Metabolically
Relevant Sensors

Complex and
Robust Circuits

Multi-Gene
Targeting

Programmable genetic circuits for pathway engineering

Allison Hoynes-O'Connor and Tae Seok Moon*

Energy, Environmental and Chemical Engineering, Washington University in St. Louis, St. Louis,
MO, 63130, United States of America

* To whom correspondence should be addressed.

Tae Seok Moon

One Brookings Dr., Box 1180

St. Louis, MO 63130, USA

Tel: +1 (314) 935-5026

Fax: +1 (314) 935-7211

Email: tsmoon@wustl.edu

Abstract

Synthetic biology has the potential to provide decisive advances in genetic control of metabolic pathways. However, there are several challenges that synthetic biologists must overcome before this vision becomes a reality. First, a library of diverse and well-characterized sensors, such as metabolite- or condition-sensing promoters, must be constructed. Second, robust programmable circuits that link input conditions with a specific gene regulation response must be developed. Finally, multi-gene targeting strategies must be integrated with metabolically relevant sensors and complex, robust logic. Achievements in each of these areas, which employ the CRISPR/Cas system, *in silico* modeling, and dynamic sensor-regulators, among other tools, provide a strong basis for future research. Overall, the future for synthetic biology approaches in metabolic engineering holds immense promise.

Keywords

Synthetic biology; metabolic pathway; genetic sensor; genetic circuit; multi-gene targeting

Introduction

Synthetic biology is a burgeoning field, with varied roots and a brief, contentious history [1,2]. The field is poised to provide meaningful technological advancements to metabolic engineering in the form of programmable circuits. These will enable metabolic engineers to exert precise, programmed control over the expression of genes, and help optimize gene expression for a wide variety of metabolic pathways. As a case in point, nitrogen fixation is a potential target process in which multi-input and multi-gene targeting circuits can be applied for advanced metabolic engineering (Figure 1). Nitrogen fixation is a high-value process due to potential agricultural applications, and efforts to engineer nitrogen fixation are already underway [3,4]. Not only is nitrogen fixation an energy intensive and potentially burdensome process, but the central enzyme, nitrogenase, is also irreversibly inactivated by oxygen. Strict regulation of gene expression through multi-input and multi-gene targeting circuits will ensure that genes are only expressed in the absence of oxygen, and when culture conditions are such that the necessary energy can be spared. Furthermore, feedback and feedforward mechanisms can allow cells to dynamically regulate expression levels based on metabolic requirements, preventing wasteful protein synthesis.

The transition from bench-scale circuit construction [5,6] to full-scale pathway optimization [7] necessitates the development of metabolically relevant sensors, complex and robust circuit behavior, and multi-gene targeting strategies that can provide genome-wide regulation. First, recent progress in the discovery or development of genetic sensors will be discussed, with some examples of the integration of these sensors into simple genetic circuits for metabolic pathway optimization. Next, we will describe achievements in circuit construction, as well as the balance between circuit complexity and robustness. Finally, multi-gene targeting strategies will be described, with a particular focus on applications to metabolic pathways.

Discovery and Development of Metabolically Relevant Sensors

Many genetic circuits have utilized inducible promoters adapted from natural systems that respond to chemical inducers. Although such inducible promoters are invaluable research tools, they are not particularly useful for large-scale metabolic engineering applications. Chemical inducers must be added exogenously by the researcher, requiring a “hands-on” approach to gene regulation. Additionally, chemical inducers can be expensive, making their use in large-scale reactors cost-prohibitive. Some researchers have tackled this problem by developing promoters that respond to metabolites within a given pathway. This strategy empowers cells to respond to an internal signal, allowing a “hands-off” approach, in addition to cutting inducer costs. Furthermore, engineering cells to be more responsive to their internal state can result in improved efficiencies. Several reviews have addressed the topic of the design, discovery, and optimization of such sensors [8-10].

The main challenge that synthetic biologists are currently facing on this front is the construction of a library of high-performing and well-characterized sensors that respond to a variety of cellular signals. Many of these sensors exist naturally. For instance, many sugar-responsive sensors can be mined from existing genomes by identifying genes involved in sugar metabolism. Synthetic biologists have made strides in characterizing and modifying these natural sensors to fit the requirements of synthetic circuits [11-13]. Another approach to natural sensor discovery was demonstrated by Dahl et al., wherein comparative whole-genome transcript arrays were used to identify promoters that would respond to farnesyl pyrophosphate (FPP), a toxic intermediate of the isoprenoid pathway [14]. As an alternative to mining for sensors in the

genome, some studies have demonstrated that sensors can be designed *de novo*. Carothers et al. demonstrated that a model-driven approach could be used to design ligand-binding RNA devices that respond to metabolic pathway intermediates [15]. Continued work in this field will allow for the development of a well-characterized library of genetic sensors.

In addition to metabolite sensors, environmental sensors have also been developed to respond to environmental signals. A potential application of such sensors is illustrated in the proposed nitrogen-fixing cyanobacterium (Figure 1). Cells growing in photobioreactors often experience overheating during the late afternoon [16]. Down-regulating an energy intensive process like nitrogen fixation during this stressful period, enabled by a heat-repressible expression system [17], will allow the organism to survive, and is likely to result in a higher overall productivity. In addition, because nitrogenase is irreversibly inactivated by oxygen, including an oxygen sensor in the circuit design will prevent futile gene expression. Coordinated implementation of both oxygen and temperature sensors can ensure that gene expression occurs only under the appropriate conditions, reducing unnecessary protein synthesis.

Metabolically Relevant Sensors in Simple Genetic Circuits

One benefit of using metabolically relevant sensors to regulate metabolic pathways is that gene expression can be tied to substrate availability. The first engineered system to link a metabolically relevant sensor with a simple circuit used the *glnAp2* promoter to control two genes related to lycopene production [18]. Because the *glnAp2* promoter is activated by high glucose flux, lycopene was produced only when there was excess glucose flux in the cell, increasing lycopene productivity three-fold and reducing waste. This study paved the way for later work in the development of dynamic sensor-regulator systems [19].

Dynamic sensor-regulators generally use intermediate-sensing systems to ensure that pathway genes are controlled by precursor availability, limiting unnecessary protein production and pushing substrates through the target metabolic pathway. Zhang et al. designed a dynamic sensor-regulator that up-regulated biodiesel-producing genes in the presence of high free fatty acid concentrations, resulting in a 3-fold increase in yield, and a titer of 1.5 g/L [20]. Other dynamic sensor-regulators have been constructed using malonyl-CoA sensitive promoters, resulting in increased fatty acid titers [21,22]. Dahl et al., after identifying FPP-responsive promoters as described above, used these promoters to construct a dynamic sensor-regulator, and successfully increased the titer of amorphaadiene [14]. These studies provide valuable examples of the integration of metabolically relevant sensors and simple genetic circuits, and will be foundational for future studies that incorporate multiple sensors and more complex circuits.

Drawing inspiration from these dynamic sensor-regulators, a similar mechanism is shown in the proposed circuit (Figure 1). An accumulation of fixed nitrogen may cause decreased flux through the nitrogen fixation pathway. However, by storing fixed nitrogen as cyanophycin, and effectively removing the product from the system, the rate of nitrogen fixation can be increased. Of course, synthesis of cyanophycin should occur only when fixed nitrogen is abundant, and accordingly, a promoter that is activated by high concentrations of fixed nitrogen is shown to control the gene involved with cyanophycin synthesis. This dynamic regulation will allow the processes of nitrogen fixation and cyanophycin synthesis to operate efficiently.

Complex and Robust Circuits

As demonstrated by the studies described above, pathways that respond to metabolically-relevant signals show higher titer, productivity, or yield. However, these studies have only demonstrated the use of a single sensor to control a metabolic pathway. Multi-input logic gates will enable metabolic engineers to design pathways that respond to several inputs. Increasing the number of inputs will result in increased specificity of the response, reducing the possibility of false positive expression in fluctuating environments, and allowing for more complete control over pathway expression. Programmable and complex logic operations are necessary to ensure that the output (gene expression) is appropriate for the given environmental or metabolic inputs (e.g. carbon flux, metabolite concentration, and nutrient availability). For instance, in the proposed nitrogen fixation circuit (Figure 1), an AND gate, consisting of transcription factor and chaperone, is utilized to ensure that genes are expressed exclusively when both temperature and oxygen are low. The construction of multi-input genetic circuits such as this one is a step towards the application of such circuits to metabolic pathways.

A wide variety of genetic circuits have been constructed, and their design and construction were recently reviewed [23-26]. Genetic circuits have been demonstrated to use a diversity of mechanisms, including transcriptional regulation [27,28] and DNA recombination [29,30], as well as a combination of transcriptional and post-translational regulation [31]. RNA circuits, which generally operate on the translational level, have also been constructed [32,33]. A recent computational study analyzed the effects of various RNA control strategies on a metabolic pathway [34]. They compared static pathway control to multi-input dynamic control, wherein RNA devices sensed key intermediates in the *p*-aminostyrene (*p*-AS) pathway, and regulated pathway gene expression accordingly. These metabolically-relevant sensors had been constructed using a model-driven design technique as discussed above [15]. Although most dynamic control systems were worse than static control, the top 5% of dynamic control constructs showed 3-fold increase in yield of *p*-AS.

As engineers design increasingly complex circuits and integrate metabolically-relevant sensors, it is imperative that circuit robustness is prioritized as a research objective. Circuit robustness is the ability of a circuit to function as designed, even in the face of environmental perturbations [35]. Most complex genetic circuits have been tested in highly controlled environments over a relatively small number of generations. An unavoidable consequence of scale-up is increased variability in environmental conditions, which, coupled with the likelihood of mutations over several generations, will contribute to variability in circuit behavior. A recent study found that circuits were prone to evolutionary instability [36]. This indicates that robustness is particularly important for complex circuits that can impose burdens on host cells, and circuits in which pathway genes are highly expressed [37,38]. Strategies to engineer biological robustness have been developed and reviewed [35,39-41], and integration of these strategies into complex genetic circuits will be essential for successful pathway engineering.

Multi-Gene Targeting Strategies

Optimization of a metabolic pathway often requires the regulation of more than one gene. This could include up-regulating genes involved in precursor generation or product export [42], down-regulating competitive pathways, or dynamically regulating genes within the target pathway. In the example of the nitrogen fixation circuit, *nifH*, *nifD*, and *nifK* are all regulated as structural components of the nitrogenase enzyme. Additionally, genes associated with glycogen synthesis are down-regulated to ensure that high levels of glucose are available to provide energy for the energy-demanding nitrogen fixation process. Finally, genes associated with

cyanophycin synthesis are dynamically regulated in order to increase flux through the nitrogen fixation pathway. As multi-input circuits transition to metabolic engineering applications, the ability to simultaneously and orthogonally regulate several different output genes is essential.

Orthogonality is a key feature of multi-gene regulation. To provide precise control over metabolic networks, it is necessary to ensure that one regulator does not inadvertently regulate an unintended target. Several studies have shown that RNA is an excellent candidate molecule for orthogonal regulation. Because RNA molecules interact through complementary base pairing, designing orthogonal RNA regulator/target pairs is relatively simple and has the potential to be implemented with a large number of RNA regulator/target pairs. Several recent studies have shown how RNA regulation can be an effective strategy for multi-gene regulation [43-46]. Another notable study used CRISPR/Cas to construct genetic circuits for multi-gene targeting with orthogonal sgRNA-promoter pairs [47]. As an alternative to RNA regulation, another recent study demonstrated orthogonal gene regulation using T7 polymerase variants [48]. By breaking the polymerase into fragments and putting the fragments under the control of a genetic circuit, they further demonstrated the integration of logical behavior and multi-gene targeting.

Certain multi-gene targeting strategies have direct implications for metabolic engineering. Callura et al. used orthogonal RNA regulator/target pairs to generate a metabolic switchboard [49]. They were able to control carbon flux in *E. coli* by orthogonally regulating four genes involved in central metabolism. In another demonstration of multi-gene targeting with CRISPR, researchers altered the sgRNA molecules so that in addition to including a recruitment site for dCas9, they also included recruitment sites for other proteins that would either activate or repress gene expression [50]. By expressing unique regulators to target three separate genes within the violacein pathway, they were able to control product distribution. These examples provide valuable strategies for multi-gene regulation, which will ultimately be combined with metabolically relevant sensors and complex, robust logic, to build programmable genetic circuits with meaningful applications in metabolic engineering.

Future Perspectives and Conclusions

Ultimately, researchers will be able to program cells to respond to a series of meaningful inputs, including, but not limited to, metabolic state, culture conditions, substrate availability, and the concentration of toxic intermediates. These inputs will be parsed by genetic circuits in a programmed manner. Circuit output will take the form of gene regulation, wherein several genes will be simultaneously targeted, and their expression will be altered so as to optimize productivity, titer, and yield of the metabolic pathway. Before this goal can be achieved, several changes must occur in how circuits are designed, constructed, and tested.

First, it will be necessary to replace canonical sensors, such as the pLac promoter, with pathway-relevant genetic devices. These devices will enable a cell to monitor input signals, and regulate gene expression accordingly. A significant amount of research has focused on developing metabolically sensitive sensors, and this research is already showing promising results. However, parts are still limited in their availability. The development of high throughput techniques to design [15] or discover [14] metabolically relevant sensors will advance their integration in multi-input circuits. Further efforts to characterize and tune the behavior of such parts will hasten the implementation of multi-input circuits in metabolic engineering applications.

Integration of metabolically relevant sensors with simple genetic circuits has already been demonstrated, and it is likely that circuits will continue to grow in complexity as more sensors and regulators are developed. However, with increased circuit complexity comes increased burden, and there is a tradeoff between the two. To function in metabolic engineering applications, multi-input and multi-gene targeting circuits must be held to a high standard of robustness. Many complex logical behaviors demonstrated by genetic circuits have been observed in highly controlled, lab-scale scenarios. Ensuring that these behaviors are consistent and reliable in fluctuating environments [51] is essential, as heterogeneous and fluctuating conditions would be expected in a large scale metabolic engineering application. Further research into the specific causes and mechanisms of metabolic burdens may point to design solutions that will allow engineers to generate optimized genetic systems.

Finally, in order to build a multi-input and multi-gene targeting circuit that can be applied to advanced pathway optimization, all three of the components listed in Table 1 will be necessary. There are many studies that provide elegant strategies for one of those three components independently, and a handful of studies that integrate more than one of those components. However, future research must not only integrate all three of these components, but also incorporate a diversity of genetic sensors, regulate a wider variety of gene targets, and apply these tools to the optimization of a metabolic pathway. In addition, there are several essential design considerations that were not discussed in this review. As circuits increase in complexity, advanced assembly techniques will be required to build constructs that consist of various sensor, circuit, and regulator components [3,52,53]. As multi-gene targeting techniques continue to advance, tools for genome-wide targeting, optimization, gene modification, and high-throughput screening will become indispensable [45,54-57]. Furthermore, metabolic engineering will continue to identify critical gene targets within central metabolism that will act as targets for genetic circuits [58,59]. As research unifies strategies for metabolically relevant sensors, complex and robust logic, and multi-gene targeting, and incorporates additional design considerations, programmable genetic circuits will provide a powerful tool for optimization of metabolic pathways.

Acknowledgements

This work was supported by the Department of Energy [DE-SC0012705 to T. M.]; the National Science Foundation [MCB-1331194 to A. H. and T. M. and CBET-1350498 to T. M.]; and the Mr. and Mrs. Spencer T. Olin Fellowship for Women in Graduate Study to A. H.

Figure Caption

Figure 1. Proposed programmable genetic circuit in a metabolic engineering application. Cells will sense their environmental conditions (temperature [17] and oxygen concentration) and metabolic state (fixed nitrogen availability). These inputs will inform “decisions” regarding gene regulation via a complex and robust genetic circuit, including the AND gate as pictured [31]. Multi-gene targeting strategies will allow for the simultaneous and orthogonal regulation of distinct gene targets. In this case, low temperature and low oxygen will allow the expression of nitrogenase, an enzyme that can fix nitrogen but is irreversibly inactivated by oxygen. At the same time, glycogen synthesis will be down-regulated to ensure that there is sufficient glucose available to provide energy to the process of nitrogen fixation. Fixed nitrogen will activate the expression of cyanophycin synthase, which will store fixed nitrogen as cyanophycin, and increase flux through nitrogenase. The truth table describes the expected outputs that will result from a given set of environmental and metabolic input signals.

Table 1. An overview of three major research areas necessary for the development of programmable genetic circuits for pathway regulation. Examples are provided for each research area, as well as for combinations of these research areas. Ultimately, all three research areas will be unified in order to achieve an effective paradigm for integrating multi-input and multi-gene targeting circuits into metabolic engineering efforts.

Genetic Devices	Description	Examples
Sensors¹	Allow cells to sense and respond to metabolic state or environmental conditions	Afroz et al., 2015 [12] Kaplan et al., 2008 [13]
Circuits²	Ensure that gene expression occurs only under a programmed set of conditions	Moon et al., 2012 [31] Rinaudo et al., 2007 [32] Win & Smolke, 2008 [33]
Multi-Gene Targeting³	Allow for simultaneous and orthogonal regulation of several different genes	Na et al., 2013 [45] Mutalik et al., 2012 [46] Callura et al., 2012 [49]
Sensors + Circuits	Circuits compile information collected by various metabolically relevant sensors and cause a programmed response	Carothers et al., 2011 [15] Hoynes-O'Connor et al., 2015 [17] Farmer & Liao, 2000 [18] Zhang et al., 2012 [20]
Circuits + Multi-Gene Targeting	Circuit output simultaneously and orthogonally regulates the expression of several different genes in a programmed manner	Temme et al., 2012 [4,28] Callura et al., 2010 [43] Lucks et al., 2011 [44] Nielsen & Voigt, 2014 [47] Segall-Shapiro et al., 2014 [48] Zalatan et al., 2015 [50]
Sensors + Circuits + Multi-Gene Targeting	Environmental or metabolic conditions orthogonally regulate expression of a variety of genes, according to programmed, logical circuit behavior	Dahl et al., 2013 [14] Xu et al., 2014 [22]

¹ References qualified for any of the “Sensors” categories if they included at least one sensor that responded to a metabolic or environmental signal.

² References qualified for any of the “Circuits” categories if they constructed genetic circuits that were able to perform logic operations, or incorporated feedback/feedforward or dynamic regulation.

³ References qualified for any of the “Multi-Gene Targeting” categories if they demonstrated the simultaneous and orthogonal regulation of at least two separate genes that were not in the same operon, and were controlled by different regulation mechanisms (e.g., regulatory elements with different sequences).

Annotated References

Outstanding Interest

** [1] Cameron DE, Bashor CJ, Collins JJ: **A brief history of synthetic biology**. *Nat Rev Microbiol* 2014, **12**:381-390.

This review provides a concise history of synthetic biology, with a particular focus on the key breakthroughs that have shaped the field.

** [14] Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Batth TS, Redding-Johanson AM, Petzold CJ, Mukhopadhyay A, Lee TS, Adams PD, et al. **Engineering dynamic pathway regulation using stress-response promoters**. *Nature Biotechnology* 2013, **31**:1039-1046.

Researchers performed a comparative transcriptome analysis to identify FPP-responsive promoters. These promoters were then used to construct a dynamic sensor-regulator system to achieve increased titers of amorpha-14:0 diene. Though there was only one input signal, this paper demonstrates all three components discussed in this review.

** [22] Xu P, Li L, Zhang F, Stephanopoulos G, Koffas M: **Improving fatty acids production by engineering dynamic pathway regulation and metabolic control**. *Proc Natl Acad Sci U S A* 2014, **111**:11299-11304.

A malonyl-CoA sensor was used to balance between cell growth and product formation, resulting in a 2.1-fold increase in fatty acid titer compared to the engineered cell with the unregulated pathway. Though there was only one input signal, this paper demonstrates all three components discussed in this review.

** [45] Na D, Yoo SM, Chung H, Park H, Park JH, Lee SY: **Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs**. *Nature Biotechnology* 2013, **31**:170-174.

Antisense RNA molecules were designed to target a variety of genes throughout the genome, including central metabolism genes, transcription factors, and transporters. These regulators were used to demonstrate increased production of tyrosine and cadaverine. This is an example of orthogonal multi-gene targeting that has the potential to be integrated with sensors and circuits.

** [50] Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS, et al. **Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds**. *Cell* 2015, **160**:339-350.

By altering sgRNA molecules to include recruitment sites for proteins known to function in gene regulation, the authors were able to construct a series of orthogonal gene regulators to affect product distribution in the violacein pathway. This is a powerful example of orthogonal gene regulation applied simultaneously to several genes.

Special Interest

* [17] Hoynes-O'Connor A, Hinman K, Kirchner L, Moon TS: **De novo design of heat-repressible RNA thermosensors in *E. coli*** *Nucleic Acids Research* 2015, **43**: 6166-6179.

By sequestering an RNase E cleavage site in a stem-loop that is exposed at high temperatures, the authors designed heat-repressible RNA thermosensors *de novo*. These thermosensors were then integrated into a 3-input AND gate, demonstrating the integration of an environmentally-relevant sensor in a genetic circuit.

* [19] Venayak N, Anesiadis N, Cluett WR, Mahadevan R: **Engineering metabolism through dynamic control**. *Curr Opin Biotechnol* 2015, **34**:142-152.

This review provides a comprehensive overview of different types of dynamic regulation that have been applied to metabolic engineering.

* [47] Nielsen AAK, Voigt CA: **Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks**. *Molecular systems biology* 2014, **10**:10.15252/msb.20145735.

This study used CRISPR/Cas to construct orthogonal regulators that were layered into logic gates. Circuits were then connected to a native transcription factor that affects multiple genes in *E. coli*. This is an example of circuits and multi-gene regulation being applied to an existing regulatory network in *E. coli*.

* [48] Segall-Shapiro TH, Meyer AJ, Ellington AD, Sontag ED, Voigt CA: **A 'resource allocator' for transcription based on a highly fragmented T7 RNA polymerase**. *Mol Syst Biol* 2014, **10**:10.15252/msb.20145299.

This study demonstrates orthogonal gene regulation using variants of the T7 RNA polymerase. The different split polymerase fragments were expressed under different promoters, enabling the construction of a genetic circuit. This paper demonstrates the integration of logical behavior and potential orthogonal multi-gene targeting.

References

1. Cameron DE, Bashor CJ, Collins JJ: **A brief history of synthetic biology**. *Nat Rev Microbiol* 2014, **12**:381-390.
2. Stephanopoulos G: **Synthetic Biology and Metabolic Engineering**. *Acs Synthetic Biology* 2012, **1**:514-525
3. Smanski MJ, Bhatia S, Zhao D, Park Y, L BAW, Giannoukos G, Ciulla D, Busby M, Calderon J, Nicol R, et al. **Functional optimization of gene clusters by combinatorial design and assembly**. *Nat Biotechnol* 2014, **32**:1241-1249.
4. Temme K, Zhao D, Voigt CA: **Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca***. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**:7085-7090.
5. Elowitz MB, Leibler S: **A synthetic oscillatory network of transcriptional regulators**. *Nature* 2000, **403**:335-338.
6. Gardner TS, Cantor CR, Collins JJ: **Construction of a genetic toggle switch in *Escherichia coli***. *Nature* 2000, **403**:339-342.
7. Nielsen J, Fussenegger M, Keasling J, Lee SY, Liao JC, Prather K, Palsson B: **Engineering synergy in biotechnology**. *Nat Chem Biol* 2014, **10**:319-322.
8. Michener JK, Thodey K, Liang JC, Smolke CD: **Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways**. *Metabolic Engineering* 2012, **14**:212-222.
9. Park M, Tsai SL, Chen W: **Microbial Biosensors: Engineered Microorganisms as the Sensing Machinery**. *Sensors* 2013, **13**:5777-5795.
10. Holtz WJ, Keasling JD: **Engineering static and dynamic control of synthetic pathways**. *Cell* 2010, **140**:19-23.
11. Bennett MR, Pang WL, Ostroff NA, Baumgartner BL, Nayak S, Tsimring LS, Hasty J: **Metabolic gene regulation in a dynamically changing environment**. *Nature* 2008, **454**:1119-1122.
12. Afroz T, Biliouris K, Boykin KE, Kaznessis Y, Beisel CL: **Trade-offs in Engineering Sugar Utilization Pathways for Titratable Control**. *ACS Synth Biol* 2015, **4**:141-149.
13. Kaplan S, Bren A, Zaslaver A, Dekel E, Alon U: **Diverse two-dimensional input functions control bacterial sugar genes**. *Mol Cell* 2008, **29**:786-792.
14. Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Batth TS, Redding-Johanson AM, Petzold CJ, Mukhopadhyay A, Lee TS, Adams PD, et al.: **Engineering dynamic pathway regulation using stress-response promoters**. *Nature Biotechnology* 2013, **31**:1039-1046.
15. Carothers JM, Goler JA, Juminaga D, Keasling JD: **Model-driven engineering of RNA devices to quantitatively program gene expression**. *Science* 2011, **334**:1716-1719.
16. Bechet Q, Shilton A, Fringer OB, Munoz R, Guieysse B: **Mechanistic Modeling of Broth Temperature in Outdoor Photobioreactors**. *Environmental Science & Technology* 2010, **44**:2197-2203.
17. Hoynes-O'Connor A, Hinman K, Kirchner L, Moon TS: **De novo design of heat-repressible RNA thermosensors in *E. coli***. *Nucleic Acids Research* 2015, **43**: 6166-6179.
18. Farmer WR, Liao JC: **Improving lycopene production in *Escherichia coli* by engineering metabolic control**. *Nature Biotechnology* 2000, **18**:533-537.
19. Venayak N, Anesiadis N, Cluett WR, Mahadevan R: **Engineering metabolism through dynamic control**. *Curr Opin Biotechnol* 2015, **34**:142-152.
20. Zhang F, Carothers JM, Keasling JD: **Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids**. *Nature Biotechnology* 2012, **30**:354-359.
21. Liu D, Xiao Y, Evans BS, Zhang F: **Negative Feedback Regulation of Fatty Acid Production Based on a Malonyl-CoA Sensor-Actuator**. *ACS Synth Biol* 2015, **4**:132-140.
22. Xu P, Li L, Zhang F, Stephanopoulos G, Koffas M: **Improving fatty acids production by engineering dynamic pathway regulation and metabolic control**. *Proc Natl Acad Sci U S A* 2014, **111**:11299-11304.
23. Brophy JAN, Voigt CA: **Principles of genetic circuit design**. *Nature Methods* 2014, **11**:508-520.

24. Nielsen AAK, Segall-Shapiro TH, Voigt CA: **Advances in genetic circuit design: novel biochemistries, deep part mining, and precision gene expression.** *Current Opinion in Chemical Biology* 2013, **17**:878-892.
25. Qi LS, Arkin AP: **A versatile framework for microbial engineering using synthetic non-coding RNAs.** *Nature Reviews Microbiology* 2014, **12**:341-354.
26. Slusarczyk AL, Lin A, Weiss R: **Foundations for the design and implementation of synthetic genetic circuits.** *Nature Reviews Genetics* 2012, **13**:406-420.
27. Daniel R, Rubens JR, Sarpeshkar R, Lu TK: **Synthetic analog computation in living cells.** *Nature* 2013, **497**:619-623.
28. Temme K, Hill R, Segall-Shapiro TH, Moser F, Voigt CA: **Modular control of multiple pathways using engineered orthogonal T7 polymerases.** *Nucleic Acids Research* 2012, **40**:8773-8781.
29. Bonnet J, Yin P, Ortiz ME, Subsoontorn P, Endy D: **Amplifying Genetic Logic Gates.** *Science* 2013, **340**:599-603.
30. Siuti P, Yazbek J, Lu TK: **Synthetic circuits integrating logic and memory in living cells.** *Nat Biotechnol* 2013, **31**:448-452.
31. Moon TS, Lou C, Tamsir A, Stanton BC, Voigt CA: **Genetic programs constructed from layered logic gates in single cells.** *Nature* 2012, **491**:249-253.
32. Rinaudo K, Bleris L, Maddamsetti R, Subramanian S, Weiss R, Benenson Y: **A universal RNAi-based logic evaluator that operates in mammalian cells.** *Nature Biotechnology* 2007, **25**:795-801.
33. Win MN, Smolke CD: **Higher-order cellular information processing with synthetic RNA devices.** *Science* 2008, **322**:456-460.
34. Stevens JT, Carothers JM: **Designing RNA-Based Genetic Control Systems for Efficient Production from Engineered Metabolic Pathways.** *ACS Synth Biol* 2015, **4**:107-115.
35. Randall A, Guye P, Gupta S, Duportet X, Weiss R: **Design and connection of robust genetic circuits.** *Methods Enzymol* 2011, **497**:159-186.
36. Sleight SC, Sauro HM: **Visualization of Evolutionary Stability Dynamics and Competitive Fitness of Escherichia coli Engineered with Randomized Multigene Circuits.** *Acs Synthetic Biology* 2013, **2**:519-528.
37. Tan C, Marguet P, You L: **Emergent bistability by a growth-modulating positive feedback circuit.** *Nat Chem Biol* 2009, **5**:842-848.
38. Ceroni F, Algar R, Stan G-B, Ellis T: **Quantifying cellular capacity identifies gene expression designs with reduced burden.** *Nature methods* 2015, **12**:415-418.
39. Afroz T, Beisel CL: **Understanding and exploiting feedback in synthetic biology.** *Chemical Engineering Science* 2013, **103**:79-90.
40. Kitano H: **Biological robustness.** *Nat Rev Genet* 2004, **5**:826-837.
41. Lee Y, Rivera JGL, Liao JC: **Ensemble Modeling for Robustness Analysis in engineering non-native metabolic pathways.** *Metabolic Engineering* 2014, **25**:63-71.
42. Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A: **Engineering microbial biofuel tolerance and export using efflux pumps.** *Molecular Systems Biology* 2011, **7**:1-7.
43. Callura JM, Dwyer DJ, Isaacs FJ, Cantor CR, Collins JJ: **Tracking, tuning, and terminating microbial physiology using synthetic riboregulators.** *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**:15898-15903.
44. Lucks JB, Qi L, Mutalik VK, Wang D, Arkin AP: **Versatile RNA-sensing transcriptional regulators for engineering genetic networks.** *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**:8617-8622.
45. Na D, Yoo SM, Chung H, Park H, Park JH, Lee SY: **Metabolic engineering of Escherichia coli using synthetic small regulatory RNAs.** *Nature Biotechnology* 2013, **31**:170-174.
46. Mutalik VK, Qi L, Guimaraes JC, Lucks JB, Arkin AP: **Rationally designed families of orthogonal RNA regulators of translation.** *Nat Chem Biol* 2012, **8**:447-454.
47. Nielsen AAK, Voigt CA: **Multi-input CRISPR/Cas genetic circuits that interface host regulatory networks.** *Molecular systems biology* 2014, **10**:10.15252/msb.20145735.

48. Segall-Shapiro TH, Meyer AJ, Ellington AD, Sontag ED, Voigt CA: **A 'resource allocator' for transcription based on a highly fragmented T7 RNA polymerase.** *Mol Syst Biol* 2014, **10**:10.15252/msb.20145299.
49. Callura JM, Cantor CR, Collins JJ: **Genetic switchboard for synthetic biology applications.** *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**:5850-5855.
50. Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS, et al.: **Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds.** *Cell* 2015, **160**:339-350.
51. Acar M, Mettetal JT, van Oudenaarden A: **Stochastic switching as a survival strategy in fluctuating environments.** *Nat Genet* 2008, **40**:471-475.
52. Shao Z, Zhao H, Zhao H: **DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways.** *Nucleic Acids Res* 2009, **37**:e16.
53. Torella JP, Boehm CR, Lienert F, Chen JH, Way JC, Silver PA: **Rapid construction of insulated genetic circuits via synthetic sequence-guided isothermal assembly.** *Nucleic Acids Res* 2014, **42**:681-689.
54. Warner JR, Reeder PJ, Karimpour-Fard A, Woodruff LB, Gill RT: **Rapid profiling of a microbial genome using mixtures of barcoded oligonucleotides.** *Nat Biotechnol* 2010, **28**:856-862.
55. Wang BL, Ghaderi A, Zhou H, Agresti J, Weitz DA, Fink GR, Stephanopoulos G: **Microfluidic high-throughput culturing of single cells for selection based on extracellular metabolite production or consumption.** *Nature Biotechnology* 2014, **32**:473-478.
56. Raman S, Rogers JK, Taylor ND, Church GM: **Evolution-guided optimization of biosynthetic pathways.** *Proc Natl Acad Sci U S A* 2014, **111**:17803-17808.
57. Wang HH, Isaacs FJ, Carr PA, Sun ZZ, Xu G, Forest CR, Church GM: **Programming cells by multiplex genome engineering and accelerated evolution.** *Nature* 2009, **460**:894-899.
58. Solomon KV, Moon TS, Ma B, Sanders TM, Prather KL: **Tuning primary metabolism for heterologous pathway productivity.** *ACS Synth Biol* 2013, **2**:126-135.
59. Solomon KV, Sanders TM, Prather KL: **A dynamic metabolite valve for the control of central carbon metabolism.** *Metab Eng* 2012, **14**:661-671.

Figure
[Click here to download high resolution image](#)