

Origin of Bistability Underlying Mammalian Cell Cycle Entry

Guang Yao^{1,2†}, Cheemeng Tan^{3§}, Mike West^{2,4}, Joseph R. Nevins^{2,5} and Lingchong You^{2,3,6}

¹*Department of Molecular & Cellular Biology, University of Arizona, Tucson, AZ 85721, USA.*

²*Institute for Genome Sciences and Policy, Duke University, Durham, NC 27708, USA.*

³*Department of Biomedical Engineering, Duke University, Durham, NC 27708, USA.*

⁴*Department of Statistical Science, Duke University, Durham, NC 27708, USA.*

⁵*Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710, USA.*

⁶*Duke Center for Systems Biology, Durham, NC 27708, USA*

§ Current address: Ray and Stephanie Lane Center for Computational Biology, Carnegie Mellon University, Pittsburgh, PA 15213

Running title: *Robust design of RB-E2F switch*

Subject Category: *Cell cycle*

Character count: *39,250*

† Correspondence should be addressed to: G.Y. (guangyao@arizona.edu)

Abstract

Precise control of cell proliferation is fundamental to tissue homeostasis and differentiation. The cellular commitment to proliferation occurs at the restriction point (R-point). It has long been recognized that the R-point is tightly regulated by the Rb-E2F signaling pathway. Our recent work has further demonstrated that this regulation is mediated by a bistable switch mechanism. Nevertheless, the essential regulatory features in the Rb-E2F pathway that create this switching property have not been defined. Here we analyzed a library of gene circuits comprising all possible link combinations in a simplified Rb-E2F network. We identified a minimal circuit that is able to generate robust, resettable bistability. This minimal circuit contains a coupled feed-forward regulation and a mutual-inhibition feedback loop, which forms an AND-gate control of the E2F activation. Underscoring its importance, experimental disruption of this circuit was found to abolish E2F bistability. Our findings suggested basic design principles for the robust control of the bistable cell cycle entry at the R-point.

Keywords:

Bistable switch / Cell cycle checkpoint / Design principle / Rb-E2F pathway / Robustness

Introduction

The Rb-E2F pathway regulates the initiation of DNA replication and plays a critical role in cell proliferation (Attwooll et al, 2004; Bartek et al, 1996; Dyson, 1998; La Thangue, 1994; Nevins, 1992; Trimarchi & Lees, 2002; Weinberg, 1995). Its importance is further corroborated by findings that the RB-E2F pathway is deregulated in almost all cancer cells (Nevins, 2001; Weinberg, 2007). Recently, we have demonstrated experimentally that the Rb-E2F pathway controls the R-point by functioning as a bistable switch (Yao *et al*, 2008). This switch converts graded growth signals into an all-or-none activation of E2F. Once switched ON by strong growth stimulation, the hysteretic E2F activity remains ON even when the growth stimulation is diminished. This long-lasting E2F activity drives the completion of the cell cycle after passing the R-point (Yao *et al*, 2008).

Interestingly, the Rb-E2F bistable switch is resettable. That is, as far as the steady-state is concerned, the final E2F level eventually returns to a monostable OFF-state under a low serum condition (Yao et al, 2008). This resettable property is intrinsic to the Rb-E2F network and does not require downstream regulatory activities (Figure S1, also see Supplementary Discussion). This resettable property of the Rb-E2F bistable switch is a steady-state property, which should not be confused with the temporal irreversibility of cell cycle progression after the R-point. Resettable bistable switches, by creating a wide hysteresis loop, can drive irreversible cellular processes in the temporal domain, as demonstrated previously on mitotic entry in frog eggs (Pomerening et al, 2003; Sha et al,

2003). The resettable bistability of the Rb-E2F switch provides tight control of cell proliferation in response to growth signals, which is disrupted by oncogenic lesions such as ablation of Rb family proteins (Dannenberg *et al*, 2000).

The resettable bistability of the Rb-E2F switch provides a mechanistic explanation of the R-point control of normal cell proliferation and quiescence (Yao *et al*, 2008). However, how does the Rb-E2F pathway generate such a switching property? Finding the answer could help us to better understand the control mechanism underlying cell cycle entry, which is likely conserved across various cell types while disrupted in most, if not all, cancer cells. To this end, mathematical models have been created to examine potential bistable mechanisms underlying the R-point control (Aguda & Tang, 1999; Hatzimanikatis *et al*, 1999; Novak & Tyson, 2004; Qu *et al*, 2003; Thron, 1997; Yao *et al*, 2008). However, these studies have not attempted to examine the essential regulatory features that create resettable bistability in mammalian cell cycle entry.

In this study, we modeled and identified the basic gene circuit underlying resettable Rb-E2F bistable switch by the criterion of robustness. Robustness is a property of a system to maintain its functionality against internal or external perturbations, such as cellular noise, genetic variation, and environmental changes (Kitano, 2004; Stelling *et al*, 2004). Biological systems often exhibit robustness as a fundamental characteristic (Alon *et al*, 1999; Barkai & Leibler, 1997; Eldar *et al*, 2002; Feinerman *et al*, 2008; Krantz *et al*, 2009; Little *et al*, 1999; Rao *et al*, 2004; von Dassow *et al*, 2000; Wang *et al*, 2008). The Rb-E2F bistable switch is also robustly observed under different cell culture conditions

(Yao *et al*, 2008), consistent with its critical roles for the R-point control. Therefore, our starting hypothesis is that if we analyze all possible gene circuit designs in the Rb-E2F network, the most robust one to generate resettable bistability likely underlies the origin of this switching property in the cell.

By modeling all 768 possible gene circuits derived from a simplified Rb-E2F network, we identified a minimal circuit that is able to generate robust, resettable bistability. Consisting of a coupled positive feedback loop and a feedforward motif, the circuit exhibits resettable bistability against a wide range of parametric and structural perturbations. Its unique characteristics reveal basic design features of the Rb-E2F bistable switch, which is commonly disrupted in cancer development.

Results and Discussions

Model simplification and construction

The Rb-E2F pathway is a complex signaling network (Figure 1A) consisting of intertwined transcriptional controls, kinase cascades, and microRNA regulations (Blagosklonny & Pardee, 2002; Sears & Nevins, 2002). To formulate a mathematically tractable system, we coarse-grained the system to reduce network complexity while keeping its essential regulatory features. To this end, we combined redundant and overlapping regulatory activities and collapsed linear signaling cascades (Figure 1). For example, all E2F activators (E2F1, 2, 3) were combined into one node EE, and all Rb family proteins (Rb, p107, p130) were combined into another node RP. The linear

signaling cascade consisting of Ras, Myc and CycD/cdk4,6 was collapsed into the node MD; the cascade consisting of E2F and CycE/cdk2 was collapsed into the node EE. Overlapping links between collapsed network modules were also combined. For example, the two original activations links (from Myc to E2F1, 2, 3, and from Myc to cdc25A and CycE/cdk2) were combined into one link #7 (from MD to EE); the two mutual-inhibition loops (between Rb family proteins and E2F activators-CycE/cdk2, and between p27 and CycE/cdk2) were combined into one mutual-inhibition loop between RP and EE (5-6) (Figure 1). This coarse-graining removed intermediate signaling steps, which could reduce accuracy of simulated temporal dynamics. It would not, however, significantly affect steady-state properties of the system (as seen in (Ma *et al*, 2006), also see “Time scale” in Methods), which makes this coarse-graining suitable for our investigation of the bistability of the Rb-E2F switch (Figure 1C).

The coarse-graining resulted in a simplified network containing three nodes and ten links (Figure 1B & Table S1). Each node represents the combined (post)transcriptional and (post)translational activities of the corresponding signaling subnetwork (grey-shaded in Figure 1A); each link represents the lumped regulatory interactions between the two neighboring nodes. The choice of the three-node simplification was mainly dictated by the network structure of the Rb-E2F pathway, especially the locations at which network modules can be collapsed (containing linear links). In addition, the three-node simplification (one input node, one output node, and one regulatory node) achieved a balance between the need to reduce network complexity

and to keep its essential regulatory properties – as noted similarly in a recent study on adaptation networks (Ma *et al*, 2009).

Next, we constructed a library of 768 mathematical models that covered all possible link combinations in the simplified three-node Rb-E2F network (see Methods). We also generated 20,000 parameter sets by randomly sampling parameter values within biologically feasible ranges (see Methods). These random parameter sets served as surrogates of varying cellular activities across different cell types and cellular environments. We note that 20,000 parameter sets were sufficient to obtain convergent rankings of model robustness in our studies (Table S2). Here, we measure robustness by the proportion of parameter sets that allow a circuit to generate the desired switching characteristics.

The most robust bistable model consists of all four positive feedback loops

As discussed earlier, the Rb-E2F switch exhibits two salient characteristics: bistability (conditions #1 & 2, Figure 2A inset) and resettability (condition #3, Figure 2A inset). First, we identified regulatory functions that underlie robust bistability. As the exact dynamics of E2F activation could vary among different cell types and growth conditions, we focused on qualitative aspects of bistability, and did not impose specific constraints on the location, width, and amplitude of bistable regions. That said, we tested and confirmed that applying these constraints (e.g., width of the bistable region, Table S3) did not affect the model predictions. Each of the 768 gene circuits was analyzed against

the same collection of 20,000 parameter sets. Many circuits were found able to generate bistability (Figure 2B & Table S4A).

We found that the most robust circuit to generate bistability was *2-3-5-6-7-9b* (Figure 2C; note that the input link #1 was omitted when referring to all gene circuits). This circuit contains all the four positive feedback loops in the simplified Rb-E2F network (Figure 1B, dashed box). This result is consistent with the notion that involving multiple positive feedback loops could boost robustness of bistable systems (Ferrell, 2008).

The gene circuit *2-3-5-6-7-9b* generated bistability in 12,907 of the 20,000 random parameter sets (Table S4A). However, only 10 of these 12,907 parameter sets required all the six links of *2-3-5-6-7-9b* to create bistability. For the other parameter sets, certain sub-circuits (e.g., *3-5-6*, *2-3-6*, *2-7*, *7-9b*) were already sufficient (data not shown). Therefore, links in the circuit *2-3-5-6-7-9b* were not equally important for bistability.

Top minimal models for bistability consists of OR/NOR-gate type positive feedbacks

To better understand the basic control mechanism underlying the Rb-E2F bistable switch, we searched for “minimal models”. Given each of the 20,000 parameter sets, we identified corresponding minimal models that fulfill two conditions: (a) they are able to generate bistability; and (b) they do not contain non-essential links for such property. That is, if a circuit is a minimal model for bistability, all of its included links would be essential, and none of its sub-circuits would generate bistability given the same parameter

set. There could be multiple non-overlapping minimal models for a given parameter set (see Table S5 for an example). In addition, minimal models obtained from one parameter set could be different from those obtained from another parameter set.

According to these criteria, the most robust minimal model in generating bistability was 3-5-6. This circuit contains a positive feedback loop between *EE* and *RP* (Figure 2D), and functions as a minimal model in generating bistability at 8,351 of the 20,000 random parameter sets (Table S4B). In addition to 3-5-6, three other minimal models (2-3-6, 2-7, 7-9b) were similarly robust in generating bistability. All these four minimal models share one common feature – containing an OR/NOR-gate type positive feedback loop (Figure 2D). In contrast, the robustness of other minimal models in generating bistability dropped at least 3-fold (Figure 2D & Table S4B).

Top minimal models for *resettable* bistability consists of AND-gate type positive feedbacks

We next investigated whether the switching dynamics of the four minimal models identified above were resettable. That is, when the growth input is removed completely, the steady state of the system output *EE* should return to the OFF state (and the cellular state resets to quiescence, see constraint #3 in Figure 2A inset). The most robust minimal model for bistability, 3-5-6, performed poorly with this criterion. Among the 8,351 bistable switches (out of the 20,000 parameter sets) generated with this circuit, only 12.4% were resettable. Even a smaller percentage of bistable switches generated by each

of the other three minimal models above were resettable: 11.8% for 2-3-6; 6.3% for 2-7, and 3.4% for 7-9b.

We found that much fewer model choices in the Rb-E2F network were able to robustly generate resettable bistability (comparing Table S6A to S4A). Of these, 3-5-6-7 was the most robust minimal model (Figure 2E). In fact, the top eight models to generate resettable bistability were all built on this minimal model 3-5-6-7 (Figure S2 and Table S6A). The gene circuit 3-5-6-7 contains a coupled positive feedback and feedforward regulation, which forms an AND-gate control (between the link #7 and the positive feedback loop 5-6) of the system output *EE*. The second most robust minimal model to generate resettable bistability was 7-9a, which also contains an AND-gate control of *EE* (between the link #7 and the positive feedback loop #9a, Figure 2E). This gene circuit 7-9a, however, was about two-fold less robust than 3-5-6-7 in creating resettable bistability (Figure 2E and Table S6B).

Basic design principles underlie a robust Rb-E2F switch

Previous studies have demonstrated that positive feedback is a necessary condition for bistability (Ferrell, 2002; Gardner *et al*, 2000; Ozbudak *et al*, 2004; Tan *et al*, 2009). The simplified Rb-E2F network contains four positive feedback circuits, 2-7, 2-3-6, 5-6, and 9 (9a/dominant link or 9b/additive link, Table S1) (Figure 2C). Only one of the positive-feedback-based circuits (3-5-6-7), however, was robust in generating resettable bistability. What is unique about this circuit?

First, we note that an AND-gate type positive feedback loop is critical for resettable bistability. Although all OR/NOR-gate based positive feedback circuits in the Rb-E2F network could robustly create bistability (Figure 2D), most of the resulting switches were non-resettable. This poor resettability was due to the lack of dominant control of system output EE by the input S . For example, in circuits 2-3-6 and 2-7 (Figure 2D), the OR-gate regulation of MD is described by (also see Methods):

$$\frac{d[MD]}{dt} = \frac{1}{\tau_{MD}} \left(\left(\frac{[S]^{n_1}}{K_1^{n_1} + [S]^{n_1}} + \beta_2 \frac{[EE]^{n_2}}{K_2^{n_2} + [EE]^{n_2}} \right) - [MD] \right)$$

When β_2 is sufficiently large, once the positive feedback was established and EE reached a high level, removal of S would not shut OFF MD for the majority of parameter space. This would result in non-resettable bistability in EE . For this reason, we found that the most robust circuits for resettable bistable switches were all derived from the two AND-gate type minimal models in the Rb-E2F network, 3-5-6-7 and 7-9a (Figure S2). In these two models, the system output EE is under an AND-gate regulation involving the dominant control of EE by the system input S , which facilitates the resettability of the generated bistable switches.

Second, we note that a mutual-inhibition-type positive feedback loop is critical for creating robust, resettable bistability. This requirement is evident in the comparison between the gene circuits 3-5-6-7 and 7-9a. Both containing an AND-gate regulation of the system output EE (between the link #7 and a positive feedback loop), the circuit 3-5-6-7 was much more robust than 7-9a in creating resettable bistability (Figure 2E). In

the model 7-9a, a positive feedback was created via *EE* auto-activation (link #9), and the activation rate of *EE* can be described as:

$$\frac{d[EE]}{dt} = \frac{1}{\tau_{EE}} \left(\frac{[MD]^{n7}}{K_7^{n7} + [MD]^{n7}} \cdot \frac{[EE]^{n9}}{K_9^{n9} + [EE]^{n9}} - [EE] \right).$$

In this case, the *EE* activation was dependent on a high level of *EE* itself, which however cannot reach a high level without first being activated. This dilemma limited the model robustness.

In comparison, the positive feedback in the model 3-5-6-7 was mediated by mutual inhibition (5-6), and the activation rate of *EE* can be described as:

$$\frac{d[EE]}{dt} = \frac{1}{\tau_{EE}} \left(\frac{[MD]^{n7}}{K_7^{n7} + [MD]^{n7}} \cdot \frac{K_6^{n6}}{K_6^{n6} + [RP]^{n6}} - [EE] \right).$$

In this case, the *EE* activation can be easily achieved by reducing the level of *RP* through the link #3, which is one arm of the feedforward motif (Figure 2E). Interestingly, this mutual-inhibition circuit structure (5-6) appears to be conserved in the regulatory network of the yeast *Start* point, where the same circuitry is implemented with non-homologous genes to generate a similar bistable switch (Charvin *et al*, 2010). This commonality makes sense given that this mutual-inhibition circuit provides a robust mechanism in generating the desired bistability for the proper control of cell cycle entry.

Altogether, our analysis suggested that the unique circuit design of 3-5-6-7, containing both a mutual-inhibition type positive feedback and an AND-gate control of the system output *EE*, was responsible primarily for generating a robust, resettable Rb-E2F bistable switch.

Disrupting the gene circuit 3-5-6-7 abolishes bistability

If the circuit 3-5-6-7 primarily accounts for the resettable bistability in the Rb-E2F signaling network, disruption of it would likely abolish the corresponding switching property. We chose to experimentally perturb this circuit by targeting the Cyclin E/Cdk2 activity (node *EE*, Figure 3A). This would block the mutual-inhibition feedback loop 5-6, which was predicted in above analysis to be essential for the Rb-E2F bistable switch.

In our experiment, we first fully turned ON the Rb-E2F bistable switch in cells. To this end, serum-starved, quiescent E2F-d2GFP reporter cells (see Methods) were stimulated with a strong serum pulse (20% serum for 5 hours, Figure 3B) that was sufficient to activate the Rb-E2F bistable switch in the majority of cell population (Yao *et al*, 2008). Cells were then switched to a low-serum medium (0.3% serum). Because of the hysteresis associated with the Rb-E2F switch, E2F continued to accumulate even in the absence of strong serum stimulation, and reached its ON-state in most cells by the 20th hour (Figure 3C).

We then tested whether disruption of the mutual-inhibition feedback loop 5-6 by inhibiting the Cyclin E/Cdk2 activity would affect E2F bistability. To this end, we utilized a small chemical inhibitor of Cdk2 (CVT-313, see Methods). This inhibitor can specifically block the cyclin-dependent kinase activity of Cdk2 (with an IC₅₀ of 430-fold lower than that for Cdk4) (Brooks *et al*, 1997), and thus reduce the strength of the link #5

(Figure 3A). Increasing doses of Cdk2 inhibitor CVT-313 were added to the culture at the 20th hour, after E2F reached the ON-state (Figure 3B). The maximum dose (5 μ M) of CVT-313 applied in this experiment was still subtoxic to the E2F-d2GFP cells; it did not fully arrest cell proliferation even after three days under otherwise normal growth condition (data not shown).

In the absence of the Cdk2 inhibitor, E2F level at the 43rd hour still maintained at the ON-state in the low-serum medium in most cells (isolated from downstream regulations by synchronizing at G2/M with nocodazole) (Figure 3C). This result demonstrates the strong intrinsic bistability of the Rb-E2F switch with intact mutual-inhibition loop 5-6. With increasing doses of the Cdk2 inhibitor, E2F level in fewer and fewer cells could still maintain at the ON-state under the same low-serum condition (Figure 3C). With 5 μ M of the Cdk2 inhibitor, E2F level in the majority of cell population returned to the OFF-state. A similar result was also observed in cells treated with different serum-pulse stimulation but the same Cdk2 inhibition (Figure S4). These results indicate that the disruption of the mutual-inhibition loop 5-6 by the Cdk2 inhibitor can indeed abolish the E2F bistability.

Our results are consistent with the model prediction that the gene circuit 3-5-6-7 is essential for a functional Rb-E2F bistable switch. That is, when the mutual-inhibition loop 5-6 was blocked by inhibiting the Cyclin E/Cdk2 activity, although the other positive feedback loops (2-7, 2-3-6, and 9, Figure 3A) were still present in the Rb-E2F network, they were not sufficient to sustain the E2F-ON state in the absence of growth

stimulation. Our study also suggests that the Cyclin E/Cdk2 activity, instead of being required for a cell to reach the R-point (Ekholm *et al*, 2001; Martinsson *et al*, 2005), is necessary for maintaining the activated Rb-E2F bistable switch (and thus, irreversible cell cycle progression after the R-point). In addition, our experimental results support the notion that the Rb-E2F bistable switch is intrinsically resettable. That is, besides inhibiting Cyclin E/Cdk2, the Cdk2 inhibitor also blocked the Cyclin A/Cdk2 negative feedback on E2F (20th-43rd hour, Figure 3B) (Brooks *et al*, 1997). As a consequence of without the Cyclin A negative feedback, the disruption of the mutual-inhibition loop 5-6 alone was sufficient to reset the E2F level to the OFF-state.

The circuit 3-5-6-7 exhibits high structural flexibility

Lastly, we found that the circuit 3-5-6-7 exhibited high structural flexibility in generating a resettable Rb-E2F bistable switch. Structural flexibility refers to the property of a system to maintain its functionality despite alterations in its network topology (Kitano, 2004; Stelling *et al*, 2004). Here we defined topological neighbors of a gene circuit as those that can be obtained by one link addition or removal (Figure 4A). We then estimated the structural flexibility of a given gene circuit by the average robustness of its topological neighbors in creating a resettable Rb-E2F bistable switch.

When we ranked the 768 gene circuits derived from the Rb-E2F network by their calculated structure flexibility (Table S7), we found that there existed a tight correlation ($\rho = 0.87$) between the structure flexibility of a given gene circuit to its parameter

flexibility, i.e, its robustness against parameter perturbations (Figure 4B). This result makes intuitive sense, as certain structure alterations can be considered as extreme forms of parametric perturbations: e.g., removing an activation link from an AND-gate is equivalent to setting the corresponding rate constant K to 0 (Table 1).

We found that the minimal model 3-5-6-7 and its derivatives held all the top ranks in structure flexibility (Figure 4C). In fact, gene circuits with one- or two-link variations from the minimal circuit 3-5-6-7 formed an interconnected network that was highly enriched for gene circuits with strong structure flexibility (Figure 4D, comparing the 3-5-6-7 vs. 7-9a based networks). This interconnected network of structurally flexible circuits could facilitate the system evolvability (de Visser et al, 2003; Kirschner & Gerhart, 1998; Little, 2010). That is, gene circuits responsible for the Rb-E2F switch could evolve continuously for new functionalities (with the additional or removal of regulatory links), while maintaining its essential property of resettable bistability. Although the specific evolutionary trajectory cannot be inferred from analyzing the formed Rb-E2F switch, the robustness of the system could aid in the initiation and maintenance of this desired property during evolution.

Conclusion

In this study, we sought to identify the basic gene circuit underlying the resettable Rb-E2F bistable switch, which controls mammalian cell cycle entry (Yao *et al*, 2008). To this end, we analyzed a library of mathematical models that covered all possible gene

circuit designs derived from a simplified Rb-E2F network. We identified one minimal gene circuit that was uniquely robust in generating resettable bistability. This minimal circuit 3-5-6-7 consists of a mutual-inhibition type positive feedback and a feedforward regulation, forming an AND-gate control of the system output. Our analysis suggested that the feedforward loop with an AND-gate is critical for the resettability of the generated bistable switch, and the mutual-inhibition feedback loop is critical for parametric robustness of bistability. Consistently, constitutive Myc expression, which insulates the feedforward loop from the extracellular growth signals, was found to inhibit cell cycle exit and cell differentiation (Bogenmann *et al*, 1995; Foley *et al*, 1998); targeted disruption of the mutual-inhibition feedback loop was found to abolish the bistability in the Rb-E2F network (Figure 3). This unique circuit topology of 3-5-6-7 also contributed to its strong structure flexibility in creating a resettable bistable switch, which could facilitate the system evolvability. Altogether, our study suggested that the gene circuit 3-5-6-7 primarily accounts for the origin of the resettable bistability in the Rb-E2F network for the proper R-point control. Consistent with this notion, the gene circuit 3-5-6-7 appears targeted and disrupted by frequent mutations in human cancers (e.g., loss of function of Rb and p16, and over-expression of CycD/cdk4, Figure S3).

We identified the basic regulatory features underlying the resettable Rb-E2F bistable switch by the criterion of robustness. Biological systems, however, need to balance between robustness and other desired functionalities. In fact, the complete Rb-E2F network (Figure 1B) did not exhibit the highest robustness in creating resettable

bistability. We suggest that the minimal gene circuit 3-5-6-7 defines the basic qualitative property of the Rb-E2F bistable switch. Additional regulatory activities could then allow quantitative fine-tuning of the bistability (e.g., the precise location, width, amplitude of its bistable region) (O'Donnell et al, 2005; Pickering et al, 2009; Qian & Cooper, 2008), the timing of its switching dynamics (Lee et al, 2010), or the introduction of other types of dynamic responses (Wong et al, 2011). These regulatory links could be systematically optimized to balance between precision and robustness, to create switching characteristics for the proper control of cell cycle entry in different cell types adapted in their specific growth environments.

Methods

The ODE model library. Our simplified Rb-E2F network contains three nodes and ten regulatory links. All links were modeled using Michaelis-Menten type functions.

Nondimensionalization was carried out to reduce the number of required parameters as in (Ma *et al*, 2006) – see Table S10 for details. For example, considering a node A that is

regulated by a positive autoregulation, we have $\frac{d[A]}{dt} = \frac{k_A * [A]^n}{K_A^n + [A]^n} - \frac{\ln 2 * [A]}{\tau_A}$. Let

$\frac{\tau_A}{\ln 2} \Rightarrow \tau'_A$, $\frac{A}{k_A * \tau'_A} \Rightarrow A'$, and $\frac{K_A}{k_A * \tau'_A} \Rightarrow K'_A$, we get

$\frac{d[A']}{dt} = \frac{1}{\tau'_A} * \left(\frac{[A']^n}{K'_A^n + [A']^n} - [A'] \right)$. Similarly, all regulatory links have either the form

$A^n/(K^n + A^n)$ (positive regulation) or $K^n/(K^n + A^n)$ (negative regulation), which can be

described by two parameters (n and K) and one variable (A). Each link was modeled as

either a dominant or an additive link based on its characteristic as described in the

literature. Please refer to Table S1 for details.

To construct a model library comprising all link combinations in the simplified Rb-E2F network, each of the links #2~10 (between the node MD – receiver to the system input S , and the node EE – system output) could be either present or absent in a given model. In addition, link #9 had two possible forms when present, plus its absence state, resulting in a total of 3 states. Therefore, a total of $2^8 * 3 = 768$ models were generated.

See Table 1 for details of the models.

Parameters. A collection of 20,000 parameter sets was generated to test model robustness. Each parameter set contained a combination of 26 parameters: K_{I-10} , n_{I-10} , τ_{MD} , τ_{RP} , and τ_{EE} , β_{MD} , β_{RP} , and β_{EE} (Table 1). Parameters were uniformly sampled in log scale in the following ranges: K_{I-10} , 0.01–1; n_{I-10} , 1–10; τ_{MD} , τ_{RP} , and τ_{EE} , 0.2–20 (hr); β_{MD} , β_{RP} , and β_{EE} , 0.1–10. The parameter ranges were chosen based on previous modeling study on the Rb-E2F pathway (Yao *et al*, 2008). We also tested and confirmed that the choice of different parameter ranges did not affect the final result – e.g., when the range of K_{I-10} was further expanded 100 fold to 10^4 (0.001-10), the same minimal model was identified as the most robust circuit for resettable bistability in the Rb-E2F network (Table S8).

Time scale. To test whether the network coarse-graining would affect the steady-state bistable behavior of the system, we focused on a specific case where more realistically, contradicting signs of regulations could act at different time scales on a given node (i.e., links #7 and #8 acting on MD , and links #9 and #10 acting on EE). To this end, 20,000 parameters sets was generated as above, but each with two additional parameters (τ_{MD_i} and τ_{EE_i}) that correspond to the half-life of a linker node (MD_i or EE_i) introduced in the middle of link #8 and link #10, respectively (Table S12). Both added parameters were uniformly sampled in log scale in the range of 0.2–20 (hr), which created variable time delays in the dynamics of link #8 and link #10 (in comparison to link #7 and link #9, respectively, which bears a closer resemblance to the real dynamics in the cell). Model

simulations were repeated with this new collection of parameter sets, which we found resulted in the same order of top models for robust Rb-E2F bistable switch (Table S9).

Simulation. For each model with a given parameter set, the *EE* steady state $[EE]_{ss}$ was simulated from 25 growth input levels $[S]$ that were uniformly distributed in the log scale between 0.01-20 (percent of serum, covering the conditions from serum starvation to saturation) (Figure 2A inset). At each growth input level, simulations were carried out from two initial conditions, corresponding to the quiescence state (*EE*-OFF: $[EE] = [MD] = 0.01$, $[RP] = 11$) and the proliferation state (*EE*-ON: $[EE] = [MD] = 11$, $[RP] = 0.01$). We note that the maximum possible value for the ON-state of a node was 11 (Table 1), and we assumed that the corresponding OFF-state value was over 1,000-fold lower than the ON-state value. All simulations were carried out by using the deterministic LSODA method in the COPASI program (Hoops *et al*, 2006), with model simulation time of 1,000 hours to ensure the system to reach steady state.

Determination of bistability and resettability. Three criteria were applied to identify the desired switching property (Figure. 2A inset).

(1) Being a switch. $[EE]_{ss-max} - [EE]_{ss-min} > \lambda ([EE]_{ss-max} + [EE]_{ss-min})$ ($[EE]_{ss-max}$ and $[EE]_{ss-min}$ denote the maximum and minimum values of the *EE* steady state simulated from the initial condition *EE*-OFF, respectively; $\lambda = 0.1$). We have tested and confirmed that the value of the arbitrary threshold value of λ did not affect the final model prediction (data not shown).

(2) Being bistable. Let $\Delta[EE]_{ss} = [EE]_{ss(EE-ON)} - [EE]_{ss(EE-OFF)}$ as the difference between the two steady-state trajectories starting from the *EE-ON* and *EE-OFF* initial conditions. A model was considered bistable if $\Delta[EE]_{ss} \geq ([EE]_{ss-max} - [EE]_{ss-min}) * 0.1$ for at least two of the 25 input levels tested (except for the saturation level $[S]_{max} = 20$, where the system should be monostable), or $\Delta[EE]_{ss} \geq ([EE]_{ss-max} - [EE]_{ss-min}) * 0.2$ for at least one input levels above.

(3) Being resettable. $\Delta[EE]_{ss} \rightarrow 0$ when $[S] \rightarrow 0$.

A model was considered as a bistable switch if criteria 1 and 2 were met, and it was considered as a resettable bistable switch if all the three criteria were met.

Minimal models. At a given parameter set, when multiple models can generate the desired switching behavior, the minimal models were identified by the criterion of “link essentiality”. In a minimal model, if any link was removed, the desired switching property would not hold. In a non-minimal model, at least one link could be removed without disrupting the desired switching property. See Table S5 for an example.

GFP reporter cell line and Cdk2 inhibition. The E2F-d2GFP reporter cell line was derived from a single cell clone (#23) as described previously (Yao *et al*, 2008). Cells were regularly maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (No. 31053, Gibco/Invitrogen) supplemented with 10% of bovine growth serum (BGS, from Hyclone) and puromycin (1.0 $\mu\text{g/ml}$). In the Cdk2 inhibition experiment, cells were first synchronized at the quiescent state by serum starvation (0.02% BGS) for two days, then stimulated with high serum (20% BGS)-containing DMEM for 5 hours. Following the

serum-pulse, cells were washed twice with DMEM and switched to low-serum (0.3% BGS) condition. The Cdk2 inhibitor CVT-313 (#238803, EMD/CalBiochem, San Diego, CA) was added to the medium at the 20th hour at indicated concentrations. Between the 20th and 43rd hour, cells were synchronized at the G2/M phase by nocodazole (0.1 µg/ml). Cells were harvested at indicated time points and subjected to flow cytometry. Approximately 5,000 cells were assayed at each test condition.

References

- Aguda BD, Tang Y (1999) The kinetic origins of the restriction point in the mammalian cell cycle. *Cell Prolif* **32**: 321-335
- Alon U, Surette MG, Barkai N, Leibler S (1999) Robustness in bacterial chemotaxis. *Nature* **397**: 168-171
- Attwooll C, Lazzerini Denchi E, Helin K (2004) The E2F family: specific functions and overlapping interests. *EMBO J* **23**: 4709-4716
- Barkai N, Leibler S (1997) Robustness in simple biochemical networks. *Nature* **387**: 913-917
- Bartek J, Bartkova J, Lukas J (1996) The retinoblastoma protein pathway and the restriction point. *Curr Opin Cell Biol* **8**: 805-814
- Blagosklonny MV, Pardee AB (2002) The restriction point of the cell cycle. *Cell Cycle* **1**: 103-110
- Bogenmann E, Torres M, Matsushima H (1995) Constitutive N-myc gene expression inhibits trkA mediated neuronal differentiation. *Oncogene* **10**: 1915-1925
- Brooks EE, Gray NS, Joly A, Kerwar SS, Lum R, Mackman RL, Norman TC, Rosete J, Rowe M, Schow SR, Schultz PG, Wang X, Wick MM, Shiffman D (1997) CVT-313, a specific and potent inhibitor of CDK2 that prevents neointimal proliferation. *J Biol Chem* **272**: 29207-29211
- Charvin G, Oikonomou C, Siggia ED, Cross FR (2010) Origin of irreversibility of cell cycle start in budding yeast. *PLoS Biol* **8**: e1000284
- Dannenberg JH, van Rossum A, Schuijff L, te Riele H (2000) Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev* **14**: 3051-3064
- de Visser JA, Hermisson J, Wagner GP, Ancel Meyers L, Bagheri-Chaichian H, Blanchard JL, Chao L, Cheverud JM, Elena SF, Fontana W, Gibson G, Hansen TF, Krakauer D, Lewontin RC, Ofria C, Rice SH, von Dassow G, Wagner A, Whitlock MC (2003) Perspective: Evolution and detection of genetic robustness. *Evolution* **57**: 1959-1972

- Dyson N (1998) The regulation of E2F by pRB-family proteins. *Genes Dev* **12**: 2245-2262
- Ekholm SV, Zickert P, Reed SI, Zetterberg A (2001) Accumulation of cyclin E is not a prerequisite for passage through the restriction point. *Mol Cell Biol* **21**: 3256-3265
- Eldar A, Dorfman R, Weiss D, Ashe H, Shilo BZ, Barkai N (2002) Robustness of the BMP morphogen gradient in *Drosophila* embryonic patterning. *Nature* **419**: 304-308
- Feinerman O, Veiga J, Dorfman JR, Germain RN, Altan-Bonnet G (2008) Variability and robustness in T cell activation from regulated heterogeneity in protein levels. *Science* **321**: 1081-1084
- Ferrell JE, Jr. (2002) Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. *Curr Opin Cell Biol* **14**: 140-148
- Ferrell JE, Jr. (2008) Feedback regulation of opposing enzymes generates robust, all-or-none bistable responses. *Curr Biol* **18**: R244-245
- Foley KP, McArthur GA, Queva C, Hurlin PJ, Soriano P, Eisenman RN (1998) Targeted disruption of the MYC antagonist MAD1 inhibits cell cycle exit during granulocyte differentiation. *EMBO J* **17**: 774-785
- Gardner TS, Cantor CR, Collins JJ (2000) Construction of a genetic toggle switch in *Escherichia coli*. *Nature* **403**: 339-342
- Hatzimanikatis V, Lee KH, Bailey JE (1999) A mathematical description of regulation of the G1-S transition of the mammalian cell cycle. *Biotechnol Bioeng* **65**: 631-637
- Hoops S, Sahle S, Gauges R, Lee C, Pahle J, Simus N, Singhal M, Xu L, Mendes P, Kummer U (2006) COPASI--a COMplex PATHway SIMulator. *Bioinformatics* **22**: 3067-3074
- Kirschner M, Gerhart J (1998) Evolvability. *Proc Natl Acad Sci U S A* **95**: 8420-8427
- Kitano H (2004) Biological robustness. *Nat Rev Genet* **5**: 826-837
- Krantz M, Ahmadpour D, Ottosson LG, Warringer J, Waltermann C, Nordlander B, Klipp E, Blomberg A, Hohmann S, Kitano H (2009) Robustness and fragility in the yeast high osmolarity glycerol (HOG) signal-transduction pathway. *Mol Syst Biol* **5**: 281

La Thangue NB (1994) DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. *Trends Biochem Sci* **19**: 108-114

Lee T, Yao G, Bennett DC, Nevins JR, You L (2010) Stochastic E2F activation and reconciliation of phenomenological cell-cycle models. *PLoS Biol* **8**: e1000488

Little JW (2010) Evolution of complex gene regulatory circuits by addition of refinements. *Curr Biol* **20**: R724-734

Little JW, Shepley DP, Wert DW (1999) Robustness of a gene regulatory circuit. *EMBO J* **18**: 4299-4307

Ma W, Lai L, Ouyang Q, Tang C (2006) Robustness and modular design of the Drosophila segment polarity network. *Mol Syst Biol* **2**: 70

Ma W, Trusina A, El-Samad H, Lim WA, Tang C (2009) Defining network topologies that can achieve biochemical adaptation. *Cell* **138**: 760-773

Martinsson HS, Starborg M, Erlandsson F, Zetterberg A (2005) Single cell analysis of G1 check points-the relationship between the restriction point and phosphorylation of pRb. *Exp Cell Res* **305**: 383-391

Nevins JR (1992) E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* **258**: 424-429

Nevins JR (2001) The Rb/E2F pathway and cancer. *Hum Mol Genet* **10**: 699-703

Novak B, Tyson JJ (2004) A model for restriction point control of the mammalian cell cycle. *J Theor Biol* **230**: 563-579

O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT (2005) c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**: 839-843

Ozbudak EM, Thattai M, Lim HN, Shraiman BI, Van Oudenaarden A (2004) Multistability in the lactose utilization network of Escherichia coli. *Nature* **427**: 737-740

Pickering MT, Stadler BM, Kowalik TF (2009) miR-17 and miR-20a temper an E2F1-induced G1 checkpoint to regulate cell cycle progression. *Oncogene* **28**: 140-145

Pomerening JR, Sontag ED, Ferrell JE, Jr. (2003) Building a cell cycle oscillator: hysteresis and bistability in the activation of Cdc2. *Nat Cell Biol* **5**: 346-351

- Qian H, Cooper JA (2008) Temporal cooperativity and sensitivity amplification in biological signal transduction. *Biochemistry* **47**: 2211-2220
- Qu Z, MacLellan WR, Weiss JN (2003) Dynamics of the cell cycle: checkpoints, sizers, and timers. *Biophys J* **85**: 3600-3611
- Rao CV, Kirby JR, Arkin AP (2004) Design and diversity in bacterial chemotaxis: a comparative study in *Escherichia coli* and *Bacillus subtilis*. *PLoS Biol* **2**: E49
- Sears RC, Nevins JR (2002) Signaling networks that link cell proliferation and cell fate. *J Biol Chem* **277**: 11617-11620
- Sha W, Moore J, Chen K, Lassaletta AD, Yi CS, Tyson JJ, Sible JC. (2003) Hysteresis drives cell-cycle transitions in *Xenopus laevis* egg extracts.[see comment]. *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 100, pp. 975-980.
- Stelling J, Sauer U, Szallasi Z, Doyle FJ, 3rd, Doyle J (2004) Robustness of cellular functions. *Cell* **118**: 675-685
- Tan C, Marguet P, You L (2009) Emergent bistability by a growth-modulating positive feedback circuit. *Nat Chem Biol* **5**: 842-848
- Thron CD. (1997) Bistable biochemical switching and the control of the events of the cell cycle. *Oncogene*, Vol. 15, pp. 317-325.
- Trimarchi JM, Lees JA (2002) Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* **3**: 11-20
- von Dassow G, Meir E, Munro EM, Odell GM. (2000) The segment polarity network is a robust developmental module. *Nature*, Vol. 406, pp. 188-192.
- Wang J, Xu L, Wang E (2008) Potential landscape and flux framework of nonequilibrium networks: robustness, dissipation, and coherence of biochemical oscillations. *Proc Natl Acad Sci USA* **105**: 12271-12276
- Weinberg RA (1995) The retinoblastoma protein and cell cycle control. *Cell* **81**: 323-330
- Weinberg RA (2007) *The biology of cancer*, New York: Garland Science.

Wong JV, Yao G, Nevins JR, You L (2011) Viral-Mediated Noisy Gene Expression Reveals Biphasic E2f1 Response to MYC. *Mol Cell* **41**: 275-285

Yao G, Lee TJ, Mori S, Nevins JR, You L (2008) A bistable Rb-E2F switch underlies the restriction point. *Nat Cell Biol* **10**: 476-482

Figure Legends

Figure 1. The Rb-E2F network.

(A) A detailed Rb-E2F signaling network (modified from Blagosklonny *et al*, 2002; Sears *et al*, 2002) that controls the *G1/S* transition of mammalian cell cycle.

Grey-shaded ovals indicate overlapping or intermediate signaling activities to be lumped.

Circled numbers indicate indexes of the regulatory links (Table S1).

(B) A simplified Rb-E2F network. Positive regulatory links are shown in green and negative regulatory links in red. Link indexes are the same as in (A).

(C) The Rb-E2F bistable switch. Once the system at the quiescence state (E2F-OFF state) is stimulated beyond the R-point, it will stay at the proliferation state (E2F-ON state) even in the absence of continuous stimulation.

Figure 2. Robust models for bistability and *resettable* bistability.

(A) Identification of robust models in generating desired switching properties, at each combination of circuit topology (T_n) and parameter set (P_n). Inset: constraints to identify switching properties. S , serum input; EE_{ss} , the steady-state level of EE . Red and green curves indicate EE_{ss} dose responses simulated from initial conditions of quiescence and proliferation, respectively. See Methods for details.

(B) Heat map of model robustness. Shown is the two-way clustering result of 768 gene circuits by 20,000 random parameter sets. Red and blue colors indicate positive and negative for bistability, respectively.

(C) The most robust model for bistability, 2-3-5-6-7-9b.

(D) Minimal models for bistability. (Left) Ranked distribution of the robustness (occurrence/20,000 parameter sets) of minimal models in generating bistability. (Right) The top 5 minimal models for bistability. The type of logic gate and the correspondingly regulated node are indicated under each circuit. AND-gate, both involved activation links are dominant links (Table S1). OR-gate, at least one of the involved activation links is an additive link (Table S1). NOR-gate, both involved inhibition links are dominant links.

(E) Minimal models for resettable bistability. (Left) Ranked distribution of the robustness (occurrence/20,000 parameter sets) of minimal models in generating resettable bistability. (Right) The top 2 minimal models for resettable bistability. The type of logic gate, the type of involved positive feedback, and the correspondingly regulated node are indicated under each circuit. AND-gate, both involved links are dominant links (Table S1).

Figure 3. Experimental disruption of the gene circuit 3-5-6-7 abolished the Rb-E2F bistable switch.

(A) The Cdk2 inhibitor CVT-313 selectively blocks the mutual-inhibition feedback loop 5-6, but not the other three positive feedback loops (9, 2-3-6, and 2-7).

(B) Experimental protocol of serum-pulse stimulation and Cdk2 inhibition. At time 0, cells were serum-starved and at quiescence. See Methods for details.

(C) The influence of Cdk2 inhibition on E2F bistability. Each curve represents the histogram of the E2F-d2GFP distribution from approximately 5,000 cells. The Cdk2

inhibitor dose and sample harvest time are as indicated. The dashed lines connecting the high and low E2F-d2GFP modes are for the guide of eyes.

Figure 4. The gene circuit 3-5-6-7 exhibited high structural flexibility.

(A) Structural flexibility of the gene circuit 3-5-6-7. Each direct topological neighbor of 3-5-6-7 is shown with its robustness (number in parenthesis over 20,000) in generating resettable bistability.

(B) The correlation between structure flexibility (number at the Y-axis over 20,000) and parameter flexibility (number at the X-axis over 20,000) of all gene circuits.

(C) Top 10 models in structural flexibility. All models were derived from the same minimal model 3-5-6-7, with corresponding link additions shown by the arrows. Number in parenthesis over 20,000 indicates the structural flexibility of each model. Number above the oval node indicates the rank of structural flexibility.

(D) Interconnected gene circuits derived from minimal models 3-5-6-7 and 7-9a. Each node represents one gene circuit. Each edge connects direct topological neighbors (models with one-link variation). Shown in each graph are gene circuits with up to two-link variations from the red circled node (circuit 3-5-6-7 or 7-9a). The diameter of each node is proportional to the structure flexibility of the corresponding gene circuit.

The layout of the nodes does not have specific meaning.

Table 1. Mathematical models of the simplified Rb-E2F network.

(A) When all links are present (see Table S10 for nondimensionalization procedure):

$\frac{d[MD]}{dt} = \frac{1}{\tau_{MD}} \left(\frac{[S]^{n1}}{K_1^{n1} + [S]^{n1}} + \beta_{MD} \frac{[EE]^{n2}}{K_2^{n2} + [EE]^{n2}} - [MD] \right)$
$\frac{d[RP]}{dt} = \frac{1}{\tau_{RP}} \left(\left(1 + \beta_{RP} \frac{[EE]^{n4}}{K_4^{n4} + [EE]^{n4}} \right) \frac{K_3^{n3}}{K_3^{n3} + [MD]^{n3}} \frac{K_5^{n5}}{K_5^{n5} + [EE]^{n5}} - [RP] \right)$
$\frac{d[EE]}{dt} = \frac{1}{\tau_{EE}} \left(f(9a 9b) \frac{K_6^{n6}}{K_6^{n6} + [RP]^{n6}} \frac{K_8^{n8}}{K_8^{n8} + [MD]^{n8}} \frac{K_{10}^{n10}}{K_{10}^{n10} + [EE]^{n10}} - [EE] \right)$

$f(9a|9b)$: kinetic term of the link #9, which can exist either in the form 9a (dominant link), or in the form 9b (additive link) (Table S1).

$$f(9a) = \frac{[MD]^{n7}}{K_7^{n7} + [MD]^{n7}} \frac{[EE]^{n9}}{K_9^{n9} + [EE]^{n9}}$$

$$f(9b) = \frac{[MD]^{n7}}{K_7^{n7} + [MD]^{n7}} + \beta_{EE} \frac{[EE]^{n9}}{K_9^{n9} + [EE]^{n9}}$$

(B) When any link l is absent:

If the link l is a dominant link (#1, 3, 5, 6, 7, 8, 9a, 10, Table S1), its kinetic term in the equation would be replaced by 1; if the link l is an additive link (#2, 4, 9b, Table S1), its kinetic term would be replaced by 0. Please refer to Table S11 for details.