Irreversible Transitions, Bistability and Checkpoint Controls in the Eukaryotic Cell Cycle: A Systems-level Understanding

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Summary. The cell division cycle is not an autonomous oscillator, like a circadian rhythm, but rather a circular sequence of events that must be carried out in a specific order. The order of events is enforced by checkpoints that control progression from one stage of the cell cycle to the next. The transitions can be made only if the prior event is properly accomplished. Cell cycle transitions are irreversible (ratchet-like) because they are implemented by bistable switches in the dynamics of the underlying molecular regulatory network. In this review we show how bistability and irreversibility are emergent properties of molecular interactions, how bistability ensures unidirectional progression around the cell cycle, and how checkpoint mechanisms use bistable switching networks to maintain genomic integrity. While emphasizing generic properties of these controls in all types of eukaryotes, we also discuss some characteristic differences between yeast cells and mammalian cells.

Introduction

The repetitive cycle of cell growth and division is fundamental to all aspects of biological growth, development and reproduction, and defects in cell growth and division underlie many human health problems, most notably cancer. For these reasons, a driving ambition of molecular cell biologists has been to discover the molecular basis of cell cycle regulation. This goal was largely achieved in the glory years of molecular biology (1980-2000), and Nobel Prizes were duly awarded in 2001 [1-3]. The end result was an appealing vision of a 'universal' molecular mechanism controlling the eukaryotic cell cycle [4]. But the initial appeal was quickly dispelled by a bewildering array of interacting genes and proteins that constitute the control system in any particular organism. For examples, see the interaction maps of cell cycle controls in mammalian cells [5] and in budding yeast cells [6,7]. Looking closely at these maps, we can see, in places, clear connections between some molecular interactions and certain aspects of cell cycle progression. But can we identify any general principles of cell cycle regulation embedded in the network? Can we see how the gene-protein interactions in any particular organism determine the unique characteristics of cell proliferation in that organism?

The cell cycle is a particularly striking example of the necessity of systems-level thinking in 21st century molecular cell biology [8]. The resolute reductionism of the last century, while necessary for identifying the molecular components of cellular control systems and their interactions with binding partners, has proven insufficient for achieving an integrative understanding of the molecular basis of cell physiology. Putting the pieces back together requires new ways of thinking about and doing molecular biology—an approach now known as molecular systems biology. In this chapter, we show how systems-level thinking

reveals deep and unexpected principles of cell cycle regulation.

Table 1 provides a glossary of technical terms used in this review.

Physiology of the cell cycle. The cell cycle is the sequence of events whereby a growing cell replicates all of its components and divides them more-or-less evenly between two daughter cells, so that the daughters receive all of the information and machinery necessary to repeat the process [9-11]. The most important components that need to be replicated and partitioned to daughter cells are the chromosomes—the cell's genetic material. In eukaryotic cells, the processes of chromosome replication and partitioning are accomplished in separate phases of the cell cycle (Fig. 1): S phase (DNA synthesis) and M phase (mitosis).

A eukaryotic chromosome consists of a linear, double-stranded DNA molecule in close association with many types of DNA-binding proteins. During S phase the DNA molecule is carefully copied to produce two identical double-stranded DNA molecules, which become similarly decorated with DNA-binding proteins, to produce a pair of 'sister chromatids'. (Slight differences in the nucleotide sequences of sister chromatids may arise from mistakes—mutations—in the copying process.) As sister chromatid pairs are created during S phase, they are physically bound together by protein bands, called 'cohesin rings' [12]. After DNA synthesis is completed, there is a gap (G2 phase) during which the cell prepares for mitosis. G2 cells are defined by having fully replicated chromosomes that have not yet started the mitotic process.

The goal of mitosis is to separate the sister chromatids, delivering one (and only one) copy of each chromosome to each of the incipient daughter cells. Mitosis is a complex and delicate process [13]. In prophase, the nuclear membrane breaks down, the cytoskeleton rearranges to form a bipolar spindle apparatus, and the repli-
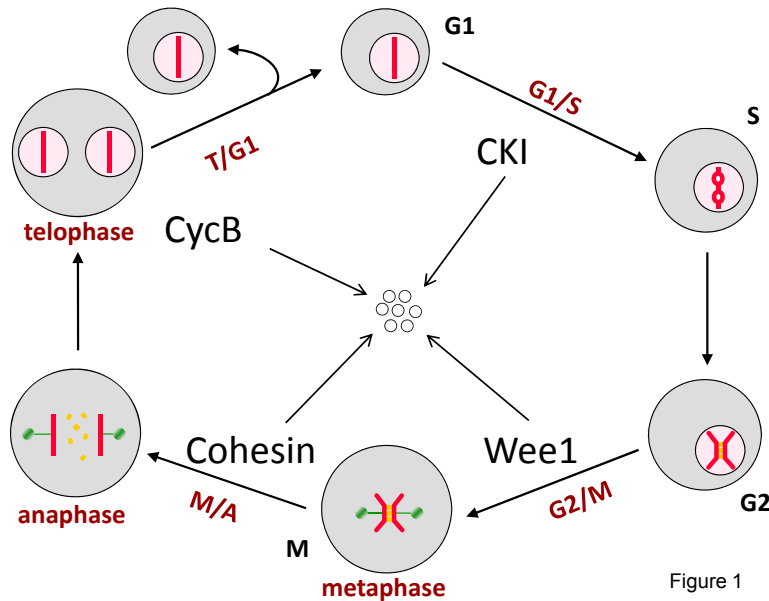
Figure 1. The eukaryotic cell cycle. Each 'cell' is bounded by a membrane enclosing cytoplasm (gray) with a nuclear compartment (pink) containing a representative chromosome (red bar). The chromosome goes through four distinct phases: G1, unreplicated; S, DNA synthesis; G2, replicated; M, mitosis. During G2 phase the sister chromatids are bound together by cohesin rings (yellow). Mitosis consists of distinct sub-phases: prometaphase (nuclear envelope breakdown, chromosome condensation, spindle assembly), prophase (alignment of replicated chromosomes on the spindle), metaphase (bi-orientation of all chromosomes on the central plate), anaphase (cleavage of cohesin rings and separation of sister chromatids to opposite poles of the spindle), telophase (reassembly of envelopes around the daughter nuclei), cell division. The four characteristic transitions of the cell cycle (G1/S, G2/M, M/A and T/G1) represent four natural stopping points ('checkpoints') where cell cycle progression can be halted if the cell detects any problems with completion of essential functions of the pre-transition state. As the cell passes each checkpoint it degrades a characteristic protein that had been inhibiting the transition (CKI, Wee1, cohesin, cyclin B). The 7 little circles represent products of protein degradation.

Table 1. Glossary

Alpha factor	A budding yeast pheromone that induces mating of haploid strains of opposite mating types (*a* and *α*).
Aneuploid	Having an unbalanced genome, with too few or too many copies of some chromosomes or sub-chromosomal regions.
Bifurcation point	A point in parameter space where a dynamical system undergoes a qualitative change in long-term behavior (e.g., from monostability to bistability).
Bistability	A dynamical system that has two stable steady states for physically realistic values of its variables ($0 \leq x_i \leq x_{i,max}$).
Centromere	The region of a replicated chromosome where the two sister chromatids are held together by cohesin rings in prometaphase.
Checkpoint	A molecular mechanism that detects problems in cell cycle processes and prevents progression to later events until the earlier processes can be successfully completed.
Coupled reactions	A chemical reaction (R1) can be made to proceed in the non-spontaneous direction ($\Delta G_1 > 0$) by coupling it to a spontaneous reaction (R2; $\Delta G_2 < 0$) in such a way that $\Delta G_{coupled} = \Delta G_1 + \Delta G_2 < 0$.

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Dynamical system	A system of interacting components (e.g., genes and proteins) that undergoes changes in time (and possibly also in space); in a continuous dynamical system, those changes can be described by a set of coupled differential equations.
Irreversibility, thermodynamic	At constant temperature and pressure, a non-equilibrium chemical reaction will proceed spontaneously (irreversibly) in the direction for which $\Delta G < 0$.
Irreversibility, dynamic	A transition in a dynamical system from one stable solution to another that is induced by a parameter change through a bifurcation point and that cannot be reversed by a small change in the parameter value in the opposite direction.
Kinetochore	The docking site for microtubules in the centromeric region of a chromatid.
Monostability	A dynamical system that has only one stable steady state for physically realistic values of its variables ($0 \leq x_i \leq x_{i,\max}$).
Nullcline	The locus of points in the phase plane where $f(x,y) = 0$ or $g(x,y) = 0$.
One-parameter bifurcation diagram	Plot of the steady state value of a dynamical variable as a function of a parameter of the dynamical system.
Parameter	A constant used to define the rate expression on the right-hand-side of a differential equation, e.g., the rate constant of a chemical reaction.
Phase plane	The state space of a two-variable dynamical system; $dx/dt = f(x,y)$, $dy/dt = g(x,y)$.
Proteosome	A multiprotein complex that functions as the cell's garbage disposal unit, capturing polyubiquitinated proteins and hydrolyzing them.
Restriction point	A point-of-no-return in G1 phase of the mammalian cell cycle with respect to removal of growth factor.
Reversibility, equilibrium	All chemical reactions are intrinsically reversible, and at equilibrium the forward and reverse reactions are occurring at the same rate.
Saddle-node bifurcation	A bifurcation point where a stable node and an unstable saddle point coalesce and annihilate each other.
Steady state	A constant solution $\{x(t) = x_o, y(t) = y_o\}$ of a dynamical system with the property that $f(x_o,y_o) = 0$ and $g(x_o,y_o) = 0$.
Steady state, stable	A constant solution of a dynamical system with the property that any small perturbation away from the steady state grows smaller as time proceeds.
Steady state, unstable	A constant solution of a dynamical system with the property that some small perturbations away from the steady state grow larger as time proceeds.
Stochastic	Random fluctuations in the number of molecules of a chemical species due to the probabilistic nature of chemical reactions.
Two-parameter bifurcation diagram	Plot of regions of distinct dynamical behaviors (e.g., monostability or bistability) in dependence on two parameters of a dynamical system.
Ubiquitin	A small polypeptide (76 amino acids) that can be covalently linked to proteins in order to label the protein for degradation by proteasomes.
Variable	A time-dependent component of a dynamical system, e.g., the activity of a cyclin-dependent kinase.

cated chromosomes condense into highly compacted, X-shaped bodies [14]. During prometaphase, microtubules emanating from the poles of the mitotic spindle search for the condensed chromosomes and bind to docking sites (kinetochores) on the sister chromatids at the mid-zone of the X, where the chromatids are still held together by 'centromeric' cohesins [15]. The goal is to attach the sister chromatids, by their kinetochore microtubules, to opposite poles of the mitotic spindle. When properly attached, each X is pulled to the midzone of the spindle (the metaphase plate) where it resides, under tension [16], until every replicated chromosome is properly aligned on the spindle (meta-phase). At this point in time, the centromeric cohesin rings are cleaved, allowing the kinetochore microtubules to pull the sister chromatids to opposite poles of the cell (anaphase). Nuclear membranes are then re-assembled around each of the segregated masses of chromosomes, forming a binucleate cell (telophase), which then divides down the middle to form two daughter cells, each with a full complement of unreplicated chromosomes (G1 phase).

Progression through the mitotic cell cycle is characterized by four crucial features [17]. First of all, to maintain a constant number of chromosomes per cell from generation to generation, it is necessary that S phase and M phase alternate, i.e., that progress through the cell cycle be unidirectional and irreversible: G1 $\rightarrow$ S $\rightarrow$ G2 $\rightarrow$ M $\rightarrow$ G1 $\rightarrow$. Second, the cycle of DNA replication and division must be coordinated with the synthesis of all other cellular components (proteins, lipids, organelles, etc.). That is, the time required to complete the cell division cycle must be identical to the mass-doubling time of cellular growth processes. If cell growth and division are not balanced in this way, then cells will get either larger-and-larger or smaller-and-smaller each generation, and eventually they will die.

Third, although the cell cycle is a periodic process, it is not governed by a clock [17]. The time spent in each phase of the cell cycle is highly variable, because progression from one phase to the next depends not on time spent in the present phase but on successful completion of the essential tasks of this phase. These completion-requirements are enforced by **checkpoints** [18-20] that guard the major transitions of the cell cycle: G1/S, G2/M, M/A (metaphase-to-anaphase) and T/G1 (telophase, cell division and return to G1, collectively known as 'exit from mitosis'). A checkpoint has three components [21]. Its surveillance mechanism looks for specific problems (incomplete replication of DNA, misalignment of chromosomes on the mitotic spindle, DNA damage). When a problem is detected, its error correction machinery is put into play (damage repair, re-attachment of microtubules to kinetochores, etc.). In the meantime, the checkpoint-proper blocks progression to the next stage of the cell cycle until the problem is resolved. These checkpoints ensure that the genome is passed down intact from generation to generation. When the checkpoints are compromised by mutations, daughter cells may inherit seriously damaged chromosomes (e.g., missing large pieces of the genome). Chromosomal abnormalities may trigger programmed cell death or malignant transformations of the damaged cell.

Fourth, the molecular mechanism controlling all the events of the cell cycle must be extremely robust: it must function perfectly under a wide variety of conditions and stresses, because mistakes can be lethal to the dividing cell and ultimately to the organism it supports. In particular, we must keep in mind that these molecular interactions are occurring within the small confines of a single cell, and the numbers of molecules participating in any aspect of the process may be extremely limiting. For example, in a haploid yeast cell, there is (in general) only one copy of

every gene, only a handful of copies of each specific mRNA, and only a few hundreds or thousands of molecules of specific regulatory proteins [22,23]. Basic laws of statistical physics demand that reactions among such small numbers of molecules must experience large stochastic fluctuations [24,25]. Yet cell cycle events are flawlessly orchestrated by the noisy molecular control system.

Any proposed explanation of the molecular basis of eukaryotic cell cycle controls must be consistent with these basic features of cell physiology. Informal, textbook explanations should not be accepted uncritically. Because the cell cycle is fundamentally a sequential process played out in time and space, the control system must be described in dynamic terms that provide insight into the general principles of temporal and spatial regulation and that account in quantitative detail for the idiosyncrasies of cell growth and division in particular organisms.

Molecular biology of the cell cycle.

In eukaryotes, the basic events of the cell cycle—DNA synthesis and mitosis—are controlled by a family of cyclin-dependent protein kinases (CDKs). As their name implies, these enzymes, in conjunction with a suitable cyclin partner, phosphorylate protein targets [26,27] and thereby initiate processes like DNA replication, nuclear envelope breakdown, chromosome condensation and mitotic spindle assembly. Hence the timing of cell cycle events depends on sequential waves of activation and inactivation of CDKs.
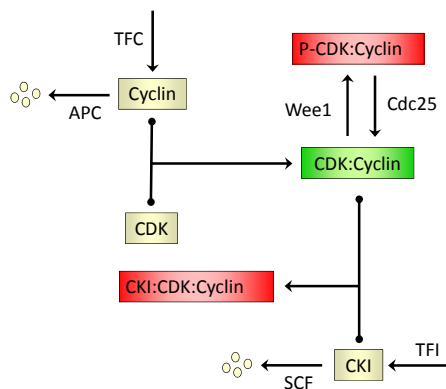

Figure 2

Figure 2. Mechanisms for regulating the activity of a CDK:cyclin heterodimer. The kinase subunit, CDK, is usually present in constant amount, in excess of cyclin subunits. The concentration of cyclin subunits is determined by the activities of its transcription factor (TFC) and its degradation machinery (APC or SCF). Active CDK:cyclin dimers can be inactivated by binding to a stoichiometric inhibitor (CKI), whose abundance is determined by the activities of its transcription factor (TFI) and its degradation machinery (SCF). In addition, the kinase subunit can be inactivated by phosphorylation (kinase = Wee1) and reactivated by dephosphorylation (phosphatase = Cdc25). The enzymes (TFC, APC, TFI, SCF, Wee1 and Cdc25) are all subject to their own regulatory interactions.
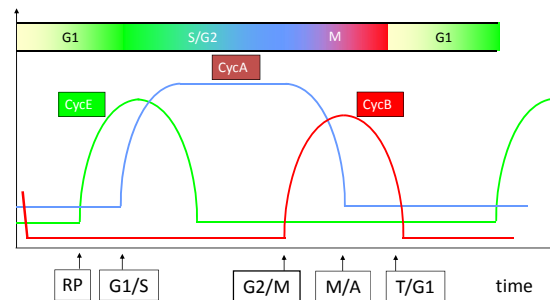

Figure 3

Figure 3. The cell cycle of a 'generic' eukaryote. We track fluctuations in three major classes of cyclins (A, B and E). In early G1 phase, all three classes of cyclins are absent. In mid-G1, CycE begins to rise at an event called the restriction point (RP) in mammalian cells (Start in yeast cells). CycA-dependent kinase is responsible for initiating DNA synthesis, so it rises at the G1/S transition. CycB-dependent kinase is responsible for mitosis, so its activity rises at the G2/M transition. CycA level falls in prometaphase, but CycB level falls later (after chromosome alignment on the metaphase plate).

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During steady proliferation of most cell types, the catalytic subunits (Cdk1, Cdk2, Cdk4 and Cdk6) are present in excess, and their activities are dependent on the availability of specific regulatory subunits (cyclin A, cyclin B, cyclin D, cyclin E) [28]. The abundance of each type of cyclin is controlled by its turnover (its rates of synthesis and degradation) (Fig. 2). Cyclin synthesis rate is determined by the activity of specific transcription factors, and cyclin degradation rate is determined by the activity of specific E3 ubiquitin-ligating complexes (APC and SCF) [29]. Polyubiquitinated cyclin molecules are rapidly degraded by proteasomes in the cell.

Specific CDK:cyclin heterodimers are active at distinct phases of the cell cycle (Fig. 3). In early G1 phase, cells are mostly devoid of cyclin molecules, except for minor amounts of cyclin D in combination with either Cdk4 or Cdk6 [28,30]. In late G1, cyclin E makes a brief appearance when, in combination with Cdk2, it turns on the transcription factor for cyclin A and turns off the ubiquitination of cyclin A [31]. Hence, cyclin A accumulates and, in combination with Cdk2, drives the cell through S phase. In G2 phase, cyclin A changes partners to Cdk1 and promotes the production of cyclin B. Cdk1:CycB heterodimers are essential for successful completion of mitosis. During prophase, most cyclin A is degraded, but cyclin B persists at high levels right up to metaphase [32]. During late metaphase and anaphase, cyclin B is rapidly cleared from the cell, leaving the daughter cells in G1 phase with only the remnant supply of cyclin D.

There are two other modes of CDK regulation that are crucial for cell cycle control (Fig. 2). First, both Cdk1 and Cdk2 can be phosphorylated on neighboring threonine and tyrosine residues in the N-terminus of the polypeptide chain [33]. These phosphorylations, which significantly inhibit the activity of the CDK:cyclin heterodimer, are carried out by members of the Wee1 family of protein kinases. To regain catalytic activity, the heterodimer must be dephosphorylated by a member of the Cdc25 family of protein phosphatases [34]. How Wee1 and Cdc25 activities are regulated will be described later. Second, there exist families of cyclin-dependent kinase inhibitors (CKIs) that bind strongly to CDK:cyclin dimers to form inactive trimers [35]. The fraction of the CDK:cyclin pool that can be inhibited in this way depends on the abundance of CKI molecules. Like cyclins, the abundance of a CKI is determined by its rates of synthesis and degradation [36].

A few other molecular components deserve special attention (see Table 2 for a summary). Cyclin D, cyclin E and CKIs are ubiquitinated by an E3 ubiquitin ligase called SCF, which recognizes its substrates only when they are properly phosphorylated. Hence, the degradation of these regulatory proteins can be controlled by specific protein kinases. Cyclins A and B are ubiquitinated by the 'anaphase promoting complex/cyclosome' (APC/C), which requires an auxiliary protein (Cdc20 or Cdh1) to target specific substrates to the APC. The phosphorylation states of the APC and its binding partners, Cdc20 and Cdh1, determine the activity of the complex [37]. In prometaphase APC:Cdc20 actively degrades cyclin A but not cyclin B. Cyclin B degradation is delayed until late metaphase [38], concurrently with securin.

Cohesin rings are cleaved by a protease called separase, which is kept inactive throughout most of the cell cycle by being bound to an inhibitor called securin [39]. During prometaphase, as the replicated chromosomes are being aligned on the mitotic spindle, the activity of APC:Cdc20 toward securin and CycB is blocked by an inhibitor, Mad2 [40]. When all chromosomes are properly aligned, the mitotic checkpoint is lifted, Mad2 is removed from APC:Cdc20, which then ubiquitinates securin, leading to its degradation by proteasomes. Free mole-

Table 2. Molecular components of the cell cycle control system

Name	Yeast Ortholog	Description	Typical Reaction[a,b]
Cdk1, Cdk2, …	Cdc28	Cylin-dependent kinase	Protein + ATP → Protein-P + ADP; Cdk$n$:CycX
CycA	Clb5, Clb6	S-phase promoting cyclin	ORC → ORC-P; Cdk2:CycA
CycB	Clb1, Clb2	M-phase promoting cyclin	Histone → Histone-P; Cdk1:CycB
CycD	Cln3	Growth-responding cyclin	RB → RB-P; Cdk4:CycD
CycE	Cln1, Cln2	Starter kinase	CKI → CKI-P; Cdk2:CycE
CAP		Counter-acting phosphatase	Protein-P + H_2O → Protein + P_i; CAP
CKI	Sic1	Cdk stoichiometric inhibitor	Cdk1:CycB + CKI → CKI:Cdk1:CycB
Cohesin	Scc1, etc.	Sister-chromatid cohesion	Xsome → Xtid:Cohesin:Xtid; S phase
Separase	Esp1	Cohesin degradation	Cohesin → DP; Separase
Securin	Pds1	Separase inhibitor	Separase + Securin ↔ Securin:Separase
APC	APC	Anaphase promoting complex	Securin:Separase → Separase + DP; APC:Cdc20
Cdc20	Cdc20	Targeting subunit of APC	APC + Cdc20 ↔ APC:Cdc20
Cdh1	Hct1	Homolog of Cdc20	Cdk1:CycB → Cdk1 + DP; APC:Cdh1
SCF	SCF	Skp-Cullin-Fbox complex	CKI-P → DP; SCF
Mad2	Mad2	Inhibitor of APC:Cdc20	APC:Cdc20 + Mad2$_A$ → MCC
	Cdc14	Exit phosphatase	APC:Cdh1-P → APC:Cdh1; Cdc14
	Net1	Inhibitor of Cdc14	Net1 + Cdc14 ↔ Net1:Cdc14
E2F	SBF	Transcription factor	*CYCE* promoter + E2F ↔ *CYCE*:E2F (active gene)
RB	Whi5	Inhibitor of E2F	RB + E2F ↔ RB:E2F
Wee1	Swe1	Tyrosine kinase	Cdk1:CycB → P-Cdk1:CycB; Wee1
Cdc25	Mih1	Tyrosine phosphatase	P-Cdk1:CycB → Cdk1:CycB; Cdc25

[a] A + B → C + D; E denotes a chemical reaction and its catalyzing enzyme. Reversible binding reactions are indicated by ↔.

[b] Abbreviations: ORC = Origin of Replication Complex; DP = Degradation Products; MCC = Mitotic Checkpoint Complex

cules of separase then cleave cohesin rings and promote anaphase.

The concurrent degradation of CycB during late metaphase and anaphase helps the cell to return to the G1 state. In budding yeast cells, as we describe later, the return to G1 is aided by the activation of Cdc14 (a Cdk counter-acting phosphatase) and Cdh1 (a Cdc20-homolog) [41].

From this brief description of the molecules that control cell cycle progression some specific features are incredibly obvious, like the roles of CDKs in triggering DNA synthesis and mitosis or the role of APC in promoting anaphase, but the subtle details of cell cycle control remain shrouded in mysteries. To understand exactly how the four fundamental properties of cell cycle progression are ensured by the underlying cell cycle machinery we must address the problem from a 'systems' point-of-view, asking two main questions. What are the basic principles of cell cycle regulation? And how are these principles implemented in molecular interactions? If we can answer these questions satisfactorily, then the whole welter of facts and speculations about the cell cycle will begin to make sense. And as we begin to understand the molecular logic of cell cycle control, we can expect to parlay this knowledge into significant advances in human health, agricultural productivity and biotechno-logical innovations.

"The chemical irreversibility of proteolysis is exploited by the cell to provide directionality at critical steps of the cell cycle." *Science* (1996)

"An obvious advantage of proteolysis for controlling passage through these critical points in the cell cycle is that protein degradation is an irreversible process, ensuring that cells proceed irreversibly in one direction through the cycle." Textbook (2004)

"Importantly, the irreversible nature of proteolysis makes it an invaluable complement to the intrinsically reversible regulation through phosphorylation and other post-translational modifications." Curr. Biol. (2004)

Irreversibility and Bistability

Irreversible progression through the cell cycle means that S and M phases always occur in strict alternation, separated by gaps—G1 and G2. When functioning properly, the cell cycle moves steadily forward like a ratchet device, not slipping backwards, say, to do two rounds of DNA synthesis without an intermediate mitosis. Of course, this happens sometimes, in cells under stress or in mutant cells [42] or even by design (in terminally differentiated polyploid cells) [43]. But in general, mitotic cell cycle transitions are irreversible. What sort of molecular interactions account for this directionality?

It is an alluring fact that at each irreversible transition of the cell cycle an important regulatory protein is degraded (Fig. 1). At the G1/S transition, the CKI that was inhibiting CDK activity throughout G1 phase is rapidly degraded. At the G2/M transition, the Wee1 kinase that was inhibiting CDK activity throughout G2 phase is rapidly degraded [44]. At the M/A transition, the cohesin rings that were holding sister chromatids together prior to metaphase are cleaved by separase. As cells exit mitosis and return to G1, cyclin B subunits, which played essential roles in orchestrating mitosis, are degraded by the APC. These observations have led many commentators to suggest that proteolysis is the basis of irreversibility (Table 3). Is proteolysis one of the fundamental principles of cell cycle regulation that we are seeking?

The hydrolysis of a peptide bond, $(AA)_N + H_2O \rightarrow (AA)_{N-1} + AA$, is a thermodynamically irreversible process, i.e., the free energy change of this chemical reaction is negative. But, come to think of it, every reaction that occurs in the cell has $\Delta G < 0$. If ΔG were positive, then the reaction would proceed in the opposite direction, as dictated by the second law of thermodynamics. Not only is protein degradation thermodynamically spontaneous but so also is protein synthesis, $(AA)_N + AA \rightarrow (AA)_{N+1} + H_2O$, because the ribosome couples this reaction to the hydrolysis of four molecules of ATP,

$$4\ ATP + 4\ H_2O \rightarrow 4\ ADP + 4\ P_i$$
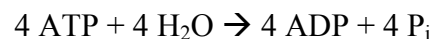
(P_i = inorganic phosphate ions). The overall reaction, polypeptide chain elongation + ATP hydrolysis, is an irreversible reaction ($\Delta G < 0$).

In the same way, protein phosphorylation and dephosphorylation are both intrinsically irreversible reactions:

Protein $+ATP \rightarrow$ Protein-P + ADP, $\Delta G < 0$.

Protein-P $+ H_2O \rightarrow$ Protein $+ P_i$, $\Delta G < 0$.

The net effect of a cycle of protein phosphorylation and dephosphorylation is the hydrolysis of one molecule of ATP to ADP + P_i. This is called a 'futile' cycle. Although futile from an energetic point of view, the cycle may be quite functional in the information-economy of the cell; corresponding, in computer language, to flipping a bit from 0 to 1 and back to 0. ATP hydrolysis is the price the cell must pay for an elementary information-processing operation. A cycle of protein

synthesis and degradation is also 'futile' in the same respect. The only difference is that it costs much more ATP to synthesize and then degrade a full protein; but, then, there is much more 'information' in a complete protein than in a phosphorylated amino acid side chain.

It is incorrect to maintain that post-translational modifications of proteins are intrinsically reversible whereas proteolysis is intrinsically irreversible (Table 3). The reverse of protein degradation is protein synthesis in the same way that the reverse of protein dephosphorylation is protein phosphorylation [45]. The only differences are 'time and money': protein turnover (synthesis and degradation) takes longer and costs more than post-translational modifications. Indeed, it is common to find that protein synthesis and degradation are dynamically balanced, so that the total level of the protein is held at a constant value. If protein turnover is rapid, then protein level can be quickly ramped up or down, as changing circumstances dictate, simply by disturbing the balance between synthesis and degradation. In such cases, proteolysis is a 'dynamically reversible' process.

It is equally true of protein synthesis-and-degradation as of phosphorylation-and-dephosphorylation that the opposing processes are thermodynamically irreversible but dynamically reversible. Dynamical irreversibility—which is the type of irreversibility of relevance to cell cycle transitions—is not to be sought in the thermodynamic properties of individual reactions but in the dynamical behavior of sets of coupled reactions with feedback. Dynamical irreversibility is a systems-level property of molecular regulatory networks [45].

Dynamical irreversibility is intimately connected to bistability of chemical reaction networks. To explain the connection, let's consider the simple example of a transcription factor, like E2F, that upregulates the expression of its own gene (Fig. 4A). According to the basic principles of biochemical kinetics, we can describe the dynamical features of this little network by a pair of ordinary differential equations (ODEs):

$$
\begin{aligned}
\frac{\mathrm{d}M}{\mathrm{d}t} &= k_{sm}H(P) - k_{dm}M \\
\frac{\mathrm{d}P}{\mathrm{d}t} &= k_{sp}M - k_{dp}P
\end{aligned}
\tag{1}
$$

where $M = $ [mRNA], $P = $ [protein], k_{sm} etc. = rate constants for synthesis and degradation of mRNA and protein, and $H(P) = $ probability that the gene encoding the transcription factor is being actively transcribed. Let's suppose that this probability = 1 when the transcription factor is bound to the gene's promoter, and = ε when not bound. Then the function $H(P)$ is commonly taken to be a Hill function,

$$
H(P) = \frac{\varepsilon K_p^n + P^n}{K_p^n + P^n}
\tag{2}
$$

where K_p is the equilibrium dissociation constant (units of concentration, say nM) of the transcription factor-promoter complex, and n is the Hill exponent, $n = 2$ or 4 depending on whether the transcription factor binds to the promoter as a homodimer or homotetramer.

At 'steady state' $\mathrm{d}M/\mathrm{d}t = \mathrm{d}P/\mathrm{d}t = 0$. Hence,

$$
H(P) = \frac{k_{dm}}{k_{sm}}M = \frac{k_{dm}k_{dp}}{k_{sm}k_{sp}}P = \varphi P
\tag{3}
$$

where $\varphi = k_{dm}k_{dp}/k_{sm}k_{sp}$ is a constant with units nM^{-1}. The steady state concentrations of mRNA and protein are determined by solutions of the algebraic equation $H(P) = \varphi P$, which is a cubic equation for $n = 2$ and a quintic equation for $n = 4$. From the graphs in Fig. 4B, it is easy to see that this algebraic equation may have one, two or three real positive roots (i.e., steady state values of P), depending on the values of n,
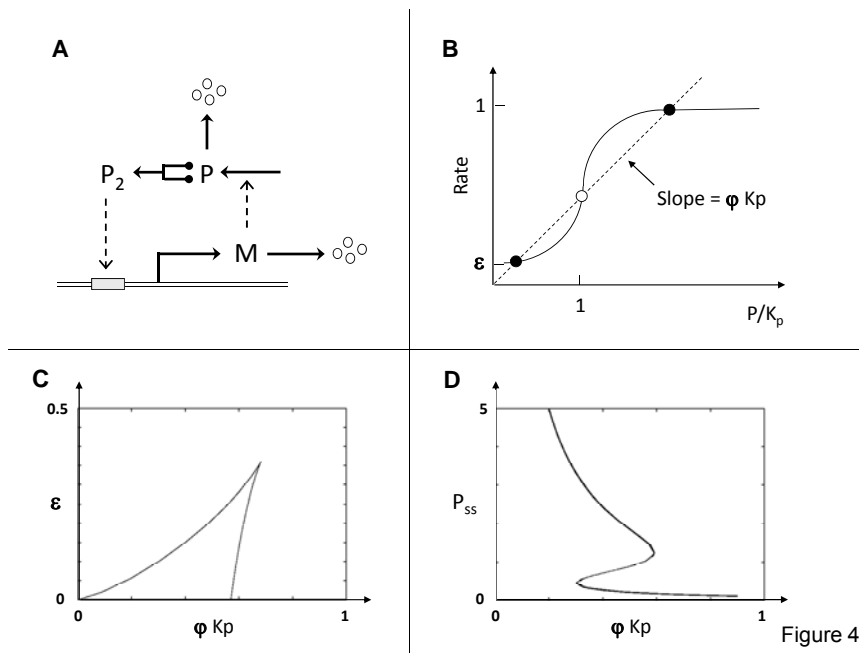
Figure 4. A simple bistable switch. (A) Wiring diagram. The synthesis of protein P is directed by mRNA M, which is transcribed from a gene controlled by a promoter (gray box on the double-stranded DNA molecule). The promoter is active when it is bound by dimers (or tetramers) of P. (B) Rates of synthesis (solid line) and degradation (dashed line) of P as functions of P/K_P. Black circles indicate stable steady states; white circle indicates an unstable steady state. Refer to Eqs. (1)-(3) in the text. (C) Domain of bistability in parameter space. (D) Signal-response curve. The steady state concentration of P is plotted as a function of $\varphi\,K_P$ for $n = 4$ and $\varepsilon = 0.1$. The response is bistable for values of $\varphi\,K_P$ in the range (0.301, 0.591).
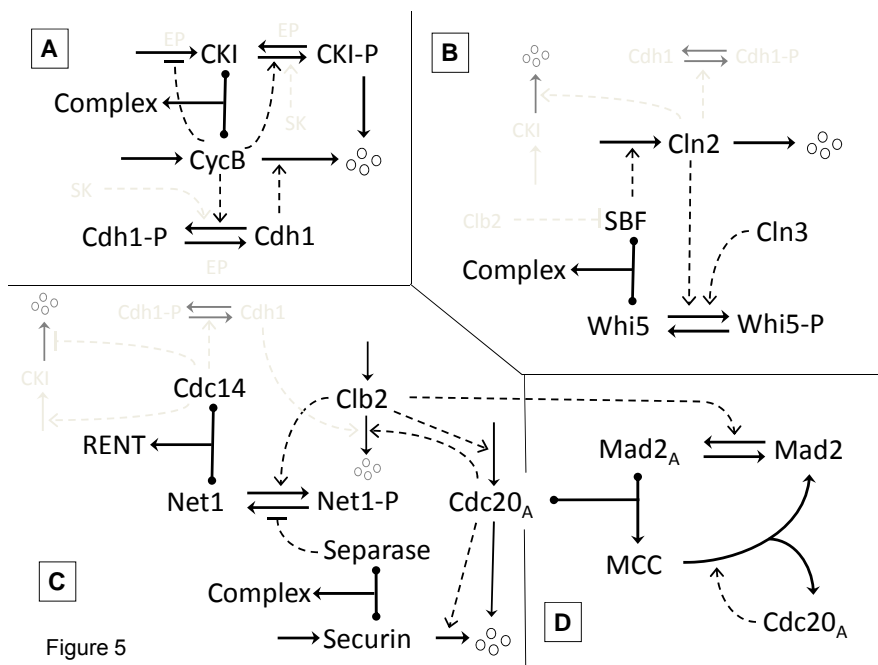

Figure 5. The molecular control systems of the budding yeast cell cycle. (A) The fundamental bistable switch, created by the antagonistic interactions between the B-type cyclins (CycB) and the G1-stabilizers (CKI and Cdh1). SK = starter kinase, EP = exit phosphatase. Four small circles are products of protein degradation. (B) The Start transition. Production of Cln2:Cdk1, the starter kinase of panel A, is controlled by the transcription factor, SBF, which is inactivated by binding to a stoichiometric inhibitor, Whi5. Phosphorylation of Whi5 releases SBF. Notice the double-negative

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feedback loop between Cln2 and Whi5. Cln3 is a 'growth indicator' that triggers the Cln2 switch. Notice also that Clb2-dependent kinase inactivates SBF, probably by direct phosphorylation. (C) Exit from mitosis. Active Cdc20:APC degrades securin and Clb2 (partially). Free separase degrades cohesin rings (anaphase) and helps to release Cdc14 from inhibition by Net1. Cdc14 is the exit phosphatase that activates Cdh1 and CKI. After Clb2 and other mitotic kinases are cleared by Cdh1:APC, Cdc20 is degraded, Cdc14 is inactivated, and the cell reverts to the **G1** steady state. (D) Metaphase checkpoint. Clb2 and Cdc20 are components of both networks C and D. The checkpoint protein, Mad2, is activated by unaligned (tensionless) chromosomes, in a reaction that requires Clb2-kinase activity. Active Mad2 binds to and sequesters Cdc20:APC in the mitotic checkpoint complex (MCC). When all chromosomes have come into alignment on the metaphase plate, the rate of activation of Mad2 drops to zero, and the MCC starts to come apart in a reaction that is accelerated by free (active) Cdc20:APC.

ε and $\varphi \cdot K_p$. In Fig. 4C we indicate how the number of steady states depends on ε and $\varphi \cdot K_p$ for $n = 2$ and $n = 4$. In Fig. 4D we show how the steady state values of P depend on $\varphi \cdot K_p$ for $n = 4$ and $\varepsilon = 0.1$.

Figure 4D is known as a one-parameter bifurcation diagram for the steady state solution, P_{ss}, of Eqs. (1) as a function of the dimensionless bifurcation parameter $\varphi \cdot K_p$. For small values of $\varphi \cdot K_p$, the gene is being actively transcribed (the gene is 'on') and P_{ss} is large. As $\varphi \cdot K_p$ increases, P_{ss} steadily decreases until the gene abruptly turns off at $\varphi \cdot K_p = 0.591$. At this value of $\varphi \cdot K_p$ the dynamical system is said to undergo a 'saddle-node' bifurcation. The upper steady state (a 'stable node') coalesces with the intermediate steady state (an unstable 'saddle point') and they both disappear, leaving only one attractor of the dynamical system, namely the lower steady state (the alternative stable node). In the lower steady state, gene expression is turned off, and it is not possible to turn it back on by a small decrease in the value of $\varphi \cdot K_p$. The transition is 'irreversible'. In order to coax the gene to turn back on, $\varphi \cdot K_p$ must be decreased below the other saddle-node bifurcation point at $\varphi \cdot K_p = 0.301$. For $0.301 < \varphi \cdot K_p < 0.591$, the reaction network is 'bistable', i.e., it can persist in one or another of two stable steady states (on or off). In the bistable zone, the two stable steady states are separated by an unstable steady state.

The bistable behavior we have illustrated with this simple model is completely representative of bistability in more complex networks. The irreversibility of transitions is related to bifurcation points in a bistable system, and bistability is a consequence of **Positive Feedback + Sufficient Nonlinearity + Rate-constant Constraints.** In our example, the 'positive feedback' is obvious: the transcription factor P up-regulates its own production. 'Sufficient nonlinearity' is reflected in the sigmoidal Hill function with $n = 2$ or 4. (If $n = 1$, there can be no bistability.) 'Rate-constant constraints' are evident in Fig. 4C: bistability is exhibited only within limited ranges of rate constant values.

Bistability can be generated by many different sorts of biochemical interactions, as we illustrate in the Appendix. All of the mechanisms considered there are involved in cell cycle regulation.

Irreversible Transitions in the Budding Yeast Cell Cycle

In Fig. 5 we sketch the molecular interactions underlying the irreversible transitions of the budding yeast cell cycle. Before describing these reaction networks in detail, we must introduce a few simplifications. In budding yeast, many cell cycle genes come in pairs: *CLN1* and *CLN2*, *CLB1* and *CLB2*, *CLB5* and *CLB6* [46]. In our diagrams and models, we lump these pairs together, i.e., 'Cln2' represents both Cln1 and Cln2 protein pools, 'Clb2' both Clb1 and Clb2, and 'Clb5' both Clb5 and Clb6. Sometimes we lump together Clb1, Clb2, Clb5 and Clb6 as 'Clb'. Also, both Sic1 and Cdc6 function as stoichiometric Cdk inhibitors in the mitotic cycle of budding yeast, so we refer to both of them together as 'CKI'. *CLN3* and

BCK2 encode proteins that are jointly responsible for growth sensitivity of the budding yeast cell cycle, and we usually are referring to these proteins jointly as 'Cln3' [7]. The expression of many cell cycle genes is controlled by regulated transcription factors, but we shall refer explicitly only to the regulation of *CLN1*, *CLN2*, *CLB5* and *CLB6* genes by SBF.

Two Alternative States. As suggested years ago by Kim Nasmyth [47,48], we can think of the budding yeast cell cycle as an alternation between two self-maintaining states: G1 is a state characterized by unreplicated chromosomes and low activity of Clb-dependent kinases; and S-G2-M is a state characterized by high Clb-kinase activity and chromosomes in the process of being replicated and aligned on the mitotic spindle. The transition from G1 to S-G2-M involves commitment to a new round of DNA replication and preparation for division. The reverse transition involves partitioning the replicated chromosomes to daughter nuclei (anaphase and telophase) and cell division.

The G1 state is stabilized by inhibitors of Clb-kinase activity, namely CKI and Cdh1. In the S-G2-M state, the G1 stabilizers are neutralized and the Clb-dependent kinases are actively promoting DNA synthesis and mitosis [7,17]. As illustrated in Fig. 5A, there is mutual antagonism between Clb-kinases and G1-stabilizers. The G1-stabilizers neutralize Clb-kinase activity (CKI binds to and inactivates Cdk1:Clb dimers, and Cdh1 promotes degradation of Clb subunits). On the other hand, active Cdk1:Clb dimers phosphorylate CKI and Cdh1, causing degradation of CKI and inactivation of Cdh1. These mutually antagonistic interactions create a basic bistable switch (Fig. 6). In the 'neutral' position (SK = 0 and EP = 0 in Fig. 6), the CKI-Clb-Cdh1 dynamical network can persist in either of two stable steady states: Clb inactive, CKI and Cdh1 active (the **G1** state), or Clb active, CKI and Cdh1 inactive (the **S-G2-M** state). This picture is the theoretical counterpart [20] of Nasmyth's intuitive notion of 'alternative self-maintaining states' [47]. (A word on our notation: G1 is a physiological state of unreplicated chromosomes, whereas **G1** is a stable steady state of a dynamical system, characterized by low activity of cyclin B-dependent kinase activities.)

In this theoretical framework, the cell physiologist's G1/S transition corresponds to a saddle-node bifurcation (Fig. 6, left), where the **G1**-branch of stable steady states ends, and the control system switches irreversibly to the **S-G2-M**-branch of stable steady states. This transition is promoted by a 'starter kinase' (Fig. 5A), which helps Clb-kinase to eliminate CKI and Cdh1. After the starter kinase has flipped the switch, it is no longer needed to maintain the system in the **S-G2-M** state. Similarly, the M/G1 transition ('exit from mitosis') corresponds to the reverse saddle-node bifurcation (Fig. 6, right), where **S-G2-M** branch ends and the control system switches irreversibly back to **G1.** An exit phosphatase opposes Clb-kinase activity and helps the G1-stabilizers to make a comeback. Thereafter, it is no longer needed to maintain the system in the **G1** state.

The theoretical picture in Fig. 6 stands or falls on the presumed bistability of the molecular interactions in Fig. 5A. Bistability at the G1/S transition has been confirmed experimentally by Cross [49], see Fig. 7. That irreversible exit from mitosis is due to feedback loops rather than cyclin B degradation has been confirmed experimentally by Uhlmann's group [50], see Fig. 8.

If we accept this theoretical picture of the G1/S and M/G1 transitions, then the next logical issues concern regulation of the starter kinase and exit phosphatase.

Start. The G1/S transition in budding yeast is guarded by a checkpoint (called 'Start' by yeast physiologists) that controls
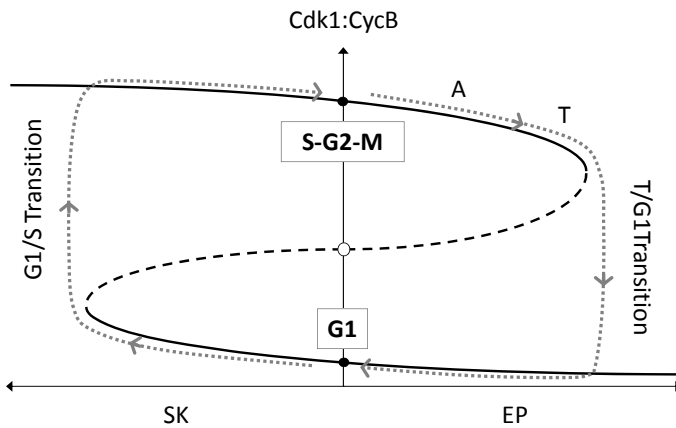
Figure 6

Figure 6. A dynamic view of cell cycle transitions. The network in Fig. 5A creates a bistable switch between a stable **G1** steady state (CycB-kinase activity low) and a stable **S-G2-M** steady state (CycB-kinase activity high). In early G1 phase, SK ≈ 0 and EP ≈ 0, and the cell is stuck in the stable **G1** state. To exit G1 phase and begin DNA synthesis, the cell requires a starter kinase to drive the bistable switch past the saddle-node bifurcation point and to induce the G1/S transition (gray dotted line). After the transition is complete, SK activity drops back to zero, but the cell is now stuck in the stable **S-G2-M** state. To leave M phase and return to G1, via anaphase (A) and telophase (T), the cell requires an exit phosphatase to drive the bistable switch past a different saddle-node bifurcation point and to induce the T/G1 transition. After the transition is complete, EP activity drops back to zero, but the cell is now returned to the stable **G1** state.
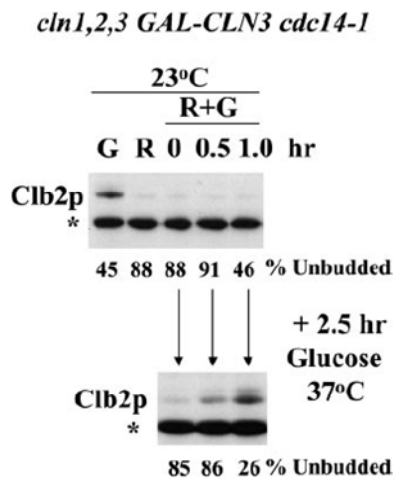

Figure 7

Figure 7. Bistability at the Start transition in budding yeast. From Cross et al. [2002]; used by permission. In this mutant strain of budding yeast, all three 'starter' cyclin genes have been knocked out (*cln1,2,3*), and a wildtype copy of one of them has been inserted under control of the galactose promoter (*GAL-CLN3*). In addition, the cell carries a temperature-sensitive allele of the *CDC14* gene (*cdc14-1*). The mutant strain is viable when grown on galactose (G) at 23°C, but inviable when grown on glucose or raffinose (no starter kinase) or when grown at 37°C (no exit phosphatase). An asynchronous population of viable cells is analyzed in the first column of the upper gel: 45% of the cells are unbudded (G1 phase), and the S/G2/M subpopulation (55%) contributes Clb2 protein to the gel. (The * indicates a loading control protein.) When the cycling population is transferred to raffinose (R) at 23°C (second column), the cells accumulate in G1 phase (88% unbudded) with negligible amounts of Clb2 protein. A population of G1-arrested cells is divided into 3 aliquots, which are

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transferred to raffinose + galactose medium (R+G) for 0, 0.5 and 1.0 h, respectively. During this time period, the cells synthesize differing amounts of Cln3 protein. After the indicated time, a sample of each aliquot is removed for analysis (columns 3,4,5), and the remaining cells are transferred to glucose medium at 37°C for 2.5 h, at which time they are analyzed as before (lower gel, columns 1,2,3). Notice that, at the end of the experiment, all cells are maintained under identical conditions without any starter kinase or exit phosphatase activities (SK = 0 and EP = 0). As the lower gel shows, these cells, at the end of the treatment, are stably arrested in two different states. The cells that were exposed to no starter kinase (0 h in R+G) remain arrested in G1 phase. The cells that were exposed to a significant amount of starter kinase (1 h in R+G) have transitioned (for the most part, 74%) to S/G2/M (they are budded and have significant quantities of Clb2 protein). The cells that were exposed to an intermediate amount of starter kinase (0.5 h in R+G) have remained (for the most part) in G1 phase (unbudded), but some cells have definitely transitioned to S/G2/M phase as witnessed by the notable amount of Clb2 protein in the second column.
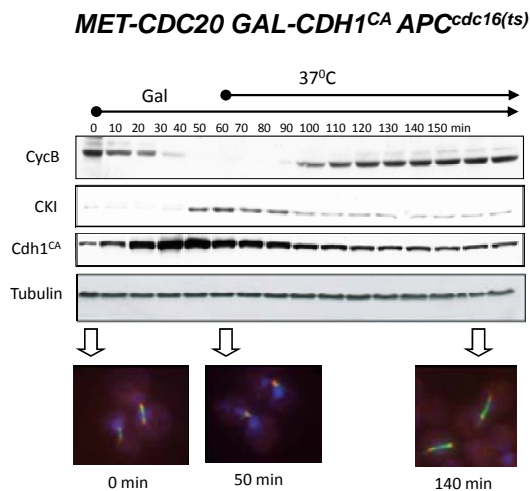

Figure 8

Figure 8. Reversible exit from mitosis in budding yeast. From Lopez-Aviles et al. [2009]; used by permission. In this mutant strain of budding yeast, the *CDC20* gene has been placed under control of a methionine-repressible promoter (*MET-CDC20*), and a non-phosphorylable version of Cdh1 protein has been inserted, under control of a galactose-inducible promoter (*GAL-CDH1*[CA]; 'CA' for 'constitutively active'). Finally, a temperature-sensitive allele (*cdc16*[ts]) of an essential component of the anaphase promoting complex (APC) replaces the wildtype gene. This strain is perfectly normal when grown in glucose at 23°C (it has Cdc20, endogenous Cdh1, and active Cdc16 proteins). When grown in glucose + methionine at 23°C (time < 0), these cells arrest in metaphase, as indicated in the first column of the gel (lots of cyclin B, no CKI, a small amount of Cdh1[CA] because the GAL promoter is slightly leaky). Furthermore, the nuclei have a metaphase morphology (micrograph at 0 min; red = spindle pole bodies, green = mitotic spindle, blue = DNA). At t=0 the cells are transferred to galactose + methionine to induce the synthesis of non-phosphorylable Cdh1 protein, as witnessed by the third row of the gel. (The fourth row is a loading control.) Because Cdh1[CA] protein cannot by phosphorylated by the high activity of CycB:Cdk1 in these cells, the cyclin B subunits are almost completely degraded by Cdh1[CA]:APC over the course of 50 min (first row of the gel), and the G1-stabilizing cyclin-dependent kinase inhibitor (CKI) begins to appear (second row). Furthermore, the nuclei have adopted an interphase morphology after 50 min of treatment. At t = 50 min the cells are transferred to 37°C to inactivate APC. Despite the fact that the cells appear to have exited mitosis and returned to G1 phase (low CycB, high CKI), these cells return to mitosis, as evidenced by the facts that CycB returns, CKI is degraded, and the nuclei return to metaphase (micrograph at 140 min). If the treatment is continued for 60 min, and then the cells are transferred to 37°C, the cells proceed into G1 phase (not shown; see original paper). This behavior is clear evidence of a separatrix between two stable steady states: after 50 min treatment, the cells are still in the domain of attraction of the stable M-phase steady state, but after 60 min treatment, the cells have moved into the domain of attraction of the stable G1-phase steady state.

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production of the starter kinase, Cln2:Cdk1 [51]. As indicated in Fig. 5B, Cln2 production is regulated by a transcription factor, SBF (a dimer of Swi4 and Swi6), which is kept inactive by binding to a stoichiometric inhibitor, Whi5 [52,53]. Phosphorylation of Whi5 and Swi6 leads to activation of SBF. In Fig. 5B we simplify these interactions by assuming that Whi5 phosphorylation causes dissociation of the SBF:Whi5 complex. Because Cln2:Cdk1 is one of the kinases that can phosphorylate Whi5, Cln2 and Whi5 are involved in a classic double-negative feedback loop that creates a bistable switch for Cln2-kinase activity. If Cln2:Cdk1 activity is low, then Whi5 is unphosphorylated and SBF is retained in inactive complexes. But if Cln2:Cdk1 activity is high, then Whi5 is phosphorylated, SBF is active, and Cln2 is steadily synthesized.

At the Start transition, this switch is flipped from the Cln2-low state to the Cln2-high state [54]. The Start switch responds to two crucial physiological signals: cell growth and mating factor. Newborn daughter cells are too small to warrant a new round of DNA synthesis [55]. They must grow to a certain critical size before they can pass Start. In addition, budding yeast cells of mating type *a* respond to pheromone (α factor) by arresting in G1 phase before Start [51]. Hence, cell growth promotes the Start transition, whereas α factor inhibits it. Both signals appear to operate through the activity of Cln3-dependent kinase (and a second protein, Bck2 [56], that is still poorly characterized). Cell growth increases the net activity of Cln3:Cdk1 in the G1 nucleus of budding yeast cells, whereas α factor stimulates production of a CKI (Far1) that specifically inhibits Cln1,2,3-dependent kinase activities.

In the presence of α factor, the Start switch is permanently arrested in the Cln2-low position. In the absence of α factor, a small cell is arrested in the Cln2-low position; but as the cell grows, Cln3:Cdk1 activity increases. By phosphorylating Whi5, Cln3-kinase partially inactivates Whi5 and helps the Cln2 self-activation loop to engage [57]. The Cln2-low state is lost by a saddle-node bifurcation (Fig. 9, left), and Cln2 begins to accumulate in a self-accelerating manner [20]. The Start switch is moving toward the Cln2-high position, but, before it reaches this state, Cln2:Cdk1 flips the G1/S switch to the Clb2-high position, and Clb2:Cdk1 inactivates SBF (see Fig. 5B). The Start switch never reaches the stable Cln2-high position, but instead drops back to the Cln2-low state (Fig. 9, left). After Cln2 has done its job as a starter kinase to activate the B-type cyclins (DNA synthesis and mitosis), it drops back to the Cln2-low state in order not to interfere with mitotic exit [58].

A word about the interpretation of Fig. 9. The left and right sides, considered separately, should be thought of as 'pseudo-phase planes'. The control system in Fig. 5 has dozens of dynamical variables, and its vector field cannot be represented on a two-dimensional phase plane. Instead we have plotted one-parameter bifurcation diagrams for subsets of the reaction network. For example, consider the left side of Fig. 9 (similar reasoning applies to the right side). The black curve is the one-parameter bifurcation diagram for the bistable network in Fig. 5A, treating Cln2-kinase as the bifurcation parameter and setting Cdc20 ≈ 0 and Cdc14 ≈ 0. The red curve is the one-parameter bifurcation diagram for the bistable network in Fig. 5B, with Cln2-kinase as the variable and Clb2-kinase as the bifurcation parameter. Strictly speaking, these bifurcation curves are not nullclines on a phase plane, but they can be thought of as pseudo-nullclines: the black curve is the Clb2 pseudo-nullcline (vector field points left or right), and the red curve is the Cln2 pseudo-nullcline (vector field points up or down). Their intersection points are certainly steady states of the full system (Fig. 5A+B, with
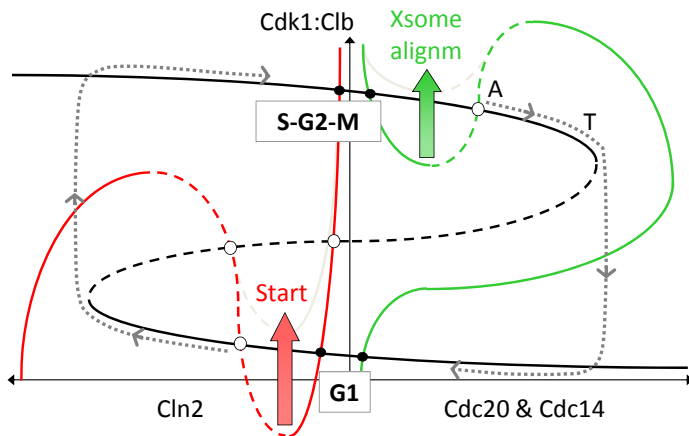
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Figure 9

Figure 9. Checkpoints in budding yeast. We identify SK with Cln2:Cdk1 and EP with a combination of activities of Cdc20 and Cdc14. Left: the G1/S transition is guarded by a checkpoint called Start (Fig. 5B), which creates a stable steady state of low Cln2 abundance. As the cell grows, the checkpoint is lifted (up arrow) and the stable **G1** state is lost by a saddle-node bifurcation. Subsequently, Cln2 production induces the G1/S transition as in panel A. Right: the M/A transition is guarded by the mitotic checkpoint. When all chromosomes are properly aligned on the metaphase plate, the checkpoint is lifted (up arrow) and the stable metaphase-arrested state is lost by a saddle-node bifurcation. Subsequently, Cdc20 and Cdc14 are activated and they drive exit from mitosis and return to G1 phase.

EP ≈ 0), and one can readily guess the stability of each steady state and the temporal evolution of the dynamical system when the steady states are removed by saddle-node bifurcations.

Mitotic Checkpoint. The M/G1 transition is guarded by the mitotic checkpoint, which controls activation of APC:Cdc20 and the exit phosphatase, Cdc14 [59]. Figure 5D diagrams the mitotic checkpoint proper, which controls activation of APC:Cdc20, and Fig. 5C indicates how active Cdc20 initiates the activation of Cdc14 and the re-establishment of cells in **G1**.

The role of the mitotic checkpoint (the 'anaphase switch') is to prevent cells from cleaving cohesin rings until all replicated chromosomes are properly aligned on the mitotic spindle [60]. Alignment is judged, it seems, by tension within the centromeric region of bi-oriented chromosomes (one kinetochore attached by microtubules to one pole of the spindle, and the other

kinetochore to the other pole). Unaligned, tensionless chromosomes generate a signal that activates Mad2, and active Mad2 binds to APC:Cdc20 [40], holding it in an inactive complex (the mitotic checkpoint complex, MCC, which is inactive with respect to degradation of separase and Clb2, but active on the degradation of Clb5). The signal from tensionless chromosomes to Mad2 depends on Clb-kinase activity, which is high in prometaphase. As soon as all chromosomes are properly aligned and under tension, the rate of Mad2 activation becomes 0, and the MCC begins to dissociate, releasing active APC:Cdc20.

The network in Fig. 5C has two positive feedback loops. (1) Clb2:Cdk1 (via tensionless chromosomes) activates Mad2, which inactivates APC:Cdc20; whereas APC:Cdc20 degrades Clb2, thereby inactivating the kinase. This double-negative feedback loop creates a bistable switch at the metaphase checkpoint [61]. (2) Active APC:Cdc20 promotes disso-

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ciation of the MCC. This self-activation loop accelerates the release from the checkpoint, so that anaphase follows soon after full chromosome alignment.

The anaphase switch has two stable steady states: (1) Clb2 level high, Mad2 active, Cdc20 inactive; and (2) Mad2 inactive, Cdc20 active, Clb2 level low. At the metaphase/anaphase transition, the switch is flipped from the Cdc20-inactive state to the Cdc20-active state. Consequently, APC:Cdc20 degrades securin, releasing separase to cleave cohesin rings and trigger anaphase (separation of sister chromatids). In addition, separase has a non-catalytic role [62], inhibiting the phosphatase that has been keeping Net1 active throughout the early stages of mitosis [63]. Inhibition of the phosphatase allows Clb2-kinase and other mitotic kinases (notably Polo kinase) to phosphorylate Net1 and release Cdc14 [63-65]. Meanwhile, APC:Cdc20 degrades Clb2, and the combination of low Clb2-kinase activity and high Cdc14-phosphatase activity silences the mitotic checkpoint [66]. (Cohesin cleavage at anaphase creates tensionless chromosomes, but they do not re-activate Mad2 because now Clb2:Cdk1 activity is low and Cdc14 activity is high.) Cdc14 promotes activation of the G1-stabilizers (CKI and Cdh1). CKI inhibits any remaining Clb-dependent kinase activity, and Cdh1 destroys Polo kinase and Cdc20.

In Fig. 9 (right), we show how chromosome alignment removes the mitotic-arrest state by a saddle-node bifurcation, allowing the anaphase switch to flip on. Cdc20 and Cdc14 activities rise, but before they can reach the upper steady state, they induce degradation of Clb proteins and activation of the G1-stabilizers. The transition to G1 phase removes Cdc20 and Cdc14, restoring the newborn cells to the beginning of the cycle.

Irreversible Transitions in the Mammalian Cell Cycle

The molecular machinery regulating progression through the mammalian cell cycle is very similar, in principle, to the yeast cell cycle. In the following paragraphs we will highlight the most important similarities and differences.

In mammalian cells, as in yeast, the G1 phase of the cell cycle is stabilized by three types of interactions that keep low the activities of S-phase promoting factor (SPF = Cdk2:CycA) and M-phase promoting factor (MPF = Cdk1:CycB): (1) High activity of APC:Cdh1, which promotes degradation of both CycA and CycB [67]. (2) High abundance of CKIs that inhibit SPF and MPF heterodimers [68]. (3) High abundance of an inhibitor (retinoblastoma protein, RB) of the transcription factors (E2F family) that promote synthesis of early cyclins (CycE and CycA) [69]. Each of these G1-stabilizers can be phosphorylated and neutralized by SPF and MPF, creating a fundamental bistable switch between steady states for **G1** and **S-G2-M**. Progression through the mammalian cycle may also be envisioned as flipping this switch on by a starter kinase (Cdk2:CycE) and off by an exit pathway (Cdc20 and CAPs).

In particular, the Start transition in budding yeast is quite analogous to the 'restriction point' (RP) in the mammalian cell cycle [70]. The molecular mechanisms of the two checkpoints are almost identical under the identification of SBF with E2F, Whi5 with RB, Cln2 with CycE, Cln3 with CycD, and Clb5 with CycA. Both checkpoints are responsive to extracellular signals. In budding yeast cells, α factor activates a MAP kinase pathway that up-regulates a stoichiometric inhibitor of Cln3 and blocks cells in pre-Start. In mammalian cells, growth factor (GF) activates a MAP kinase pathway that up-regulates a transcription factor for CycD and promotes passage through the RP. The logic differs because, for yeast cells, the

default state of the cell cycle is vegetative growth and division, and yeast cells need a definite signal (α factor) to block progression through the cell cycle and start the mating process. For mammalian somatic cells, on the other hand, the default state is G1 arrest; only special cells under special circumstances are permitted to grow and divide. The permission is granted by specific GFs that promote passage through the RP.

Bistability at the mammalian RP has been demonstrated experimentally in elegant experiments by Yao et al. [71], see Fig. 10. In a later paper [72], the same authors showed experimentally that RP-bistability is due to the double-negative feedback loop (RB --| E2F → CycE --| RB).

Bistability in the mitotic exit mechanism of mammalian cells is still a matter of some disagreement [73,74], although in our opinion the evidence is definitely in favor of a bistable switch [45,75]. Our description of a bistable anaphase switch in budding yeast, based on experiments by Uhlmann and colleagues [66], is confirmed by similar experiments with fruit flies [76], see Fig. 11, and mammalian cells [77].

Budding yeast cells differ from most other types of organisms (including fission yeast cells, plant cells, fruit fly embryos, frog embryos and mammalian cells) in lacking a checkpoint at the G2/M transition. Budding yeast cells are unique in having many small chromosomes that need not undergo much condensation during mitosis; hence, they can go almost directly from S phase into mitosis [78]. Other organisms, on the contrary, need a gap phase (G2) between the end of S and the onset of M, during which chromosomes are replicated and available for transcription. When these cells enter mitosis, their chromosomes become so highly condensed that all transcription ceases. The duration of G2 is determined by the G2/M checkpoint. During G2 phase, mitotic cyclins accumulate in

complexes that are inactivated by tyrosine phosphorylation of the Cdk subunit (Fig. 2). To enter mitosis, these phosphate groups must be removed. The relevant phosphorylation and dephosphorylation reactions are:

Cdk1:CycB → P-Cdk1:CycB (less active);
    Enzyme = Wee1

P-Cdk1:CycB→Cdk1:CycB (more active);
    Enzyme = Cdc25-P

Wee1 → Wee1-P (less active);
    Enzyme = Cdk1:CycB

Wee1-P → Wee1 (more active);
    Enzyme = CAP

Cdc25 → Cdc25-P (more active);
    Enzyme = Cdk1:CycB

Cdc25-P → Cdc25 (less active);
    Enzyme = CAP

Clearly, Cdk1:CycB and Cdc25 are involved in a positive feedback loop (mutual activation) [79,80], and Cdk1:CycB and Wee1 are involved in a double-negative feedback loop (mutual antagonism) [81,82]. This network controlling the G2/M transition is strongly bistable, as first pointed out by Novak and Tyson [83]. The theoretical predictions of that paper were confirmed ten years later by two groups independently and simultaneously [84,85], see Fig. 12.

Additional Checkpoints

It should be obvious now that additional checkpoints can be created by realigning the curves in Fig. 7. For instance (Fig. 13, left), the cell can create a new checkpoint in late G1, with high level—but low activity—of starter kinase (CycE), by synthesizing a stoichiometric inhibitor (CKI) of CycE:Cdk2. This is exactly the strategy used by mammalian cells to block entry into S phase if DNA damage is detected in G1. A surveillance mechanism up-regulates a master transcription factor, p53, which induces synthesis of repair enzymes and of a CKI (p21^{WAF1}) that inhibits CycE:Cdk2 and blocks cell cycle progression in late G1. If the damage can
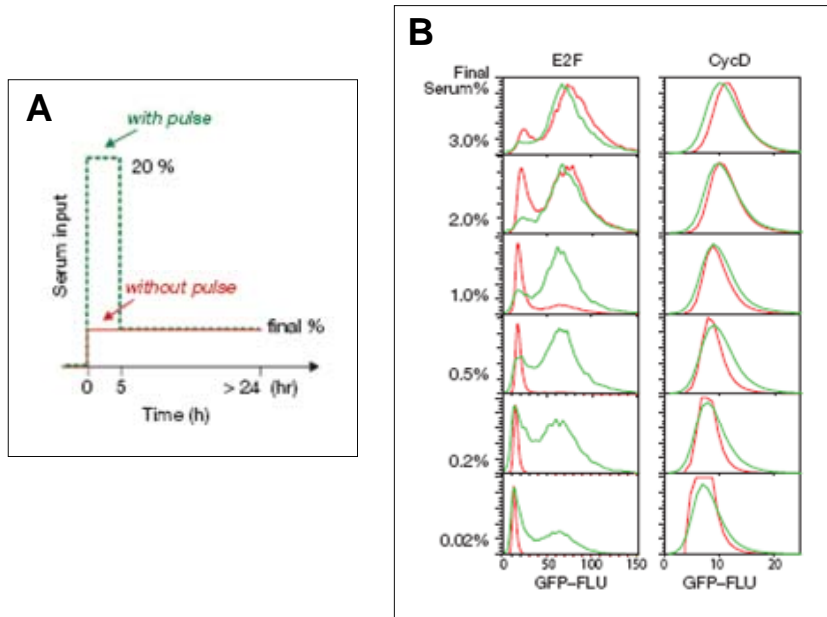
Figure 10

Figure 10. Bistability at the restriction point in mammalian cells. From Yao et al. [2008]; used by permission. Rat embryonic fibroblasts are prepared with a reporter (green fluorescent protein, GFP) expressed from either the E2F promoter or the CycD promoter. The cells are arrested in early G1 phase by serum deprivation (t < 0). At t = 0, they are provided with fetal calf serum (a source of growth factors) according to two protocols (panel A). Red (without pulse): serum is provided at a fixed final percentage, which is varied from one treatment to the next. Green (with pulse): serum is initially provided at 20% for 5 hours to induce all cells to pass the restriction point, and then serum is lowered to a final percentage identical to the other protocol. After 24 h in serum, cells are harvested and analyzed by flow cytometry. The red and green curves in panel B indicate the number of cells expressing increasing amounts of GFP (measured by fluorescence, GFP-FLU), color coded according to the treatment protocol used and displayed in a matrix indicating final serum percentage (rows) and the promoter (E2F or CycD) driving GFP production (columns). In the second column we see that CycD expression responds continuously and monotonously to final serum percentage; and the final distribution is independent of whether serum is pulsed up (red curve) or down (green curve). By contrast, the E2F reporter (first column) shows clear evidence of bistability in response to serum pulses. If serum is pulsed up from 0 directly to its final level (red curves), then E2F expression is not turned on until the final serum concentration is 1% or higher, and even so the cells divide into subpopulations of expressing and non-expressing cells (the two peaks in the histogram). On the other hand, if the serum concentration is brought down from 20% to the same final percentage, then E2F expression shows evidence of bistability even down to 0.02% serum. These results provide clear evidence of hysteresis in the E2F response (but not in CycD expression).
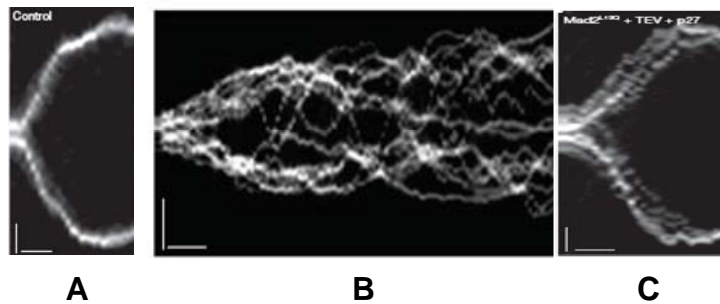
A   B   C

Figure 11

Figure 11. Reversibility of the anaphase switch in fruit fly nuclei. From Oliveira et al. [2010]; used by permission. Chromosome movements in nuclei of *Drosophila* syncytial embryos are tracked by live cell imaging and displayed as kymographs, i.e., traces of centrosome positions (vertical axis) as functions of time (horizontal axis) for all chromosomes in the nucleus. The aim of this experiment is to induce anaphase separation of sister chromatids in the absence of Cdc20 activity. To silence Cdc20, embryos are injected with a mutant protein $Mad2^{L13Q}$ that effectively blocks the activity of Cdc20. To allow for sister chromatid separation when Cdc20 and hence separase are inactive, the cohesin proteins of the embryo are engineered with cleavage sites recognized by tobacco etch virus (TEV) protease, which can be injected into metaphase-arrested embryos to induce anaphase. (A) Control. Normal movement of chromosomes during anaphase, as they are partitioned to opposite poles of the spindle. Scale bars: vertical = 2 μm; horizontal = 30 s. (B) Reversibility of the mitotic checkpoint. Nuclei are arrested in metaphase with low activity of Cdc20 by injection with $Mad2^{L13Q}$ and then treated with TEV protease to induce anaphase. The chromosomes begin to move toward the poles, but before long they appear to lose attachment to the spindle microtubules and are left behind in the middle of the cell. Random reattachments and detachments seem to cause back-and-forth movements of the chromosomes between the spindle poles. Under these conditions (low activity of Cdc20, high activity of cyclin B-dependent kinase), the mitotic checkpoint appears to be reactivated. Scale bars: vertical = 5 μm; horizontal = 60 s. (C) Reestablishing proper segregation of chromosomes. Same experiment as in panel B with the additional injection into the embryo of human p27, a stoichiometric inhibitor of cyclin B-dependent kinase. In this case the sister chromatids segregate normally, confirming that loss of cyclin B-dependent kinase activity in anaphase is crucial to silencing the metaphase checkpoint and making the anaphase switch irreversible. Scale bars: same as panel A.
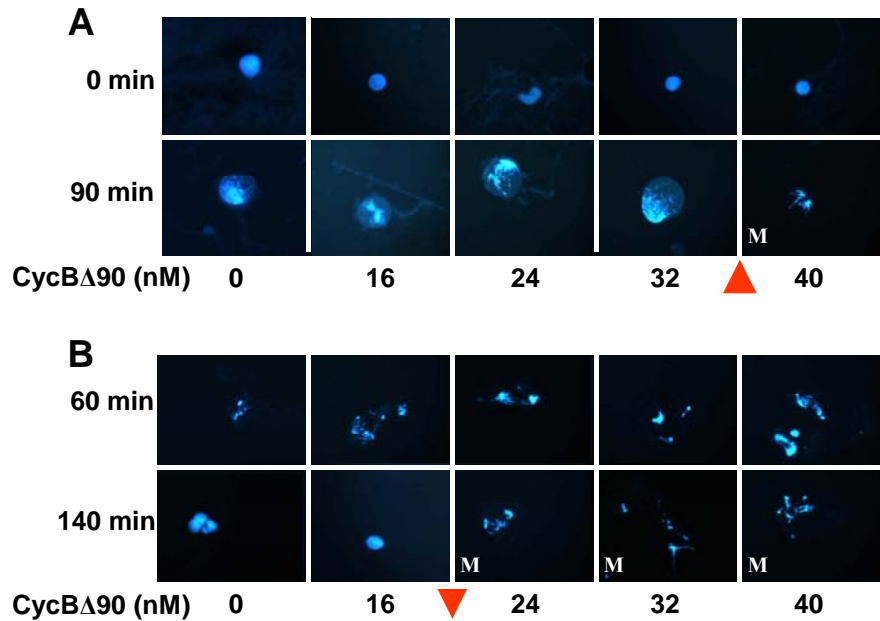
Figure 12

Figure 12. Bistability at the G2/M transition in frog egg extracts. From Sha et al. [2003]; used by permission. Cytoplasmic extracts of frog eggs are used to measure the amount of cyclin B necessary to induce or sustain the kinase activity of CycB:Cdk1 heterodimers (referred to in this paper as MPF, mitosis promoting factor). Sperm nuclei in the extract (stained blue for chromatin) are photographed to stage the extracts. Interphase: round nuclei, dispersed chromatin, intact nuclear membrane, low activity of MPF (confirmed in separate experiments, not shown). Mitosis: highly condensed chromatin, no nuclear membrane, high activity of MPF. (A) Cyclin threshold for activation of MPF. Extracts are prepared in interphase (t = 0) in the presence of cycloheximide, to block all protein synthesis, including the synthesis of endogenous cyclin B. Samples of the extract are injected with increasing amounts of non-degradable cyclin B (CycB∆90) at t = 0 and photographed at intervals thereafter (t = 90 min time point shown here). Extracts containing 0—32 nM CycB∆90 have insufficient MPF activity to enter mitosis, but 40 nM CycB∆90 or larger is enough to activate MPF and drive the nuclei into mitosis. (B) Cyclin threshold for inactivation of MPF. Extracts are prepared in interphase (t = 0) in the absence of cycloheximide and injected with increasing amounts of CycB∆90. Whether the injected amount is small or large, synthesis of endogenous cyclin B drives the extract into mitosis by t = 60 min. At t = 60 min the extracts are treated with cycloheximide to prevent any further synthesis of endogenous cyclin B. As the extracts try to exit from mitosis, they activate Cdc20 and degrade the endogenous cyclin B proteins but leave behind the non-degradable CycB∆90 molecules. Extracts containing 24 nM CycB∆90 or higher retain MPF in the active form and block the nuclei in mitosis. Extracts containing 16 nM CycB∆90 or lower have inactive MPF (confirmed in separate experiments, not shown) and return to interphase. For CycB∆90 concentrations in the range 24—32 nM, the MPF control system is bistable: it can persist in an MPF-inactive state (panel A) or in an MPF-active state (panel B) under identical conditions on cyclin B concentration in the extract. Which state the extract adopts depends on whether it was initially in the MPF-inactive state (above) or in the MPF-active state (below).
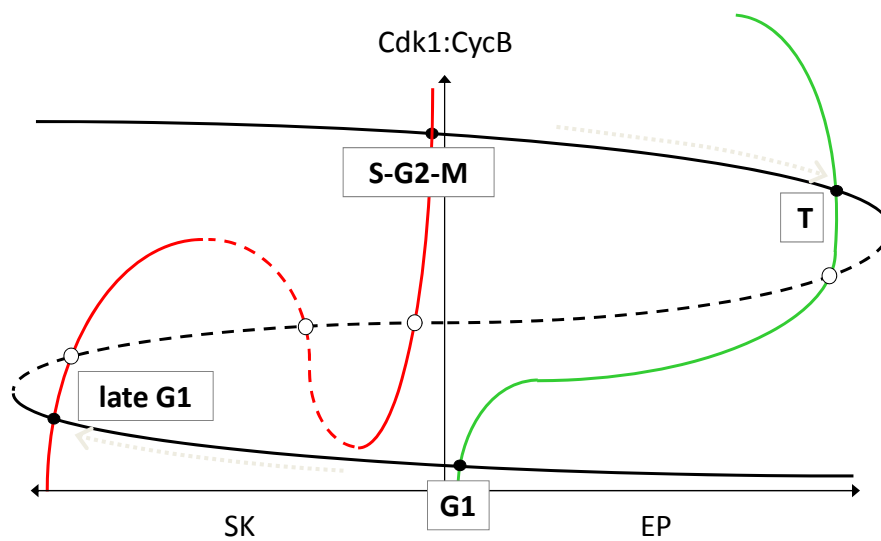
22

Figure 13

Figure 13. Additional checkpoints in late G1 and telophase. Left: a cell that has passed the restriction point (aka, Start) can still be blocked in late G1, with high levels of starter kinase, if a new checkpoint is created by a saddle-node bifurcation at the far left. Right: similarly, a cell that has completed the M/A transition can still be blocked in telophase (T), if a new checkpoint is created by a saddle-node bifurcation at the far right.

be repaired, then the CKI is removed and the cell can proceed into S phase, as usual. If the damage cannot be repaired, then p53 induces synthesis of pro-apoptotic proteins and the damaged cell commits suicide.

Similarly (Fig. 13, right), the cell can create a checkpoint in telophase (T), with partially degraded CycB and incompletely released Cdc14, if the mitotic exit pathway (Fig. 5C) is compromised. This strategy is employed by budding yeast cells to implement a 'spindle alignment' checkpoint [59]. Budding yeast cells determine the location of the cell division plane early in the cell cycle, at the point of bud emergence [78]. At the end of the cycle, the bud must separate from the mother cell at the neck between the two. Cell division will be successful only if the mitotic spindle is properly aligned with one pole in the mother-half of the cell and the other pole in the daughter-half. If this is the case, then, as the spindle elongates in late anaphase and pushes one mass of chromosomes into the bud, the spindle

pole comes into contact with the bud cortex. This physical proximity brings together a G-protein (Tem1) and its GEF (Lte1), and the activated form of Tem1 activates a kinase that provides additional phosphorylation of Net1 and complete release of Cdc14 [86]. If the spindle is not properly aligned, then Tem1 does not get activated and Cdc14 is not fully released. Cell cycle progression blocks in telophase, to give the cell time to re-orient the spindle.

These checkpoints are imposed and lifted by saddle-node bifurcations; hence, just like the restriction point and exit from mitosis, they are dynamically irreversible transitions. On the other hand, unlike RP and exit, they are not universal. For instance, in contrast to budding yeast, mammalian cells do not have a telophase checkpoint. In mammalian cells, as in most cells, the division plane is determined late in the cell cycle, by the location of the mid-zone of the late anaphase spindle (which is the position where the metaphase

plate assembled). Hence, as long as the M/A transition has been successfully completed, then cell division will automatically separate the two new daughter nuclei. There is no need for a telophase checkpoint, as in budding yeast, where exit from mitosis occurs in two stages.

Furthermore, the DNA damage checkpoint in budding yeast works completely differently than in mammalian cells. Damage in G1 phase causes a specific phosphorylation of Swi6 that blocks transcription of Cln2, the starter kinase [87]. Hence, young yeast cells with damage block at the early G1 checkpoint rather than the late G1 checkpoint. DNA damage received later in the cycle causes a block at the M/A transition in budding yeast cells [88], whereas most other cell types block at the G2/M transition.

Unicellular eukaryotes also differ from metazoan cells in the strength, duration and consequences of checkpoints. The purpose of checkpoints is to block progression through the cell cycle if problems arise that compromise successful replication of the cell and its genome. If the problem can be repaired, then the checkpoint can be lifted and the cell can proceed with the replication-division cycle. But what should the cell do if the problem cannot be repaired? For a unicellular organism, the best strategy is to bypass the checkpoint after some time and proceed to cell division. The worst thing that can happen is that the problem is lethal and the cell dies. But in many cases, the problem is not lethal, the cell survives and reproduces, and the daughter cells 'get on with life'. Maybe they carry some new mutations, maybe they are aneuploid or polyploid…but at least they are alive. For metazoans, on the contrary, these damaged cells inhabit a larger organism, and the mutations they carry may prove advantageous for the cell but fatal for the whole organism (think of malignant cancer cells). Hence, mammalian cells (which have been most thoroughly studied in this regard) tend to have stronger checkpoints, and if the damage cannot be repaired then the surveillance mechanism redirects the cell toward programmed cell death [89]. Better that the damaged cell be destroyed than that its progeny destroy the whole organism.

Conclusions

If our vision of the eukaryotic cell cycle control system is correct, then it should account naturally for the four characteristic features of mitotic cell division enumerated in the introduction. The first feature, that cell cycle progression is unidirectional and irreversible, and the third feature, that checkpoints guard the major transitions of the cell cycle, are the fundamental ideas behind our theory. We have shown how these features are based on the dynamics of the interacting genes and proteins that govern cell cycle progression.

The second feature, balanced growth and division, is a special case of the checkpoint paradigm. In unicellular eukaryotes, growth to a minimal size is a requirement for passing one of the checkpoints of the cell cycle. In budding yeast, the size requirement is enforced at Start [55,90]. In fission yeast, it is enforced at the G2/M transition [91], as is also true of the acellular slime mold, Physarum polycephalum [92]. Strict size control is also evident in the physiology of *Stentor* [93] and *Amoeba* [94,95], although the molecular details have never been worked out. By mutations, the size checkpoint can be moved to a different transition point; for example, *wee1Δ* mutants of fission yeast are size-regulated at the G1/S transition. In budding yeast, it should be noted, size control is strong in small daughter cells but weak in large mother cells [90]. It matters little that mother cells get progressively larger from generation to generation, because they eventually senesce and die.

Size control is less evident in metazoans than in yeast [96,97]. Unlike yeast cells,

which maintain a stable size distribution over many generations of growth and division [98], cell lineages in metazoans are more variable in size, especially lineages of restricted proliferative potential. We would expect size control to be more evident in cells with high proliferative capacity (germ line cells and stem cells).

Eggs and early embryos are interesting cases. Eggs grow very large and arrest in meiosis II with a single, haploid nucleus. After fertilization, the diploid zygote undergoes a series of rapid mitotic cycles (without growth) to create a ball of small, mono-nucleated cells (the blastula). Although details of these early embryonic divisions vary from one organism to another, in general the cell cycle checkpoints are not operating as usual. Around the mid-blastula stage, the mitotic division cycles change dramatically, acquiring G1 and G2 phases and checkpoint controls, including growth controls. At this point the embryo starts to grow and develop, using resources stored in the egg or provided by the placenta.

The fourth feature, that the control system must be robust in the face of unavoidable molecular noise in the small environs of a cell, is beyond the scope of this review. Suffice it to say that in recent publications we have studied realistic stochastic models of bistability and irreversibility in the cell cycle engine and found that the control system shows exactly the same sort of robustness-variability exhibited by proliferating yeast cells [99-101].

If we have done our job well in this review, then everything we have said should seem natural and intuitively appealing. If everything is so obvious, the skeptical reader might ask, "Why do we need mathematical models, saddle-node bifurcations and pseudo-phase planes? It's all right there in the reaction networks!" We hope most readers will not be so jaded. These ideas were not so obvious to us until we started thinking about cell cycle regulation in terms of mathematical models. We are convinced that a mathematical, systems-level approach to regulatory networks is absolutely essential to a correct understanding of physiological control systems like the eukaryotic cell cycle.

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Appendix: Biochemical Mechanisms Underlying Bistability

John J. Tyson[1*] and Béla Novák[2]

1. Mutual Antagonism

The dynamical properties of the molecular regulatory system in Figure A1A can be described by a differential equation for $B = [\text{CycB:Cdk1}]$,

$$\frac{\mathrm{d}B}{\mathrm{d}t} = k_{sb} - k_{db}B - k_{dbc}C_A B, \tag{A1.1}$$

where k_{sb}, k_{db} and k_{dbc} are rate constants. The first subscript, 's' or 'd', refers to the type of reaction, 'synthesis' or 'degradation', the second subscript, 'b' in this case, refers to the chemical being synthesized or degraded, and the third subscript, 'c', refers to the enzyme catalyzing the reaction (whenever relevant). In Eq. (A1.1) C_A is the activity of the Cdh1:APC complex, which is given by the steady state solution of the multisite phosphorylation chain in Figure A1A. According to Kapuy et al. {, 2009 #149}, C_A is given by the function

$$C_A = \frac{1 - (B/H)^{q+1}}{1 - (B/H)^{N+1}}. \tag{A1.2}$$

where q is the threshold number of phosphate groups above which Cdh1P$_i$ is inactive. In Eq. (A1.2), H = activity of the Cdk-counteracting phosphatase.

A representative set of parameter values for this dynamical system is given in Table A1. For this parameter set we plot C_A as a function of B in Figure A1B. In Figure A1C we plot, as functions of B, the two rate curves:

$$V_{\text{synthesis}} = k_{sb}, \text{ and } V_{\text{degradation}} = (k_{db} + k_{dbc}C_A)B. \tag{A1.3}$$

The points of intersection of these two curves (where $V_{\text{synthesis}} = V_{\text{degradation}}$) are steady state solutions of Equation (A1.1). Clearly, the dynamical system may exhibit bistability, depending on the relative values of its parameters. In Figure A1D we indicate how the steady state values of B depend on H, with all other parameters fixed at their values in Table A1.

Table A1. Parameter values for the CycB-Cdh1model.

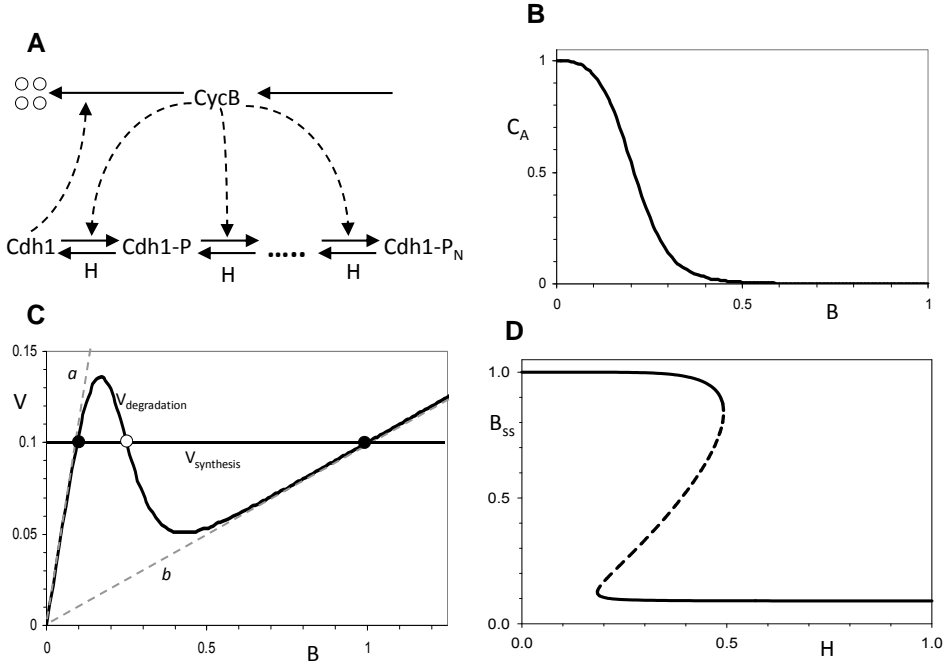| Parameter	Value	Parameter	Value
k_{sb}	0.1	q	2
k_{db}	0.1	N	9
k_{dbc}	1	H	0.25

A

B

C

D

Figure A1

Figure A1. Bistability in a model of mutual antagonism. (A) Molecular regulatory network. 'CycB' represents the CycB:Cdk1 heterodimer (a protein kinase) and 'Cdh1' represents the Cdh1:APC complex (an E3 ubiquitin ligase). Cdh1 has multiple sites (N) of phosphorylation by CycB:Cdk1. The first q states of phosphorylation (Cdh1, Cdh1-P, …, Cdh1-P_q) are assumed to be active, and states Cdh1-P_{q+1}, …, Cdh1-P_N inactive. H is a CDK-counteracting phosphatase. (B) Steady-state activity of Cdh1 as a function CycB-dependent kinase activity, from Eq. (A1.2). (C) Rates of synthesis and degradation of CycB, as functions of CycB-dependent kinase activity, from Eq. (A1.3). The intersection points correspond to two stable steady states of the dynamical system (black circles) and one unstable steady state (white circle). The slope of line a is $k_{db} + k_{dbc}C_T$, and the slope of line b is k_{db}. (D) Bifurcation diagram. The steady state values of B, from panel C, are plotted as functions of H, the activity of the counteracting phosphatase. For $0.184 < H < 0.492$, the regulatory network has three steady states, two stable (solid lines) and one unstable (dashed line). The turning points, at $H = 0.184$ and 0.492, are called saddle-node bifurcation points.

2. Stoichiometric Inhibitor

The dynamical properties of the molecular regulatory system in Figure A2A can be described by a pair of differential equations for $B_T = [CycB]_{total}$ and $I_T = [CKI]_{total}$

$$\frac{dB_T}{dt} = k_{sb} - k_{db}B_T \,, \tag{A2.1A}$$

$$\frac{dI_T}{dt} = k_{si} - \left(k_{di} + k_{dis} \cdot S + k_{dib} \cdot B\right) \cdot I_T \,. \tag{A2.1B}$$

In Eq. (A2.1B), $S = $ [Starter Kinase] = adjustable parameter, and $B = [CycB]_{free} = $ active CycB-dependent kinase uncomplexed with CKI. If we assume that the CycB:CKI complex is in equilibrium with the unbound ('free') forms of the components, then

$$K_{diss} \cdot X = B \cdot I = \left(B_T - X\right) \cdot \left(I_T - X\right), \tag{A2.2}$$

where $X = $ [Complex], $B = B_T - X$, $I = I_T - X$, and $K_{diss} = $ equilibrium dissociation constant of the complex (units = concentration). Assuming that B_T quickly reaches the steady state value determined by Eq. (A2.1B), we can set $B_T = k_{sb}/k_{db}$. In this case, X is the root of the quadratic equation that lies between 0 and $\min(B_T, I_T)$, namely,

$$X = \frac{2B_T I_T}{B_T + I_T + K_{diss} + \sqrt{\left(B_T + I_T + K_{diss}\right)^2 - 4B_T I_T}} \,. \tag{A2.3}$$

A representative set of parameter values for this dynamical system is given in Table A2. For this parameter set we plot B as a function of I_T in Figure A2B. In Figure A2C we plot, as functions of I_T, the two rate curves:

$$V_{synthesis} = k_{si}, \text{ and } V_{degradation} = (k_{di} + k_{dis}S + k_{dib}B)I_T \,. \tag{A2.4}$$

The points of intersection of the two rate curves are steady state solutions of Equation (A2.1B). Clearly, the dynamical system may exhibit bistability, and in Figure A2D we indicate how the steady state values of B depend on S, with all other parameters fixed at their values in Table A2.

Table A2. Parameter values for the CycB-CKI model.

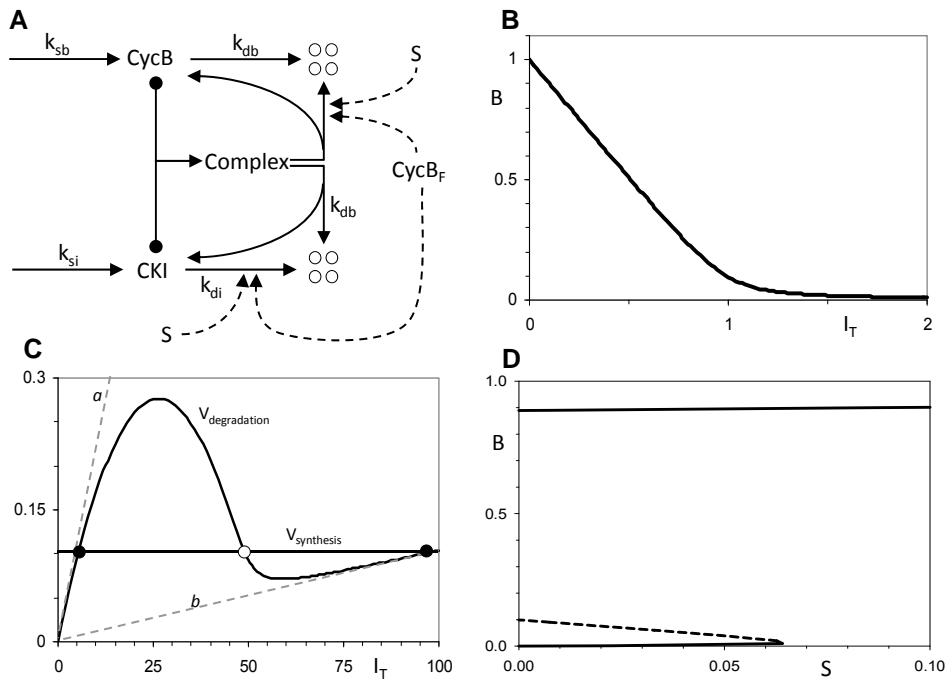| Parameter	Value	Parameter	Value
$B_T = k_{sb} / k_{db}$	1	k_{di}	0.01
$k_{dis} = k_{dib}$	1	K_{diss}	0.002
k_{si}	0.1	S	0.04

Figure A2

Figure A2. Bistability in a model of stoichiometric inhibition. (A) Molecular regulatory network. 'CycB' = CycB:Cdk1 heterodimer (a protein kinase) and 'CKI' = cyclin-dependent kinase inhibitor (i.e., the trimeric complex, CKI:CycB:Cdk1, is inactive). Active CycB-dependent kinase can phosphorylate CKI, priming it for poly-ubiquitination and subsequent degradation. S is a 'starter kinase'. The T-shaped arrow with black dots on the crossbar represents the reversible association and dissociation of the two components on the crossbar to form the complex at the end of the arrow. (B) Concentration of free CycB:Cdk1 dimer as a function of total concentration of CKI, $B = B_T - X$, with X calculated from Eq. (A2.3). (C) Rates of synthesis and degradation of CKI as functions of $[CKI]_{total}$, from Eq. (A2.4). The intersection points correspond to stable (black circles) and unstable (white circle) steady states of the dynamical system. The slope of line a is $k_{di} + k_{dib}B_T$, and the slope of line b is k_{di}. (D) Bifurcation diagram. The steady state values of B are plotted as functions of S, the activity of starter kinase. For $0 < S < 0.065$, the regulatory network has three steady states, two stable (solid lines) and one unstable (dashed line).

3. Restriction Point

The dynamical properties of the molecular regulatory system in Figure A3A can be described by a pair of differential equations for $E = $ [CycE] and $R_P = $ [RB-P]

$$\frac{dE}{dt} = k_{se} + k_{sef}F - k_{de}E \,, \tag{A3.1A}$$

$$\frac{dR_P}{dt} = \left(k_{prs} \cdot S + k_{pre} \cdot E\right) \cdot \left(R_T - R_P\right) - k_{hrh} \cdot H \cdot R_P \,, \tag{A3.1B}$$

where k_{pre} and k_{prs} are rate constants for the phosphorylation of RB by CycE-dependent kinase and by a starter kinase (parameter S), and k_{hrh} is the rate constant for the dephosphorylation of RB-P by a counteracting phosphatase (parameter H). In Eqs. (A3.1), F = [E2F]$_{free}$ = [E2F]$_{total}$ – [RB:E2F], and R_T = [RB]$_{total}$ = [RB]$_{free}$ + [RB:E2F] + [RB-P].

Solving Eq. (A3.1B) for the steady state value of R_P, we find

$$\frac{R_P}{R_T} = \frac{k_{prs} \cdot S + k_{pre} \cdot E}{k_{prs} \cdot S + k_{pre} \cdot E + k_{hrh} \cdot H} = \frac{S + E}{S + E + H} \,, \tag{A3.2}$$

where the latter equality derives from the fact that, without loss of generality, we can choose the units of S and H so that $k_{prs} = k_{pre} = k_{hrh}$. Next, we substitute $R_T - R_P = R_T H/(S+E+H)$ into the quadratic equation

$$K_{diss} \cdot X = R \cdot F = \left(R_T - R_P - X\right) \cdot \left(F_T - X\right) \,, \tag{A3.3}$$

that determines the concentration of the RB:E2F complex, X, in terms of the concentrations of free E2F and free RB, given K_{diss} = equilibrium dissociation constant of the complex. We do not display the algebraic form of X as a function of E, but we plot it in Figure A3B, along with $F = F_T - X$ as a function of E. The functions are plotted for a representative set of parameter values given in Table A3.

In Figure A3C we plot, as functions of E, the two rate curves:

$$V_{synthesis} = k_{se} + k_{sef}F, \text{ and } V_{degradation} = k_{de}E. \tag{A3.4}$$

The points of intersection of the two rate curves are steady state solutions of Equations (A3.1A,B). Clearly, the dynamical system may exhibit bistability, and in Figure A3D we

indicate how the steady state values of E depend on S, with all other parameters fixed at their values in Table A3.

Table A3. Parameter values for the RB-E2F model.

Parameter	Value	Parameter	Value
k_{se}	0.002	H	0.06
k_{sef}	0.2	S	0.05
k_{de}	0.3	F_T	1
$k_{prs} = k_{pre}$	1	R_T	2
k_{hrh}	1	K_{diss}	0.002
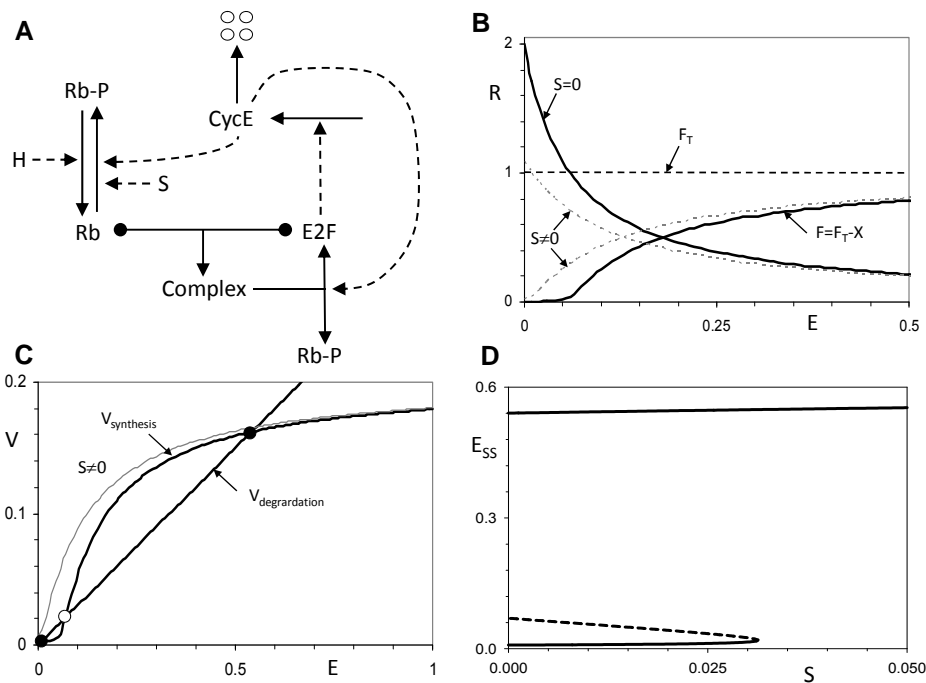
Figure A3

Figure A3. Bistability in a model of the restriction point. (A) Molecular regulatory network. 'CycE' = CycE:Cdk2 heterodimer (a protein kinase active in late G1 phase of the cell cycle), 'E2F' = transcription factor for CycE, and 'RB' = retinoblastoma protein (a stoichiometric inhibitor of E2F). Active CycE-dependent kinase can phosphorylate RB, causing it to release E2F. S is a 'starter kinase'. (B) Concentration of active RB (free RB + RB:E2F complexes) and concentration of free (active) E2F, as functions of CycE-dependent kinase activity. (C) Rates of synthesis and degradation of CycE as functions of CycE-dependent kinase activity, from Eq. (A3.4). The intersection points correspond to stable (black circles) and unstable (white circle) steady states of the dynamical system. The lower asymptote of the synthesis curve is k_{se}, and the upper asymptote is $k_{se} + k_{sef}F_T$. (D) Bifurcation diagram. The steady state values of E are plotted as functions of S, the activity of starter kinase. For $0 < S < 0.031$, the regulatory network has three steady states, two stable (solid lines) and one unstable (dashed line).

4. Anaphase Switch

The dynamical properties of the molecular regulatory system in Figure A4A can be described by a set of differential equations for B = [CycB], M_{AT} = [Mad2$_A$]$_{free}$ + [Mad2$_A$:Cdc20], and X = [Mad2$_A$:Cdc20],

$$\frac{dB}{dt} = k_{sb} - k_{db}B - k_{dbc}C \cdot B , \qquad (A4.1A)$$

$$\frac{dM_{AT}}{dt} = k_{amb}L \cdot B \cdot M_I - k_{imh}H \cdot M - k_{break}C \cdot X , \qquad (A4.1B)$$

$$\frac{dX}{dt} = k_{assoc}M \cdot C - k_{dissoc}X - k_{break}C \cdot X , \qquad (A4.1C)$$

where C = [Cdc20]$_{active}$ = $C_T - X$, M_I = [Mad2]$_{inactive}$ = $M_T - M_{AT}$, and M = [Mad2$_A$]$_{free}$ = $M_{AT} - X$. As usual, C_T = [Cdc20]$_{total}$ and M_T = [Mad2]$_{total}$. The rate constants k_{amh} and k_{imh} refer to the activation and inactivation of Mad2, k_{assoc} and k_{dissoc} refer to the association and dissociation of the Mad2$_A$:Cdc20 complex, and k_{break} refers to the breakdown of the Mad2$_A$:Cdc20 complex induced by active Cdc20 itself. The parameter L = fraction of tensionless chromosomes (i.e., replicated chromosomes that are not properly aligned on the metaphase spindle), and the H = activity of a counteracting phosphatase.

The steady state solution of Eq. (A4.1A) is $B = B_T C_0/(C_0+C)$, where $B_T = k_{sb}/k_{db}$ and $C_0 = k_{db}/k_{dbc}$. The steady state solution of Eq. (A4.1C) satisfies the quadratic equation

$$K_{diss}X + \kappa_{break}\left(C_T - X\right) \cdot X = \left(M_{AT} - X\right) \cdot \left(C_T - X\right). \qquad (A4.2)$$

where $K_{diss} = k_{dissoc}/k_{assoc}$ and $\kappa_{break} = k_{break}/k_{assoc}$. Equation (A4.2) can be solved for X as a function of C_T and M_{AT}, as in Eq. (A2.3). In Fig. A4B we plot X as a function of M$_{AT}$, along with $C = C_T - X$ and $B = B_T C_0/(C_0+C)$.

Substituting the results of the previous paragraph into Eq. (A4.1B), we obtain

$$\frac{dM_{AT}}{dt} = k_{amb}L \cdot \frac{B_T C_0}{C_0 + C_T - X} \cdot \left(M_T - M_{AT}\right) - \left[k_{imh}H \cdot \left(M_{AT} - X\right) + k_{break}\left(C_T - X\right) \cdot X\right]$$

$$= \qquad V_{production} \qquad - \qquad V_{removal} \qquad (A4.3)$$

In Figure A4C we plot the two rate laws, $V_{\text{production}}$ and V_{removal}, as functions of M_{AT}, for a representative set of parameter values given in Table A4. The points of intersection of the two rate curves are steady state solutions of Equations (A4.3). Clearly, the dynamical system may exhibit bistability, and in Figure A4D we indicate how the steady state values of M_{AT} depend on L, with all other parameters fixed at their values in Table A4. Notice that Mad2 remains active until $1 - L$, the fraction of aligned chromosomes, gets very close to 1, and then Mad2 is abruptly inactivated as the last chromosome aligns on the metaphase plate and the cell proceeds to anaphase.

Table A4. Parameter values for the Mad2-Cdc20 model.

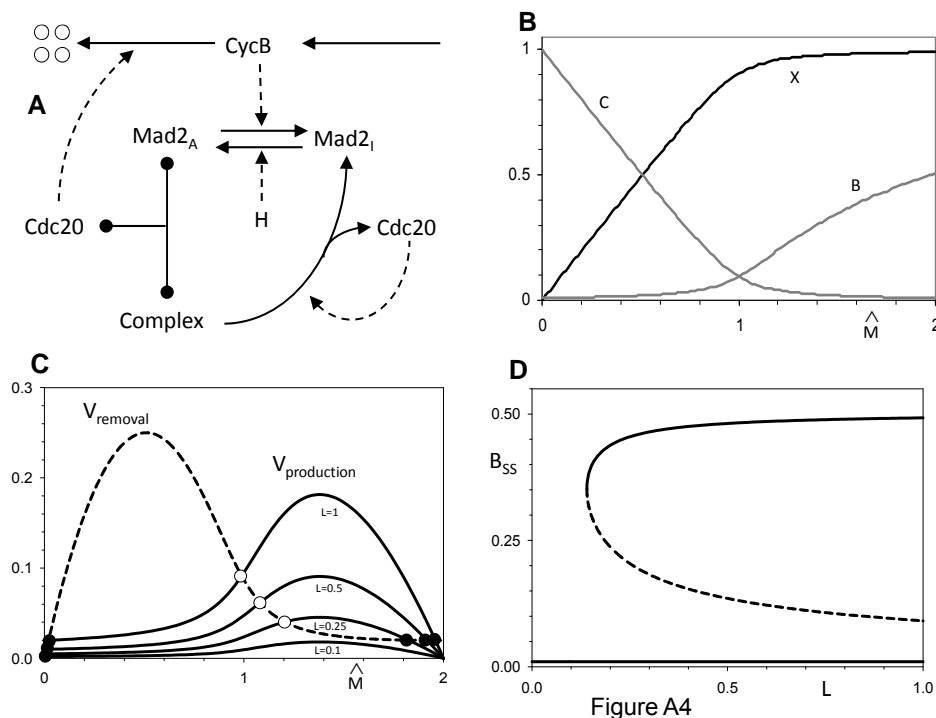| Parameter	Value	Parameter	Value
k_{amb}	1	$K_{\text{diss}} = k_{\text{diss}}/k_{\text{assoc}}$	0.01
k_{imh}	0.01	$K_{\text{break}} = k_{\text{break}}/k_{\text{assoc}}$	0.001
k_{break}	1	H	1
$B_T = k_{\text{sb}}/k_{\text{db}}$	1	M_T	2
$C_0 = k_{\text{db}}/k_{\text{dbc}}$	0.01	C_T	1

Figure A4. Bistability in a model of the anaphase switch. (A) Molecular regulatory network. 'CycB' = CycB:Cdk1 heterodimer, 'Cdc20' = Cdc20:APC complex (an E3 ubiquitin ligase), 'Mad2' = stoichiometric inhibitor of Cdc20:APC. Active Cdc20:APC primes CycB for degradation. CycB-dependent kinase activates Mad2, in conjunction with 'tensionless' chromosomes, L. H = Clb2-counteracting phosphatase. Notice that active Cdc20:APC promotes dissociation of the Mad2:Cdc20:APC complex. (B) The concentration, X, of the Mad2$_A$:Cdc20 complex is plotted as a function of $M_{\text{AT}} = [\text{Mad2}_A]_{\text{free}} + [\text{Mad2}_A:\text{Cdc20}]$. In

addition, we plot $C = [\text{Cdc20}]_{\text{active}} = C_T - X$ and $B = [\text{CycB}]_{\text{ss}} = B_T C_0/(C_0+C)$ as functions of M_{AT}. (C) Rates of production and removal of active Mad2 as functions of M_{AT} from Eq. (A4.3). The production rate curve is drawn for several values of L = fraction of tensionless chromosomes. The intersection points correspond to stable (black circles) and unstable (white circle) steady states of the dynamical system. (D) Bifurcation diagram. The steady state values of M_{AT} are plotted as functions of L. For $0.13 < L < 1$, the regulatory network has three steady states, two stable (solid lines) and one unstable (dashed line).

5. G2/M Checkpoint

The dynamical properties of the molecular regulatory system in Figure A5A can be described by three differential equations for $B = [\text{MPF}] = [\text{CycB:Cdk1}]$, $W = [\text{Wee1}]$ and $D_P = [\text{Cdc25-P}]$:

$$\frac{dB}{dt} = \left(k_{ab} + k_{abd}D_P\right)\cdot\left(B_T - B\right) - \left(k_{ib} + k_{ibw}W\right)\cdot B, \quad \text{(A5.1A)}$$

$$\frac{dD_P}{dt} = k_{adb}B\cdot\left(D_T - D_P\right) - k_{idh}H\cdot D_P, \quad \text{(A5.1B)}$$

$$\frac{dW}{dt} = k_{awh}H\cdot\left(W_T - W\right) - k_{iwb}B\cdot W, \quad \text{(A5.1C)}$$

where $k_{a...}$ and $k_{i...}$ are rate constants for activation and inactivation of the corresponding proteins, and the parameter H is the activity of the MPF-counteracting phosphatase. Without loss of generality, we can choose the units of H so that $k_{idh} = k_{adb}$.

The steady state solutions of Eqs. (A5.1B,C) are $D_P = D_T B/(H+B)$ and $W = W_T \varepsilon H/(\varepsilon H+B)$, where D_T and W_T are the total concentrations of Cdc25 and Wee1, as usual, and $\varepsilon = k_{awh}/k_{iwb}$. D_P and W as functions of B are plotted in Figure A5B, for the representative parameter values in Table A5. The rate curves,

$$\frac{dB}{dt} = \left(k_{ab} + k_{abd}D_T\frac{B}{H+B}\right)\cdot\left(B_T - B\right) - \left(k_{ib} + k_{ibw}W_T\frac{\varepsilon H}{\varepsilon H + B}\right)\cdot B,$$
$$= \qquad V_{\text{activation}} \qquad - \qquad V_{\text{inactivation}} \qquad \text{(A5.2)}$$

are plotted in Figure A5C. The points of intersection of these two curves are steady state solutions of Eq. (A5.2). Clearly, the dynamical system may exhibit bistability, and in Figure A5D we indicate how the steady state values of B depend on B_T, with all other parameters fixed at their values in Table A5.

Table A5. Parameter values for the Wee1-MPF-Cdc25 model.

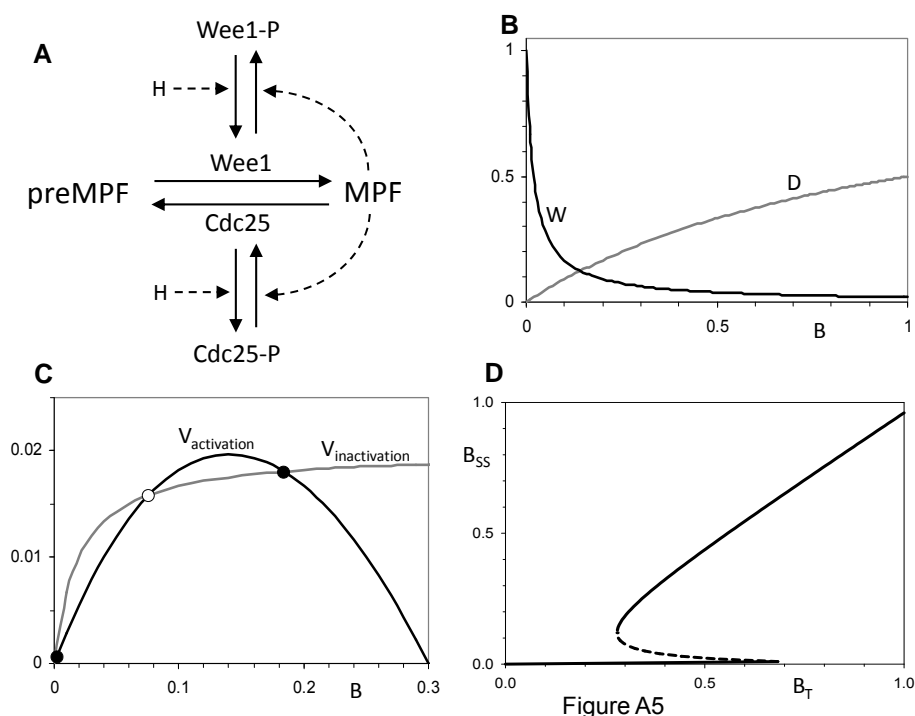| Parameter	Value	Parameter	Value
k_{ab}	0	H	1
k_{abd}	1	B_T	0.3
k_{ib}	0	D_T	1
k_{ibw}	1	W_T	1
k_{awh}	0.02	k_{iwb}	1

Figure A5. Bistability in a model of the G2/M transition. (A) Molecular regulatory network. 'MPF' = active CycB:Cdk1 kinase, 'preMPF' = inactive CycB:Cdk1-P dimer, 'Wee1' = tyrosine kinase (less active in the phosphorylated form), 'Cdc25' = tyrosine phosphatase (more active in the phosphorylated form). H = Clb2-counteracting phosphatase. (B) Active forms of Wee1 and Cdc25 as functions of active MPF. (C) Rates of activation and inactivation of MPF as functions of MPF activity, from Eq. (A5.2). The intersection points correspond to stable (black circles) and unstable (white circle) steady states of the dynamical system. (D) Bifurcation diagram. The steady state values of active MPF are plotted as functions of total cyclin B, B_T = [MPF] + [preMPF]. For $0.28 < B_T < 0.69$, the regulatory network has three steady states, two stable (solid lines) and one unstable (dashed line).