Cell

Making Cellular Memories

Devin R. Burrill[1] and Pamela A. Silver[1,*]
[1]Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA
*Correspondence: pamela_silver@hms.harvard.edu
DOI 10.1016/j.cell.2009.12.034

The induction of a protracted response to a brief stimulus is a form of cellular memory. Here we describe the role of transcriptional regulation in both natural and synthetic memory networks and discuss the potential applications of engineered memory networks in medicine and industrial biotechnology.

Biological memory can be defined as a sustained cellular response to a transient stimulus. To understand this phenomenon, we must consider how the properties of different biological systems achieve memory of a stimulus, essentially permitting a cell to produce a lasting response. One way that cells accomplish this task is through transcriptional states, which involve populations of molecules regulating gene expression. If the transcriptional response is bistable, a chemical state becomes defined as *on* or *off* and, given certain parameters, this state can be inherited through DNA replication and cell division. In this way, a cell can produce a lasting memory of a biological response.

Synthetic biologists are especially interested in transcriptional responses as a means of cellular memory because (1) much of a cell's information processing is performed through transcription, and (2) the basic machinery for such biological behavior is well-understood. As such, transcription provides us with a set of characterized genetic units, including promoters, activators, and repressors that can be recombined to create new transcriptional circuits. Furthermore, the way nature combines these biological parts to produce specific outputs, including cellular memory, has been extensively studied. Thus, we have at our fingertips the tools with which to design synthetic memory systems.

The construction of synthetic memory circuits will improve our understanding of natural networks, further aiding the creation of useful, new biological tools. For example, a device capable of remembering a biological experience might be utilized in the long-term study of particular cells within a heterogeneous population following a defined event or applied in industry for the sustained production of desired proteins after induction by a brief stimulus. Such bioengineered networks exemplify a primary objective of synthetic biology: to advance simple synthetic devices into expertly constructed circuits with important applications.

How Cells Make Memories

Nearly 50 years ago, François Jacob and Jacques Monod determined qualitatively how a cell might achieve biological memory through its transcriptional circuitry (Monod and Jacob, 1961). However, a quantitative understanding of these circuits has been achieved only recently (Alon, 2006). Synthetic biology bridges the gap between biology and mathematics, requiring our understanding of cellular memory to encompass more than half a century of scientific work. Here, we will briefly address the concepts that are fundamental to the achievement of biological memory through transcription.

The Hill Function

Cells must sense and dynamically respond to both internal and external signals. This often requires the synthesis

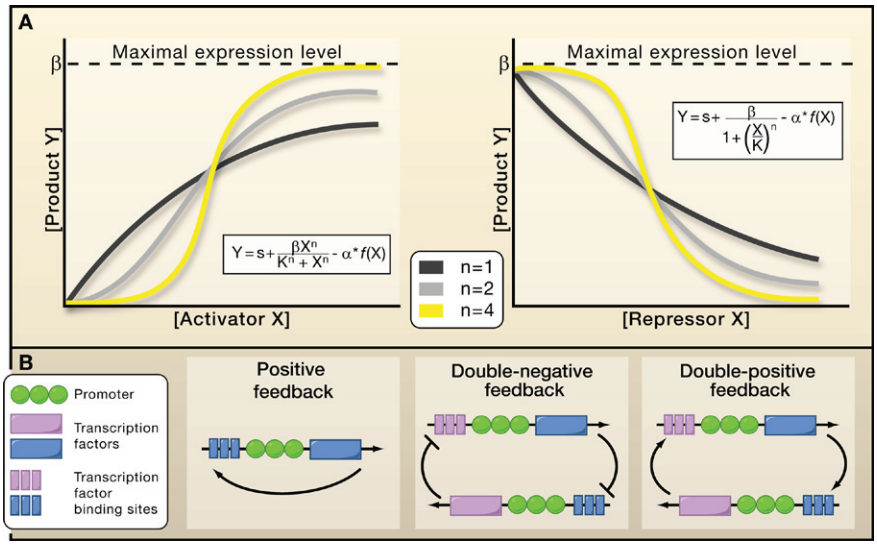

Figure 1. Gene Circuits for Cellular Memory
(A) Dynamics of regulated gene circuits described by Hill functions. (Left) The concentration of product Y is plotted as a function of the concentration of activator X, as described by Hill functions with n = 1, 2, and 4. β is the maximal expression level from Y's promoter when X is bound, and K defines the concentration of X needed to reach the threshold activation of Y. (Right) The concentration of product Y is plotted as a function of the concentration of repressor X, as described by Hill functions with n = 1, 2, and 4. β is the maximal expression level from Y's promoter when X is unbound, and K defines the concentration of X needed to reach the threshold for repression of Y.
(B) Network motifs that achieve biological memory.

or modification of transcription factors, which form an interaction network whose design determines the speed and sustainability of protein output in response to an environmental input (Monod and Jacob, 1961; Alon, 2006). Depending on whether transcription factor X is an activator or repressor, the concentration of gene product Y increases or decreases from a basal level as a function of the concentration of X binding to Y's promoter [$f(X)$]. The production of Y is further balanced by Y's degradation and dilution rates (defined as α, with units of 1/time):

$$Y = s + f(X) - \alpha \bullet f(X) \quad (1)$$

To more thoroughly understand how a transcriptional input produces specific gene outputs, one can use the Hill function in the above equation to define and describe the equilibrium binding of a transcription factor to its target promoter (Alon, 2006). Although a Hill function for a transactivating transcription factor (Equation 2) varies slightly from that of a transrepressor (Equation 3), the components of each are similar (Figure 1A):

$$Y = s + \frac{\beta X^n}{K^n + X^n} - \alpha \bullet f(X) \quad (2)$$

$$Y = s + \frac{\beta}{1 + \left(\frac{X}{K}\right)^n} - \alpha \bullet f(X) \quad (3)$$

There are three key parameters: K, β, and *n*. K is the activation/repression coefficient, defining the concentration of X needed to reach the threshold for activation or repression of Y; K's value is largely related to the chemical affinity between X and its binding site on Y's promoter (Alon, 2006). The term β defines the maximal expression level from Y's promoter (in units of mRNAs/time), obtained when an activator is bound or a repressor is unbound. The most pertinent parameter is *n*, the Hill coefficient. This value governs how a network responds to transcriptional input: a smaller *n* (n = 1) results in a more graded response, whereas a larger *n* (n = 4) produces a bistable, switch-like response (Figure 1A). The latter behavior is essential to biological memory because a bistable response allows a system to shift to an alternative steady state that might persist over time.

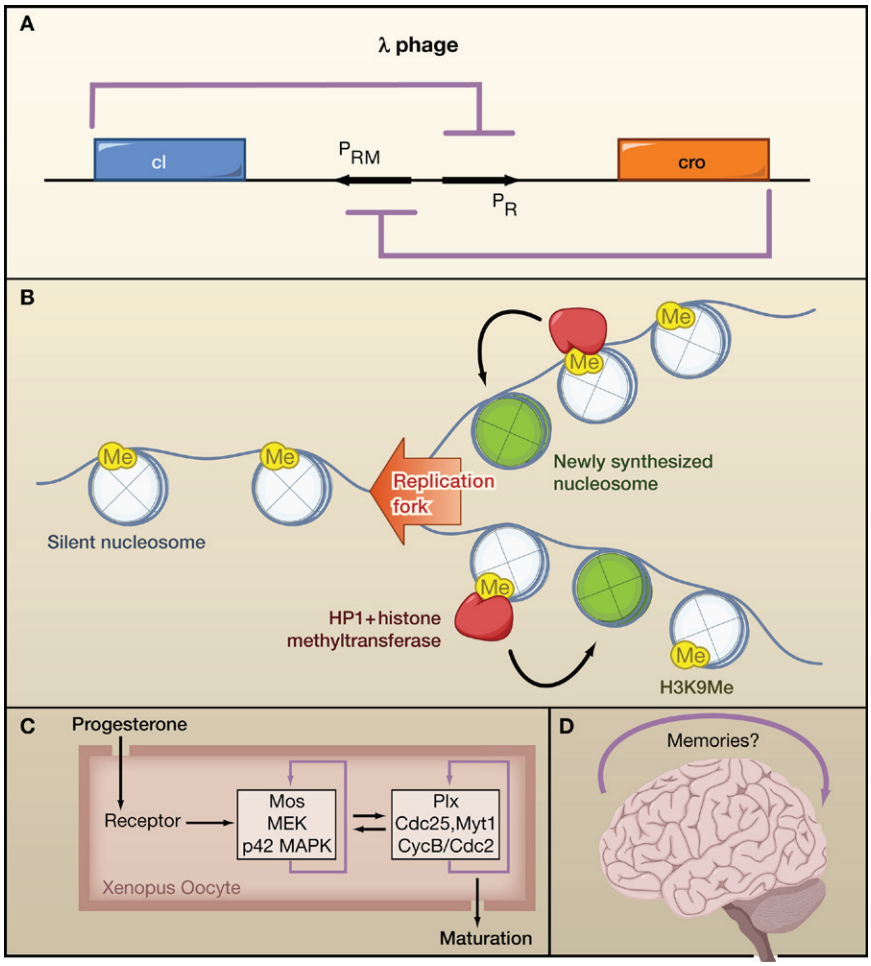

Figure 2. Natural Mechanisms of Memory

(A) The phage lambda system switches between two states based on a mutal repression loop between lambda repressor and Cro (Ptashne, 2004).

(B) Replication-coupled nucleosome assembly maintains silent chromatin. Some silent nucleosomes (gray) contain histone $(H3\text{-}H4)_2$ tetramers with histone H3 lysine 9 methylation marks (H3-K9me, yellow). Positive feedback can arise if newly synthesized nucleosomes (green) become methylated by histone methyltransferases bound to heterochromatin-associated protein 1 (red) on adjacent nucleosomes, allowing methylated regions to persist through DNA replication and cell division (adapted from Vermaak et al., 2003).

(C) Bistable networks based on positive feedback govern maturation of oocytes from the frog *Xenopus* (adapted from Xiong and Ferrell, 2003).

(D) Information storage in the brain is thought to involve bistable feedback networks (Ogasawara and Kawato, 2009).

Where Does That "n" Come From?

To achieve a sufficiently high Hill coefficient, biological systems employ a number of mechanisms. First, there must be nonlinearity within a transcriptional circuit, meaning a functional element that guarantees that a threshold-like response to a stimulus is required (Ninfa and Mayo, 2004). This effect can be achieved through the affinity, cooperativity, or multimerization of transcription factors at their binding sites within target gene promoters. Transcription factors vary widely in their degrees of cooperativity and affinity for their binding sites, and the degree of binding can also differ depending on the number of binding sites present in a given promoter. These factors help to ensure a lasting response to a transient stimulus and are especially effective at reducing stochastic fluctuation between steady states, an undesirable behavior in a transcriptional circuit designed to produce a lasting response.

Another requirement for attaining a large Hill coefficient is that the genetic elements we have touched upon thus far

must be arranged in specific motifs that permit bistable responses (Monod and Jacob, 1961). To achieve this goal, nature often employs transcriptional positive feedback (Ferrell, 2002; Alon, 2006). In this network design, production of protein Y only increases once the concentration of X approaches the expression threshold for Y's promoter, resulting in a sigmoidal response curve (Demongeot et al., 2000). Positive feedback can be produced by either a single transcription factor self-activating in response to a stimulus (positive autofeedback) or two transcription factors regulating each other through two positive or negative interactions (double-positive/double-negative feedback) (Figure 1B). When protein concentrations reach certain thresholds, all three motifs result in a switch between two steady states. If the switch is sharp enough, a gene can become locked into an alternate steady state, even in the absence of the original inducer (Ferrell, 2002).

Finally, to achieve the desired Hill coefficient, it is important to remember that transcription-based memory is derived from proteins that are degraded at some natural rate and diluted through cell growth. If the rates of degradation and dilution are faster than protein production rates, levels of transcription factor X may not be sufficiently high to achieve or maintain the desired bistable output. As this would result in a failure to achieve memory once the initial stimulus is removed, it is a critical parameter for the bioengineer to keep in mind.

Biological Memory in Nature

Many natural examples of biological memory have been discovered to possess feedback, bistability, and cooperativity. We will highlight some of this work and include examples that are not strictly transcription based but nonetheless employ parallel mechanisms. These natural systems inform the design of synthetic memory circuits, and the diversity of mechanisms highlights the general importance of this biological phenomenon.

Phage Lambda, the Lac *Operon, and the Cell Cycle*

Some of the earliest work in molecular genetics elucidated the role of bistable networks in the regulatory system of the phage lambda, the *lac* operon, and the cell cycle. For example, Eisen et al. (1970) described a multiply mutated lambda lysogen in which the toxic functions of the phage were eliminated, leaving only the regulatory essence of lambda's two-state system; considering that this was prior to the use of restriction enzymes in genetic constructions, this work arguably represents an early example of synthetic biology limited by the technology available at that time. The phage lambda system switches between two states based on a repression loop between the mutual antagonists lambda repressor and Cro (Ptashne, 2004) (Figure 2A) and involves both positive autoregulation and double-negative feedback. Likewise, in the bacterium *Escherichia coli*, the *lac* operon (required for lactose metabolism) can exist in *on* or *off* states mediated through the positive feedback loop of lactose transport (Muller-Hill, 1996). Work in oocyte extracts from the frog *Xenopus* has revealed that positive feedback loops govern cell-cycle progression by creating a bistable system wherein levels of specific proteins alternate between two steady states, determining discrete phases and forward movement through the cycle (Novak and Tyson, 1993; Thron, 1996).

Nucleosomal Modification

Positive feedback is also thought to be utilized in nucleosomal modification. The mechanism is believed to allow chromosomal regions to adopt stable and heritable states, resulting in bistable gene expression that persists through DNA replication and cell division (Dodd et al., 2007; Barrett and Wood, 2008). Positive autoregulation can arise if nucleosomes carrying a specific modification recruit enzymes that catalyze similar modifications of neighboring nucleosomes, allowing a nucleosomal cluster to stably maintain itself in a particular modification state (Stefanko et al., 2009). This phenomenon may be involved in the silencing of H3-H4 histones by methylation marks (di- or trimethylation of histone H3 lysine 9), to which heterochromatin-associated protein 1 (HP1) can bind (Vermaak et al., 2003) (Figure 2B). It is hypothesized that histone methytransferases bound to HP1 can transmit methylation marks to adjacent, newly replicated nucleosomes, creating an epigenetic feedback loop of silenced chromatin.

Cell Differentiation

Given the involvement of feedback motifs in epigenetic determination, it is not at all surprising to find this mechanism further used in eukaryotic cell differentiation, a process determined by epigenetic patterning. For example, transition between two stages of *Xenopus* oocyte maturation is induced by progesterone. Once the progesterone has dissipated, commitment to maturation is maintained via positive feedback within a mitogen-activated protein (MAP) kinase cascade (Figure 2C). The role of positive feedback in binary cell-fate switches has been further observed in mouse and human embryonic stem (ES) cells. Genome-wide transcriptional studies have identified OCT4, SOX2, and NANOG as critical players in the circuitry responsible for cell differentiation (Boyer et al., 2005; Loh et al., 2006). Dynamic modeling has revealed that these transcription factors interact via positive feedback loops, resulting in a bistable switch that regulates when and how an ES cell differentiates (Chickarmane et al., 2006).

The Immune and Nervous Systems

Finally, a discussion of memory storage in the immune and nervous systems naturally arises. These systems are alike in many ways, including their capacity to manage vast quantities of information. The immune system is required to consistently recognize foreign antigens from countless sources, and each individual neuron is required to receive information from numerous synaptic connections. To file this data, each system employs mechanisms for molecular memory. One tool used by the immune system is somatic V(D)J recombination, which bistably rearranges genes in response to specific foreign antigens, permitting lymphocytes to produce the necessary proteins for mounting immune responses. This mechanism results in both an immediate immune response and a population of long-lived memory cells that can mount stronger responses if the initial pathogen is ever again detected (Muotri and Gage, 2006). To handle the brain's computing load and to store information despite molecular turnover, one mechanism used by neurons might be positive feedback (Tanaka and Augustine, 2008; Ogasawara and Kawato, 2009). This activity may serve as a general method for perpetuating signal transduction net-

works in the brain, thus further establishing memory networks as remarkable biological tools (Figure 2D).

Synthetic Memory Circuits, Thus Far

Using natural memory circuits as guides, a variety of synthetic memory pathways have been engineered from transcription-based parts in bacterial, yeast, and mammalian cells.

Bacterial Memory Devices

One of the first simple bistable devices was constructed in *E. coli* by Gardner et al. (2000). The modules consist of double-negative feedback systems using well-described prokaryotic gene repressor proteins. This device demonstrates bistability: once the switch is flipped toward one steady state, it remains there in the absence of the original stimulus, until a second stimulus shifts the system to an alternate steady state. This behavior is ensured by cooperativity in the binding of repressors to DNA and by trial-and-error testing of promoters of different strengths (Gardner et al., 2000; Xiong and Ferrell, 2003). The Gardner circuit was the first demonstration that bistable responses can indeed be engineered into a synthetic system. Others have extended this work by building similar networks that are more robust due to further quantitative understanding of the modular components that constitute such systems (Atkinson et al., 2003; Isaacs et al., 2003).

These preliminary memory switches allowed for the later construction of bacterial memory networks with novel functions. This was best demonstrated when Kobayashi et al. (2004) used a *lacI*/lambda repressor toggle switch as a memory circuit embedded within a larger circuit based on the *E. coli* SOS signaling pathway, enabling the memory circuit to sense DNA damage and retain memory of this event (Kobayashi et al., 2004). The group further constructed a strain in which biofilm formation is induced post-DNA damage, thereby demonstrating that artificial regulatory circuits can be used to produce permanent phenotypic changes.

Yeast Memory Devices

The early successes in bacteria laid the groundwork for similar projects in the budding yeast *Saccharomyces cerevisiae.* Work on yeast memory modules largely began with Becskei and colleagues, who constructed a simple switch in which a tetracycline-dependent activator turns on its own synthesis (Becksei et al., 2001). This design permits cells to switch between *on* and *off* states in response to increasing levels of tetracycline, largely due to the inherent cooperativity of the activator and eukaryotic transcription (Becksei et al., 2001).

Ingolia and colleagues sought to determine how easily a monostable signaling pathway might become bistable by engineering positive feedback into the system (Ingolia and Murray, 2007). To address this question, they chose the budding-yeast mating-pheromone response, a well-studied MAP kinase system stimulated by exogenous pheromone (Ingolia and Murray, 2007). Using the natural phosphorylation cascade, the authors expressed a dominant active allele of a key pathway protein. Once triggered, the pathway can sustain itself because the dominant protein is able to feed back into the natural pathway, thereby producing a positive autoregulatory circuit. Furthermore, the tunability of the feedback loop was demonstrated via mutations that alter the basal and induced expression levels of the feedback promoter.

The application of quantitative approaches to reliable circuit design is well-illustrated by the work in yeast by Ajo-Franklin et al. (2007). Using quantitative modeling of transcription dynamics, the authors constructed a synthetic memory circuit based on transcriptional positive feedback. The device bistably responds to a pulse of galactose by producing a transcriptional activator that induces a downstream autofeedback loop. Given certain system parameters, the loop activity persists in the absence of galactose and the inducing transactivator, such that the circuit imparts memory of galactose exposure onto cells and their progeny. Computational modeling using quantitative descriptions of various tested transactivators suggested that low basal expression coupled with switch-like activation is required to maintain memory; growth rate was also found to significantly impact memory loop protein sustainability following cell division.

Mammalian Memory Devices

The design strategies and critical parameters discovered in nature and tested synthetically in bacteria and yeast have been further applied toward the engineering of mammalian cells. Of note is the toggle switch in Chinese hamster ovary cells, the design of which is largely based on the bacterial toggle switch (Kramer et al., 2004). Using transrepressors dependent on streptogramin and macrolide antibiotics, the system responds in a bistable manner to specific inducers. This response allows for switch-specific expression of a human glycoprotein both in culture and mice, impressively demonstrating the possibility of epigenetic transgene control through bistable circuits. Fussenegger's group has designed a number of such modules, primarily using antibiotics to control positive or double-negative feedback networks (Kramer et al., 2003; Weber et al., 2007; Tigges et al., 2009). This work lays the groundwork for the further bioengineering of mammalian systems and their application in research and clinical settings.

Transcriptional Memory Devices versus Circuits Based on DNA Recombination

The synthetic circuits discussed thus far all employ transcriptional circuitry that mimics natural networks to produce biological memory. It should be noted that memory devices have also been constructed using systems based on DNA recombinases (such as Cre or Flippase-mediated recombination), which leave a permanent mark in the genome (Mortensen, 2006; Friedland et al., 2009). Although this approach is useful, there are potential problems as DNA rearrangements and recombinase expression can have undesirable consequences if not properly regulated. Additionally, transgene expression via recombination is more difficult to tune or reverse due to its permanency, allowing less flexibility for a synthetic biologist interested in exploring the design capabilities of network-based memory. Both methods are entirely valid. Each is appropriate in different situations and should be applied with careful consideration.

Why Make Memories?

Having observed the successful integration of nature's tools into a variety of synthetic memory devices, we might now ask, what can we do with this? The ability to construct robust, synthetic circuits enables us to engineer cells capable of recording stimulus exposure

or maintaining desired levels of gene expression over time, in the absence of inducer. Advances in synthesis and new recombinant DNA technology allow the production of such devices from highly interchangeable units, permitting responses to an array of stimuli in a variety of cell types (Shetty et al., 2008). A modular approach to the induction of a high-fidelity memory device might allow researchers to identify cell populations responsive to specific events and track their progression through the cellular response. This would be of particular use if a device employs fluorescent markers that permit quantitative, single-cell tracking of cells within a responding population, as suggested by Ajo-Franklin et al. (2007). These device characteristics may address whether a response to a defined event correlates with later cell behavior. Capturing this cellular phenomenon could have great impact on the study of any disease involving the inheritance of a cellular state, such as cancer.

In addition to being readouts of cellular experience, memory modules can potentially use their output as regulatory input to perform new functions. Harnessing the ability to achieve long-term maintenance of desired relative protein levels, memory circuits might precisely regulate output or rapidly alternate between multiple outputs. Along these lines, one can imagine a memory module contributing to gene therapy or the synthetic differentiation of mammalian stem cells in a certain fashion after experiencing a brief stimulus.

Importantly, given the parallels to natural mechanisms of cell-based inheritance, such circuits may increase our understanding of biological processes such as cellular differentiation and tissue formation. For example, at decreased levels of activation, a positive feedback module can spontaneously switch between steady states. This can lead to variable gene expression and possibly undesired phenotypes including disease; in fact, unstable autocrine feedback loops have been linked to some cases of tumorigenesis (Schulze et al., 2001). By studying the engineering of feedback loops, we may better understand how their malfunction affects biological events.

Finally, memory modules are potentially useful in industrial biotechnology. The feedback loop permits sustained induction of recombinant proteins without massive quantities of inducer. Promoters that respond to a plethora of stimuli (such as specific small molecules, pH, temperature, or anaerobiosis) already exist or can be engineered, allowing for a variety of production conditions. Of course, for these ventures to be successful, certain factors will have to be considered, including the impact of induction on protein yields and how recombinant gene expression affects cell growth and physiology. If properly engineered, however, memory modules may help overcome high production costs associated with requiring large quantities of chemical inducer.

The precise design and implementation of systems exhibiting complex dynamic behavior remains a major goal of synthetic biology. An educated choice of network components and their fluid assembly into constructs with predictable behavior may enable synthetic circuits to increase our understanding of biology and improve our ability to engineer cells. The potential lying within memory modules may help progress the field of synthetic biology toward a new phase of device production. This phase will ideally incorporate detailed quantitative and qualitative approaches into the creation of highly robust, reliable memory circuits with important, applicable functions. In sum, both nature's wisdom and previously designed memory modules can provide bioengineering insight to accelerate our progress toward the creation of devices capable of producing cellular memories that can last a lifetime.

ACKNOWLEDGMENTS

Special thanks to the Silver lab, J. Way, F. Winston, and A. Salic for helpful discussion. D.R.B. is supported by the NSF Synthetic Biology Engineering Research Center, and P.A.S. by grants from the NIH.

REFERENCES

Ajo-Franklin, C.M., Drubin, D.A., Eskin, J.A., Gee, E.P., Landgraf, D., Phillips, I., and Silver, P.A. (2007). Genes Dev. *21*, 2271–2276.

Alon, U. (2006). An Introduction to Systems Biology: Design Principles of Biological Circuits (Boca Raton, FL: Chapman & Hall/CRC Press).

Atkinson, M.R., Savageau, M.A., Myers, J.T., and Ninfa, A.J. (2003). Cell *113*, 597–607.

Barrett, R.M., and Wood, M.A. (2008). Learn. Mem. *15*, 460–467.

Becksei, A., Seraphin, B., and Serrano, L. (2001). EMBO J. *20*, 2528–2535.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., et al. (2005). Cell *122*, 947–956.

Chickarmane, V., Troein, C., Nuber, U.A., Sauro, H.M., and Peterson, C. (2006). PLoS Comput. Biol. *2*, e123.

Demongeot, J., Kaufman, M., and Thomas, R. (2000). C. R. Acad. Sci. III *323*, 69–79.

Dodd, I.B., Micheelsen, M.A., Sneppen, K., and Thon, G. (2007). Cell *129*, 813–822.

Eisen, H., Brachet, P., Pereira da Silva, L., and Jacob, F. (1970). Proc. Natl. Acad. Sci. USA *66*, 855–862.

Ferrell, J.E. (2002). Curr. Opin. Chem. Biol. *6*, 140–148.

Friedland, A.E., Lu, T.K., Wang, X., Shi, D., Church, G., and Collins, J.J. (2009). Science *324*, 1199-1202.

Gardner, T.S., Cantor, C.R., and Collins, J.J. (2000). Nature *403*, 339–342.

Ingolia, N.T., and Murray, A.W. (2007). Curr. Biol. *17*, 668–677.

Isaacs, F.J., Hasty, J., Cantor, C.R., and Collins, J.J. (2003). Proc. Natl. Acad. Sci. USA *100*, 7714–7719.

Kobayashi, H., Kaern, M., Araki, M., Chung, K., Gardner, T.S., Cantor, C.R., and Collins, J.J. (2004). Proc. Natl. Acad. Sci. USA *101*, 8414–8419.

Kramer, B.P., Weber, W., and Fussenegger, M. (2003). Biotechnol. Bioeng. *83*, 810–820.

Kramer, B.P., Viretta, A.U., Daoud-El-Baba, M., Aubel, D., Weber, W., and Fussenegger, M. (2004). Nat. Biotechnol. *22*, 867–870.

Loh, Y.-H., Wu, Q., Chew, J.-L., Vega, V.B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., et al. (2006). Nat. Genet. *38*, 431–440.

Monod, J., and Jacob, F. (1961). Cold Spring Harb. Symp. Quant. Biol. *26*, 389–401.

Mortensen, R. (2006). Curr. Protoc. Mol. Biol. *23*, 1.1-1.12.

Muller-Hill, B. (1996). The Lac Operon: A Short History of a Genetic Paradigm (Berlin, Germany: Walter de Gruyter).

Muotri, A.R., and Gage, F.H. (2006). Nature *441*, 1087–1093.

Ninfa, A.J., and Mayo, A.E. (2004). Sci. STKE *232*, pe20.

Novak, B., and Tyson, J.J. (1993). J. Cell Sci. *106*, 1153–1168.

Ogasawara, H., and Kawato, M. (2009). Sci. Signal. *35*, pe7.

Ptashne, M. (2004). A Genetic Switch: Phage Lambda Revisited (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schulze, A., Lehmann, K., Jefferies, H.B.J., McMahon, M., and Downward, J. (2001). Genes Dev. *15*, 981–994.

Shetty, R.P., Endy, D., and Knight, T.F., Jr. (2008). J. Biol. Eng. *2*, 1–12.

Stefanko, D.P., Barrett, R.M., Ly, A.R., Reolon, G.K., and Wood, M.A. (2009). Proc. Natl. Acad. Sci. USA *106*, 9447–9452.

Tanaka, K., and Augustine, G.J. (2008). Neuron *59*, 608–620.

Thron, C.D. (1996). Biophys. Chem. *57*, 239–251.

Tigges, M., Marquez-Lago, T.T., Stelling, J., and Fussenegger, M. (2009). Nature *457*, 309–312.

Vermaak, D., Ahmad, K., and Henikoff, S. (2003). Curr. Opin. Cell Biol. *15*, 266–274.

Weber, W., Kramer, B.P., and Fussenegger, M. (2007). Biotechnol. Bioeng. *98*, 894–902.

Xiong, W., and Ferrell, J.E., Jr. (2003). Nature *426*, 460–465.