem cell field indicating that the adoption of a predictive framework would represent a large methodological step forward because the most informative measurements will reveal the core of the cell's decision-making network.

Temporal order of transcription within the G1-to-S transition

In both mammalian and yeast cells, the temporal order of transcription within the G1-to-S transition has been analyzed [60•]. Genes involved in positive feedback loops are transcribed before other genes regulated by the same transcription factor. In yeast, the activation of *CLN1* and *CLN2* precedes other SBF and MBF regulated genes. In mammalian cells, the positive feedback components cyclin E1, cyclin E2, Skp2, and E2F1 are all among the earliest transcribed E2F targets at the G1-to-S transition. Notably, the MBF target *NRM1* that inactivates MBF [61] is activated much later than other G1-S genes ensuring enough time for regulon expression before its inactivation. That commitment precedes activation of the 5–10% of the genome that is cell cycle regulated in both yeast and mammals suggests the principle that cells tend to make a decision before synthesizing machinery associated with the downstream cell fate.

Time integration of signals in a decision-making process

Accurate cellular decisions may be complicated by the fact that within a population of genetically identical cells grown in identical environments, there is substantial cell-to-cell variation in protein concentration. To overcome molecular noise, cells might base their decisions not only on the current strength of an input signal, but on its history [62•]. For example, by taking the integral of the signal, cells could formulate a response robust to fluctuations that are removed by time averaging. Integrated responses may be important for p53-dependent regulation in mammalian cells, where p53 may be spontaneously and transiently activated under unstressed conditions [63]. Cells respond differently to transient DNA damage that occurs during normal growth than to damage requiring a full apoptotic response. p53 targets that induce cell cycle arrest, such as p21, respond to persistent but not transient p53 activity perhaps through a temporal integration mechanism yet to be elucidated.

The temporal integration of pathway activity has also been observed in pathways governing cell cycle progression in both yeast and mammals. In human mammary epithelial cells, EGF stimulation leads to the activation of discrete, asynchronous ERK pulses, which increase in frequency with increased concentrations of EGF [64••]. That proliferation is proportional to the frequency of ERK pulses suggests that a temporal integration of ERK activity is the relevant input for cell cycle progression [44•,50]. In yeast, temporal integration of a signal results in the accumulation of a cell cycle inhibitor rather than an activator. Pheromone activates MAP kinase signaling that arrests the cell cycle by activating transcription of the CDK inhibitor Far1 [65•]. The amount of Far1 reflects a temporal integration of pheromone pathway activity, and this corresponds to the amount of cyclin-CDK needed to drive cells into the cell cycle. A similar method of measuring signal dynamics may be employed for the differentiation response in mammalian cells, where the accumulation of cell cycle inhibitors may result from the temporal integration of differentiation signals [66,67].

Conclusion

Cell cycle commitment is an important process, and knowledge of the underlying regulatory networks has been crucial for beginning to uncover the principles governing cellular decisions. Recent advances in single-cell studies have allowed unprecedented temporal resolution of the molecular mechanisms of *Start* in yeast. Since the Whi5-SBF/MBF-Cln1/2 pathway bears remarkable similarity to the Rb-E2F-cyclin E/A mammalian pathway, we expect that some of the principles underlying commitment in yeast can be usefully applied to mammals. The next few years should be an exciting period for defining how molecular events establish commitment across eukaryotes.

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