Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability

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Cell signaling systems that contain positive-feedback loops or double-negative feedback loops can, in principle, convert graded inputs into switch-like, irreversible responses. Systems of this sort are termed 'bistable'. Recently, several groups have engineered artificial bistable systems into *Escherichia coli* and *Saccharomyces cerevisiae*, and have shown that the systems exhibit interesting and potentially useful properties. In addition, two naturally occurring signaling systems, the p42 mitogen-activated protein kinase and c-Jun amino-terminal kinase pathways in *Xenopus* oocytes, have been shown to exhibit bistable responses. Here we review the basic properties of bistable circuits, the requirements for construction of a satisfactory bistable switch, and the recent progress towards constructing and analysing bistable signaling systems.

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Abbreviations

Cdk	cyclin-dependent kinase
ERK	extracellular-signal-regulated kinase
GFP	green fluorescent protein
IPTG	isopropylthiogalactoside
JNK	c-Jun amino-terminal kinase
MAPK	mitogen-activated protein kinase
rtTA	tetracycline-responsive transactivator

Introduction

Most, perhaps all, of the biochemical reactions involved in cell signaling are reversible. Proteins are phosphorylated and dephosphorylated; G proteins cycle between GTP- and GDP-bound forms; second messengers are synthesized and degraded, or released and sequestered; proteins are imported into the nucleus and exported back out; and on and on. Even proteolysis is ultimately reversible, since degraded proteins are replaced by new synthesis.

But many biological transitions are essentially irreversible. For example, under most circumstances the differentiated state is stable, and cells remain differentiated for years or decades after the stimulus that initially triggered their differentiation has been withdrawn. Likewise, most cell cycle transitions are irreversible; cells can go from G2 phase to M phase, but not back. How might the reversible activation of cell signaling pathways lead to practically irreversible changes in cell fate, given that phosphorylations turn over on a time scale of minutes and proteins turn over on a time scale of hours?

This question was addressed by Monod and Jacob 40 years ago, in an influential (and rather formidably titled) paper published in a Cold Spring Harbor Symposium on Quantitative Biology [1]. They proposed that the answer lay in the way gene regulatory systems are wired. They devised six specific types of signal transduction circuits that could be capable of 'remembering' a transient differentiation stimulus long after the stimulus was removed. Each of the circuits was built out of regulatory elements known from studies of gene regulation in prokaryotes, and each was some variation of a 'double-negative' feedback circuit.

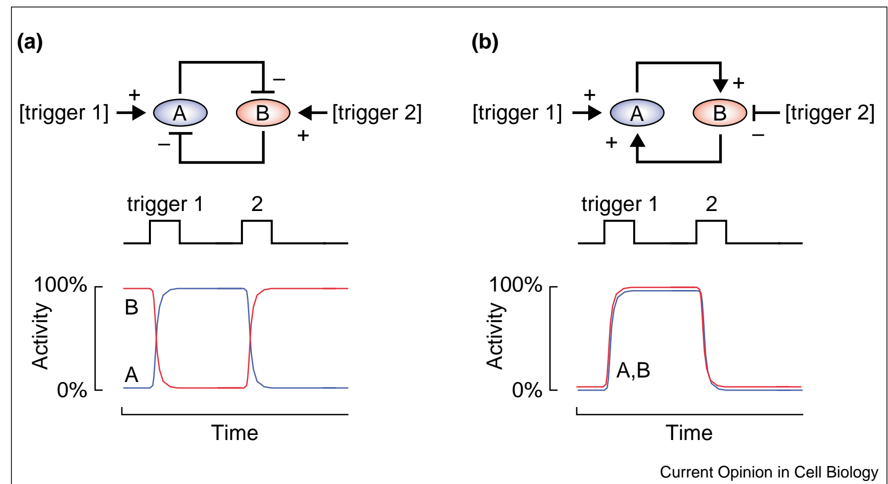
A simple example of such a circuit is shown in Figure 1a. Suppose that there are two gene products, A and B, each of which inhibits the other's transcription. Then, under the right circumstances (see 'The nuts and bolts of bistability' section below), the system could have a stable state with A on and B off, or an alternative stable state with A off and B on. Once either state has been established, it could persist indefinitely, being reinforced by the double-negative feedback loop, until some trigger stimulus forces the system to the other state. These circuits were originally envisioned as ways of achieving self-sustaining patterns of gene expression, but it is easy to see how double-negative feedback at a post-translational level could produce self-sustaining patterns of protein activity.

Self-sustaining patterns of gene expression or protein activity could also be achieved through positive feedback (Figure 1b). In this case the system would toggle back and forth between a state with both A and B off and a state with both A and B on.

The most daring and prescient aspect of Monod and Jacob's paper was probably not the supposition that feedback loops are present in eukaryotic signal transduction systems, although little was understood about eukaryotic gene regulation at the time. Rather, it was the idea that feedback loops, and not any of the myriad other conceivable mechanisms for producing biological irreversibility, might be the way differentiation is maintained and irreversibility achieved. These ideas remained largely untested beyond the confines of a few familiar prokaryotic systems — most notably the λ phage lysis/lysogeny switch and the *Escherichia coli lac* operon — until recently.

Over the past few years, there has been a resurgence of interest in feedback loops and self-perpetuating states in

Figure 1

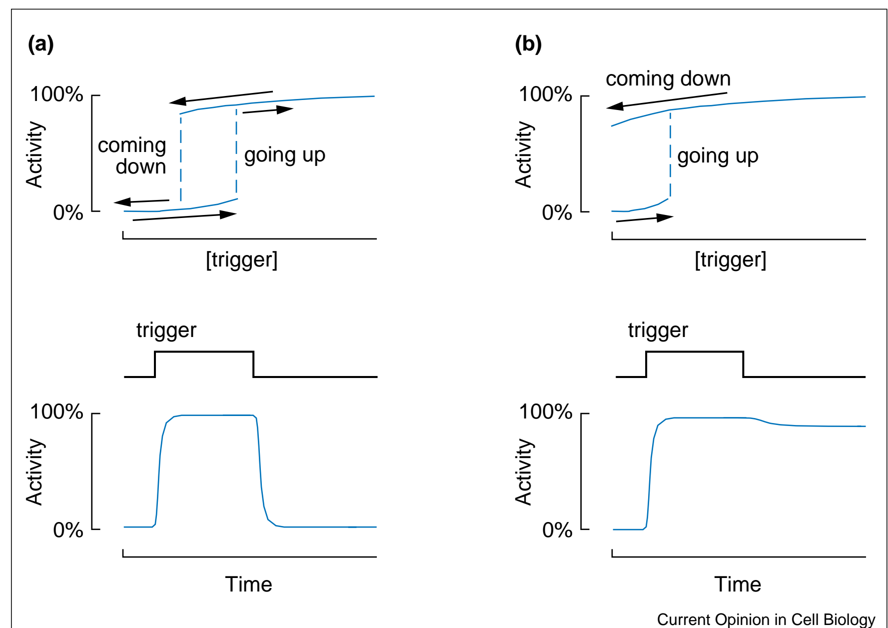


Bistable signal transduction circuits. **(a)** A double-negative feedback loop. In this circuit, protein A (blue) inhibits or represses B (red), and protein B inhibits or represses A. Thus there could be a stable steady state with A on and B off, or one with B on and A off, but there cannot be a stable steady with both A and B on or both A and B off. Such a circuit could toggle between an A-on state and a B-on state in response to trigger stimuli that impinge upon the feedback circuit. **(b)** A positive feedback loop. In this circuit, A activates B and B activates A. As a result, there could be a stable steady state with both A and B off, or one with both A and B on, but not one with A on and B off or *vice versa*. Both types of circuits could exhibit persistent, self-perpetuating responses long after the triggering stimulus is removed.

cell signaling. In part, this may represent a natural next step from the pre-genomic era's focus on individual gene products towards the post-genomic era's desire to understand the incredibly complex networks present in the whole cell. There is the hope that small modular circuits, including those envisioned by Monod and Jacob, may be sophisticated enough to yield interesting behavior, yet still simple enough to understand at an intuitive level [2]. Perhaps by studying the properties of individual signaling elements, then elementary circuits, then more complex networks, biologists can work their way up to an understanding of cellular behavior in the same way that electrical engineers work their way up from the properties of resistors, capacitors and diodes, to those of simple circuits and, finally, complex devices.

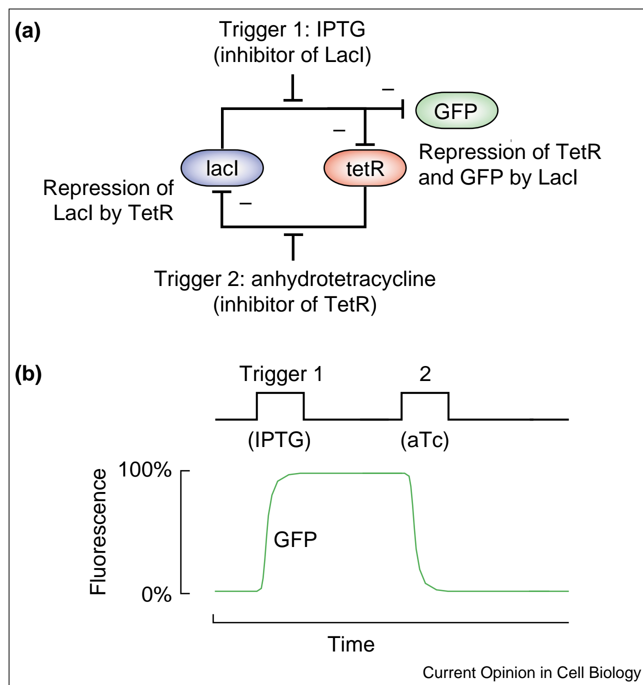
Here we will briefly review what is required to produce a bistable system, which is defined as a system that can toggle between two alternative stable steady-states but cannot rest in intermediate states, and under what circumstances a bistable system will convert a transient trigger stimulus into an irreversible response. We will then review recent experimental work on bistable biological systems. These include the engineering of simple bistable circuits in *E. coli* and *S. cerevisiae*, as well as the identification and analysis of natural bistable signaling circuits. Both types of

Figure 2



Hysteresis and irreversibility in bistable signaling circuits. **(a)** Hysteresis. Any bistable circuit should exhibit some degree of hysteresis, meaning that different stimulus/response curves are obtained depending upon whether the system began in its off or its on state. **(b)** Irreversibility. If the feedback in a bistable circuit is sufficiently strong, the circuit may exhibit true irreversibility, so that the system stays in its on state indefinitely after the triggering stimulus is removed.

Figure 3



An artificial bistable system in *E. coli*. **(a)** Design of the system. Gardner *et al.* [9••] engineered two double-negative feedback systems into *E. coli*. In the system shown here, LacI represses the expression of TetR (and GFP, used as a reporter of the status of *tetR* transcription), and TetR represses the expression of LacI. **(b)** Response of the system. The authors showed that the system could be made to toggle between the TetR-off and TetR-on states by the addition of external trigger stimuli: IPTG to disinhibit *tetR*, and anhydrotetracycline (aTc) to disinhibit *lacI*.

studies provide important tests of our understanding of signal transduction circuits.

The nuts and bolts of bistability

The ingredients required for bistability include some sort of feedback — positive feedback (Figure 1b), double-negative feedback (Figure 1a), autocatalysis, or the equivalent — but feedback alone does not guarantee that a system will be bistable [3,4•–6•]. A bistable system must also possess some type of non-linearity within the feedback circuit. That is, some of the enzymes in the feedback circuit must respond to their upstream regulators cooperatively, or, more generally, in an 'ultrasensitive' manner [7,8]. In addition, the two legs of the feedback loop must be properly balanced for the circuit to exhibit bistability; if either leg is too strong or too weak, the circuit will be monostable rather than bistable. Thus, feedback is required for bistability, but does not guarantee it.

In addition, bistability does not guarantee irreversibility. A bistable circuit will always exhibit some degree of hysteresis, meaning that it will be harder to flip the system from one state to the other than it is to maintain the system in its flipped state (Figure 2a). The limiting case where even zero stimulus is sufficient to maintain the flipped state corresponds to the type of irreversibility Monod and Jacob envisioned (Figure 2b). Irreversibility is achieved when a bistable system has very strong feedback.

Engineering artificial bistable systems: a synthetic toggle switch in *E. coli*

To test the practicality of simple bistable systems, several laboratories have now engineered artificial bistable systems in *E. coli* and yeast. The first of these were described by Gardner *et al.* [9••]. They designed double-negative feedback systems using well-described prokaryotic gene repressor proteins — the *lac* repressor (LacI), the *tet* repressor (TetR), and the lambda repressor (λcI). Pairs of these repressors (LacI and TetR, or LacI and λcI) were arranged so that each repressor inhibited the transcription of the other, and these circuits were expressed in *E. coli*. One of the engineered circuits is shown schematically in Figure 3a: TetR inhibits expression of LacI, and LacI inhibits expression of both TetR and a green fluorescent protein (GFP) reporter. The other ingredients required for bistability — some source of ultrasensitivity and a proper balance between the two legs of the circuit — were provided by cooperativity in the binding of the repressors to DNA and by trial-and-error balancing of promoter strengths.

Gardner *et al.* [9••] found that populations of bacteria expressing the circuit shown in Figure 3a turned on their GFP expression fairly abruptly once the concentration of the trigger stimulus isopropylthiogalactoside (IPTG) exceeded about 30–40 μM. At the level of the individual bacterium, the expression of GFP was even more switch-like; flow cytometry showed that individual bacteria from the 30 μM IPTG cultures expressed either very low or very high levels of GFP, with few bacteria expressing intermediate levels. Both the GFP-on state and GFP-off state persisted for many hours after removal of the trigger stimulus (IPTG for the on state; anhydrotetracycline for the off state) (Figure 3b). Thus, both states appeared to be self-perpetuating, and the engineered circuit displayed all of the behaviors expected of a bistable system.

Single- and triple-negative feedback circuits in *E. coli*

A bistable response can arise from circuits with two negative-feedback loops, or four, or any even number, but a circuit with an odd number of negative-feedback loops would be expected to exhibit different properties. A single negative-feedback loop will often produce a stable, autoregulatory, adaptive response, and indeed such a system was engineered recently into *E. coli* and shown to have the expected characteristics [10••]. Negative-feedback circuits can also, under the right circumstances, give rise to long-standing oscillations. Elowitz and Leibler [11••] engineered a triple-negative feedback circuit (with TetR repressing λcI and a GFP reporter, λcI repressing LacI, and LacI repressing TetR), which they termed the 'repressilator,' and showed that *E. coli* expressing the repressilator did, in fact, often

exhibit oscillatory GFP fluorescence. The oscillations of the repressilator were noisy and varied in character from cell to cell; it will be interesting to determine to what extent these fluctuations are stochastic effects due to the small numbers of molecules in an individual *E. coli*, or are inherent to this particular type of oscillator circuit.

A fluctuating toggle switch in *S. cerevisiae*

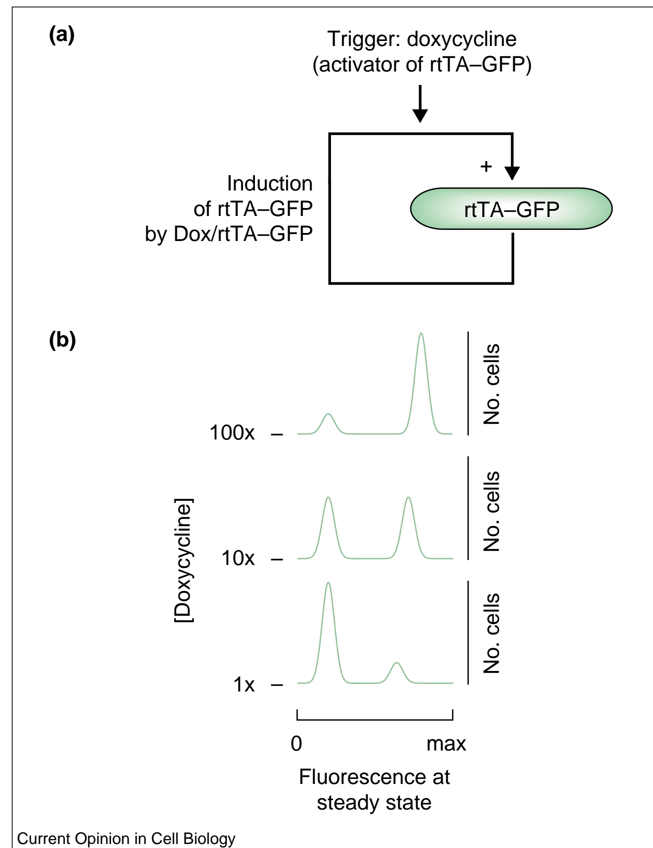
Becskei *et al.* [12••] were the first to engineer an artificial bistable circuit in a eukaryote, *S. cerevisiae*. Their circuit was a positive-feedback loop, based on a tetracycline-responsive transactivator rtTA [13] fused to a GFP reporter. The design of this circuit is shown in Figure 4a. In the absence of tetracycline analogues (doxycycline was used here), only basal concentrations of rtTA–GFP are present; this is the bistable system's 'off' state. When doxycycline is added, the low concentration of rtTA–GFP becomes activated, which induces more rtTA–GFP, which drives the formation of more doxycycline–rtTA–GFP complexes, which induce still more rtTA–GFP, and so on. This positive feedback continues until the system comes into a new balance of rtTA–GFP synthesis and destruction and rtTA–GFP/doxycycline binding and dissociation; this is the 'on' state.

In principle, this system could have behaved very similarly to the double-negative feedback circuit characterized by Gardner *et al.* [9••], with graded changes in the trigger stimulus (doxycycline) translated into a sharp transition between alternative self-perpetuating states. However, the behavior observed by Becskei *et al.* [12••] was different in several important respects. First, the on state was not self-sustaining; withdrawing the doxycycline caused all of the on-state cells to revert to the off state. This is probably because doxycycline is not just a trigger, but an actual part of the feedback loop in this case; without doxycycline present, even high levels of rtTA–GFP might not promote rtTA–GFP transcription. It would be interesting to determine if there was any hysteresis in the doxycycline response. Second, at intermediate concentrations of doxycycline, individual cells clustered in two discrete states, an off state and an on state, as expected; but they also sometimes switched from on to off or off to on. The authors inferred that they had constructed a bistable system with two discrete, alternative steady states, but that stochastic fluctuations within individual cells were sufficient to allow the cells to flip back and forth between the states [12••].

Summary of artificial bistable systems

These studies demonstrate the feasibility of expressing simple signaling circuits in *E. coli* and *S. cerevisiae*, thereby endowing the cells with novel regulatory properties. These studies have important implications from a biotechnology viewpoint: circuits of this sort could be useful in engineering useful new microorganisms or in gene therapy. These studies also hold promise for better understanding of the basic logic of natural signaling circuits.

Figure 4

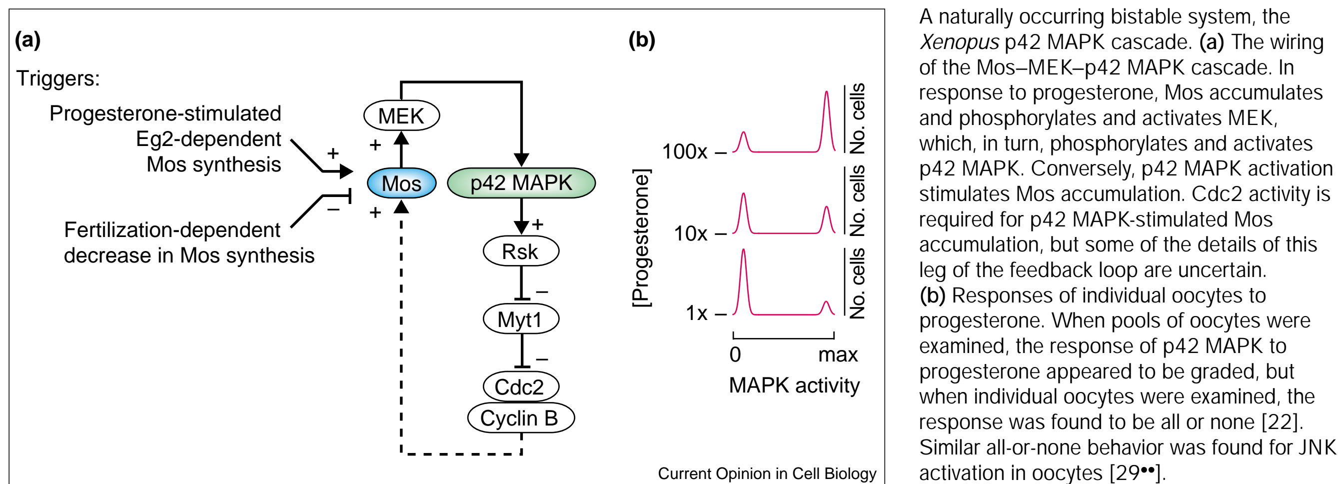


An artificial bistable system in *S. cerevisiae*. **(a)** Design of the system. Becskei *et al.* [12••] engineered several constructs where, in the presence of tetracycline analogues, rtTA–GFP stimulates its own transcription, and expressed them in yeast. **(b)** Response of the system. At the level of a population of yeast cells, the system exhibited a graded response to increasing concentrations of doxycycline (Dox), but at the level of individual cells, the responses appeared to be all or nothing.

Bistability in natural signaling systems: the MAPK cascade in frog oocytes

The best-documented example of a natural system of signal transduction proteins that functions as a bistable switch is probably the Mos–mitogen-activated protein kinase (MAPK) kinase (MEK)–p42 MAPK cascade in *Xenopus* oocytes. This cascade is activated when oocytes are induced to mature — to leave a prolonged G2-phase arrest state, complete the first meiotic division, and arrest in metaphase of meiosis II, with active Cdc2 and active p42 MAPK — by the steroid hormone progesterone [14,15•]. The normal biology of oocyte maturation is all or nothing, and irreversible in character; progesterone-treated oocytes either mature or they don't, and once they *do* mature they do not 'de-mature' if the progesterone is removed. Thus, at some level in the signal transduction process, the oocyte must convert a graded, reversible triggering stimulus — the hormone progesterone — into an all-or-nothing, irreversible cell-fate decision.

Figure 5



A naturally occurring bistable system, the *Xenopus* p42 MAPK cascade. **(a)** The wiring of the Mos–MEK–p42 MAPK cascade. In response to progesterone, Mos accumulates and phosphorylates and activates MEK, which, in turn, phosphorylates and activates p42 MAPK. Conversely, p42 MAPK activation stimulates Mos accumulation. Cdc2 activity is required for p42 MAPK-stimulated Mos accumulation, but some of the details of this leg of the feedback loop are uncertain. **(b)** Responses of individual oocytes to progesterone. When pools of oocytes were examined, the response of p42 MAPK to progesterone appeared to be graded, but when individual oocytes were examined, the response was found to be all or none [22]. Similar all-or-none behavior was found for JNK activation in oocytes [29••].

This is exactly the sort of situation for which bistable circuits might be useful, and indeed the *Xenopus* MAPK cascade appears to possess all of the required ingredients for bistability. First, the cascade is embedded in a positive-feedback loop analogous to that shown in Figure 1b, although more complicated (Figure 5a). The activation of p42 MAPK stimulates the accumulation of its upstream activator, the Mos oncoprotein, probably through both an increase in the rate of Mos translation and a decrease in the rate of Mos proteolysis [16–19]. The exact mechanisms through which p42 MAPK stimulates Mos accumulation are not well understood, although it is clear that Cdc2 is an essential intermediate [20,21]. Second, the cascade generates an ultrasensitive response even when the positive feedback loop is inactivated [22,23]. Some of this ultrasensitivity arises from the two-step non-processive dual phosphorylation mechanism for the activation of p42 MAPK by MEK [24,25]; however, it is clear that other mechanisms must contribute as well. The combination of ultrasensitivity and positive feedback could allow the cascade to function as a bistable system, provided that the legs of the feedback loop are balanced properly. The trigger that turns on this putative bistable system appears to be the Aurora-family kinase Eg2, which, in response to progesterone, activates translation of Mos [26•]. After fertilization, activation of calmodulin-dependent protein kinase II somehow turns off Mos translation and breaks the positive-feedback loop [27].

One way of determining whether the cascade functions as a bistable system is to examine how steep the stimulus/response curve is for the response of p42 MAPK to progesterone. It turns out that when populations of oocytes are examined, the response of p42 MAPK appears to be quite graded: a low concentration of progesterone produces a small p42 MAPK response, and a higher concentration produces a bigger response [22]. However, when individual oocytes are examined, the responses are all or nothing — an intermediate concentration of progesterone causes near-complete activation of p42 MAPK in some oocytes, and near-zero activation in others (Figure 5b) [22]. This is just the sort of behavior seen by Gardner *et al.* and Becskei *et al.* in their artificial bistable systems [9••,12••]. Eliminating the positive-feedback loop by blocking protein synthesis allows individual oocytes to exhibit graded (though still ultrasensitive), rather than all-or-none, responses [22]. The most likely interpretation of these findings is that the MAPK cascade of oocytes exhibits feedback-dependent bistability. An important unanswered question is whether the oocyte MAPK cascade exhibits hysteresis, as expected of all bistable systems, and whether any such hysteresis is extreme enough to make p42 MAPK activation self-perpetuating and irreversible. If so, the bistability of the p42 MAPK could contribute to the irreversibility of oocyte maturation.

Bistability in the JNK cascade

The *Xenopus* oocyte possesses at least one other MAPK cascade, a c-Jun amino-terminal kinase (JNK) cascade. Like p42 MAPK, *Xenopus* JNK is activated during oocyte maturation, but JNK remains active for a longer period of time during early embryogenesis, and JNK can be activated by stresses (such as hyperosmolarity) that do not activate p42 MAPK [28]. The physiological role of JNK activation in this context is unknown, but it has been hypothesized that JNK might keep the embryo poised for apoptosis.

The *Xenopus* JNK cascade provides a second example of bistability in a MAPK cascade. The response of JNK to progesterone or hyperosmolar sorbitol is all or nothing at the level of the individual cell (and, like the p42 MAPK response, is more graded at the population level) [29••]. Moreover, JNK is embedded in a positive-feedback loop: cytoplasm from stressed oocytes will cause the appearance of 'JNK-activation promoting factor' in recipient oocytes [29••], and expression of constitutively active JNK causes the

endogenous JNK to become activated (CP Bagowski *et al.*, unpublished data). Finally, JNK remains active long after the trigger of its activation (progesterone or sorbitol) has been washed away. All of these observations are consistent with the hypothesis that the *Xenopus* JNK cascade is a bistable system set up by positive feedback.

At first it might seem that the *Xenopus* JNK cascade is a carbon copy of the p42 MAPK cascade. However, the positive feedback in the JNK cascade is post-translational rather than translational [29••]. Thus, the same end — a bistable, all-or-none response — is achieved by the two MAPK cascades through completely different mechanisms. This suggests that bistability has arisen in the two systems through convergent evolution, and suggests that bistability may be relatively easy to evolve and may prove to be a recurrent theme in signal transduction.

ERK and JNK cascades in mammalian cell lines

Bhalla and Iyengar have pointed out that the extracellular-signal-regulated kinase (ERK1/ERK2) MAPK cascade may be embedded in a positive feedback loop in various mammalian cell lines [30]. The way the loop might function is as follows: MAPK activation leads to the activation of phospholipase A2, which produces arachidonic acid, which, in turn, can activate protein kinase C (PKC) isoforms; the active PKC can then feed into the Ras–Raf–MAPK cascade [30]. Thus, in principle these MAPK cascades could function as bistable systems. However, in most mammalian cell lines, the response of the ERK1 and ERK2 MAPKs to mitogenic stimuli appears to be graded and transient, not switch-like and irreversible, at least when analysed in populations of cells. Little is known yet, though, about the temporal and spatial dynamics of ERK activation in individual cells. This could be an interesting area for future study.

Graded MAPK responses in the yeast mating pheromone pathway

Poritz *et al.* [31•] recently reported a careful quantitative study of MAPK responses in the budding yeast mating pheromone pathway. This MAPK cascade consists of Ste11, Ste7 and Fus3 (the three kinases) plus the Ste5 scaffold protein [32•–34•]. Poritz *et al.* made use of an integrated Fus1–GFP chimera as an indicator of Fus3 activation; Fus1 transcription is induced by Fus3 activation, and the GFP chimera allows the level of Fus1 accumulation to be quantified at the single-cell level using flow cytometry. They found that Fus1–GFP accumulation was a graded function of the pheromone concentration in several strain backgrounds [31•]. There was no evidence for bistability or ultrasensitivity in the Fus1–GFP response. One potential problem is that only the steady-state responses of a bistable system would be expected to be discontinuous — on the way from one steady state to another, any intermediate state is possible — and it is not clear that a long-lived Fus1–GFP protein would reach steady state during a mating pheromone induction experiment. A simpler interpretation (and the one favored by the authors) is that the Ste11–Ste7–Fus3 cascade acts more like a rheostat than a switch, with the all-or-none aspects of mating imposed somewhere downstream of the cascade. If so, it would underscore the idea that homologous signaling modules can give rise to very different systems-level properties in different contexts.

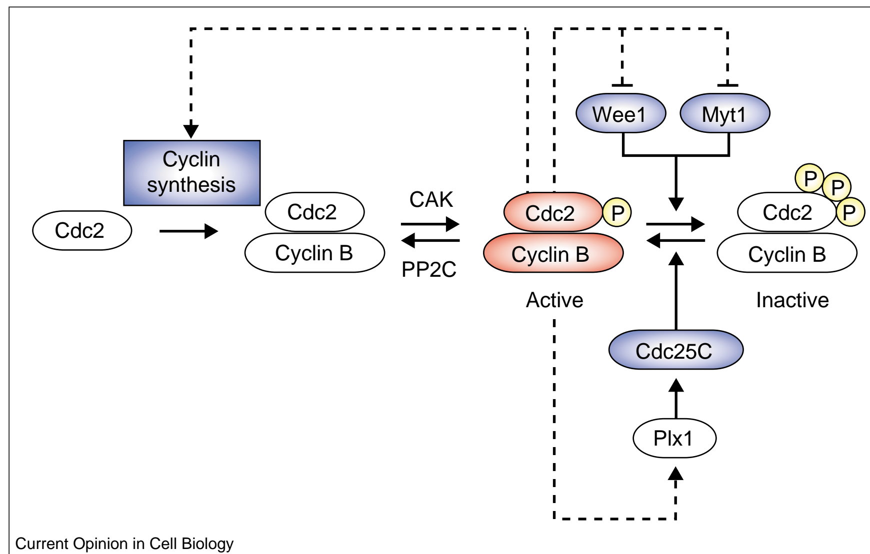
Cdc2 activation and interlocking feedback loops

Finally, we will conclude by examining a system long-hypothesized to be bistable, but for which there is as yet no definitive experimental evidence for bistability: the activation of Cdc2–cyclin B, which drives progression from interphase to mitosis, at the G2/M transition. Feedback in the Cdc2 activation system has been hypothesized to contribute towards the switch-like character of Cdc2 activation, and towards the irreversibility of the G2/M transition.

Activation of Cdc2–cyclin B is a multistep process whose broad outlines are well understood [35]. First, the B-type cyclin is synthesized and associates with enzymatically inactive Cdc2 monomers. Cyclin binding results in a substantial increase in the activity of Cdc2; the best quantitative studies on this point come from studies of the analogous cyclin-dependent kinase 2 (Cdk2)–cyclin A complex, where the binding of cyclin A to Cdk2 causes a ~400,000-fold increase in Cdk2 activity [36]. Next, the Cdk subunit of the complex undergoes three phosphorylation reactions. One of the phosphorylations occurs at Thr161, which is situated in the activation loop of the kinase, and this further increases the activity of the complex (in the Cdk2–cyclin A complex, estimates of the activation caused by this phosphorylation range from 80-fold to 100,000-fold [36,37]). The other two phosphorylations occur at Thr14 and Tyr15, and they render the Cdc2–cyclin B complex inactive. These reactions are summarized in Figure 6.

The basic features of the positive feedback loops in this system were described by Solomon *et al.* [38] more than a decade ago, through a series of experiments in *Xenopus* egg extracts. Solomon showed that in interphase extracts, which have inactive Cdc2–cyclin B, the rate of the inactivating Tyr15 phosphorylation is high and the rate of Tyr15 dephosphorylation is low; conversely, in mitotic extracts with active Cdc2–cyclin B, the rate of the inactivating Tyr15 phosphorylation is low and the rate of Tyr15 dephosphorylation is high [38]. Thus, Cdc2 directly or indirectly activates a Cdc2 activator — a positive feedback loop — and inactivates a Cdc2 inactivator — a double-negative feedback loop. Subsequent work has established that Cdc2 activation brings about the activation of the phosphatase Cdc25C [39–41] and the inactivation of two kinases, Wee1 [42] and Myt1 [43] (Figure 6). The polo-like kinase Plx1 is a required intermediary in the Cdc2-dependent activation of Cdc25C [44–47].

Figure 6



Cdc2 activation. Activation of Cdc2 depends upon the synthesis of cyclin B, the binding of cyclin B to Cdc2, and the phosphorylation of the Cdc2–cyclin B complex by Cdk activating kinase (CAK). Protein phosphatase 2C (PP2C) can reverse the CAK-mediated phosphorylation. Active Cdc2–cyclin B can be inactivated by the Wee1 and Myt1 protein kinases (through inhibitory phosphorylations at Thr14 and Thr15), whose effects are reversed by the activating phosphatase Cdc25C. There are a number of potential positive and double-negative feedback loops in this system, four of which are illustrated here: activation of Cdc25C by Cdc2, through the intermediacy of Plx1; inactivation of Wee1 by Cdc2; inactivation of Myt1 by Cdc2; and activation of cyclin translation by Cdc2. The presence of these feedback loops led to the hypothesis that Cdc2 activation is bistable.

It was quickly recognised that these feedback loops could make Cdc2 activation a bistable process [48–50]. That is, the graded synthesis of cyclin (or any other graded mitotic stimulus) could make the cell toggle from a stable interphase state with Cdc2 phosphorylated at Thr14 and Tyr15, Plx1 and Cdc25C off, and Wee1 and Myt1 on, to a distinct, stable mitotic state with Cdc2, Plx1 and Cdc25C on and Wee1 and Myt1 off. This mitotic state would persist until the Cdc2-dependent activation of the anaphase-promoting complex forces the system to return to its interphase state. Bistability in the activation of Cdc2 could ensure that the G2 and M phases are discrete states, not a continuum of states, could establish the irreversibility of the G2–M transition, and could suppress ‘chatter’ during the transition between G2 and M. A mitotic oscillator built out of a succession of bistable switches would have interesting properties. Rather than functioning like a pendulum (a good example of an analogue oscillator), swinging back and forth between two extremes, it would function more like a washing machine timer, tripping different digital all-or-none switches at different times. A digital oscillator might also be less likely to peter out than would an analogue oscillator.

It remains to be shown, however, whether Cdc2 activation really is bistable, as envisioned. As mentioned above, feedback loops (even multiple intertwined feedback loops) do not guarantee a bistable response; the loops also must possess ultrasensitivity and be properly balanced. It seems plausible that many of the activation processes in the Cdc2 system are in fact ultrasensitive — for example, the multistep dephosphorylation of Cdc2 and the multistep phosphorylation of Cdc25C, Wee1, and Myt1 could produce ultrasensitivity [8,51,52••] — but this ultrasensitivity has never been demonstrated experimentally. Moreover, it is not clear that bistability is the only way to construct a satisfactory mitotic switch, and computational studies argue that positive feedback can have potentially useful consequences (such as sensitivity amplification) even when it is not strong enough to produce bistability (JR Pomerening and JE Ferrell Jr, unpublished data). Thus, the bistability of Cdc2 activation is an attractive hypothesis but not a proven fact, and experimental tests of this hypothesis could provide important insights into the basic logic of the mitotic control system.

One of the remarkable features of the Cdc2 system is that there are so many positive and double-negative feedback loops. We have mentioned three of them (regulation of Cdc2 of/by Cdc25, regulation of Cdc2 of/by Wee1, and regulation of Cdc2 of/by Myt1), but there are others as well. Activation of Cdc2 in *Xenopus* egg extracts and entry into mitosis in various mammalian cell lines leads to activation of p42 MAPK [53–55], and p42 MAPK activation, in turn, can suppress cyclin destruction [56–58], and inhibit the Cdc2 inhibitor Myt1 [59], providing two more double-negative feedback loops. In *Xenopus* oocytes, Cdc2 activation promotes cyclin translation, another positive feedback loop (Figure 6). Finally, transcriptional positive-feedback loops are a recurring theme in cell cycle regulation in budding yeast [60–62]. One possible reason for the presence of so many interlocking positive-feedback loops is to make the bistability of Cdc2 (if, indeed, Cdc2 activation proves to be bistable) more robust — that is, to allow Cdc2 activation to be bistable even when the concentrations and activities of various components of the Cdc2 regulatory system are off a bit. This hypothesis could be tested either through computational studies or through experimental approaches in systems where the various feedback loops can be individually manipulated.

Conclusions

There are numerous other examples of cell signaling systems and other cell biological processes where it is clear that positive feedback or double-negative feedback is present — myoblast differentiation, Notch–Delta signaling, chemotaxis, and prion propagation. The concept of bistability provides a useful framework for thinking about all of these processes, but as yet there is little experimental evidence for or against bistability virtually anywhere in biology (the work reviewed above notwithstanding). Future experimental work will help us understand whether bistability is an interesting peculiarity of a few special biological systems, or a common recurring theme and unifying principle of cell biology.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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These papers (Laurent and Kellershohn [1999] [3]), Smolen *et al.* [2000] [4•] and Ferrell and Xiong [2001] [5•] and Hasty *et al.* [6•]) are all highly readable, up-to-date introductions to bistability in particular and computational cell biology in general. The issue of *Chaos* in which [5•] appears is a special theme issue devoted to molecular, metabolic and genetic control. The 20 review articles provide an excellent comprehensive introduction to computational cell biology.

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This article reports the successful expression and characterization of an artificial double-negative feedback bistable system in *E. coli*. Together with Becskei and Serrano (2000) [10••], Elowitz and Leibler (2000) [11••] and Becskei *et al.* (2001) [12••], this article is an important first step towards the engineering of modular signaling circuits.

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This article shows that a single-negative feedback loop can provide an artificial signaling system with enhanced stability.

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In this very elegant paper, the authors expressed an artificial triple-negative-feedback loop (the 'repressilator') in *E. coli*. Significant findings include the fact that the repressilator worked at all, which demonstrates that it is possible to engineer rather sophisticated biochemical behavior in living cells; and the fact that it exhibited noisy, highly variable oscillations.

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