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Synthetic biology approaches in cancer immunotherapy, genetic network engineering, and genome editing

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Investigations into cells and their contents have provided evolving insight into the emergence of complex biological behaviors. Capitalizing on this knowledge, synthetic biology seeks to manipulate the cellular machinery towards novel purposes, extending discoveries from basic science to new applications. While these developments have demonstrated the potential of building with biological parts, the complexity of cells can pose numerous challenges. In this review, we will highlight the broad and vital role that the synthetic biology approach has played in applying fundamental biological discoveries in receptors, genetic circuits, and genome-editing systems towards translation in the fields of immunotherapy, biosensors, disease models and gene therapy. These examples are evidence of the strength of synthetic approaches, while also illustrating considerations that must be addressed when developing systems around living cells.

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Insight, innovation, integration

Investigations into cells and their contents have provided evolving insight into the emergence of complex behaviors. Capitalizing on this knowledge, synthetic biology seeks to manipulate the cellular machinery towards novel purposes, extending basic discoveries from basic science to new applications. While these developments have demonstrated the potential of building with biological parts, the complexity of cells can pose numerous challenges. We will highlight the role that the synthetic biology approach has played in applying fundamental biological discoveries in receptors, genetic circuits, and genome-editing systems towards translation in the fields of immunotherapy, biosensors, disease models and gene therapy. These examples illustrate the strength of synthetic approaches, as well as considerations that must be addressed when developing systems around living cells.

Introduction

Building basic tools with synthetic biology

Cells regularly perform immense tasks, processing signals from many sources to gauge and execute a proper response. Over several decades, our knowledge of the components and connections that underlie these calculations has expanded in many organisms, providing the foundation to engineer novel behaviors into cells. One of the major aims of synthetic biology and parallel approaches is to translate these discoveries into well-characterized and reproducible components of molecular engineering.

While many such engineering parts have been developed with synthetic biology, this review will focus on three major areas: sensors, genetic circuits, and genome controls. Sensors allow engineered cells to detect and respond to a variety of extracellular and intracellular signals, such as cancer antigens,[1,2] light,[3] and small molecules.[4,5] Since the seminal works of the synthetic toggle switch[6] and oscillator,[7] many synthetic genetic circuits have been built to program both prokaryotes and eukaryotes with synthetic, complex decision-making systems such as logic gates,[8] classifiers,[9–11] edge detectors,[12] counters,[13] feedback controllers,[14] and finite state machines.[15] These works have been complemented by the advancement of genome editing tools that have made rapid genome modification feasible in many species.[16–19] The results from work in all three of these areas have produced tools that can be applied towards both the understanding and engineering of biology.

Interrogating biology using synthetic tools

The construction of synthetic molecules and circuits has enabled scientists to explore the complex networks underlying

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cellular behavior. These studies often fall into either a ''reverse'' or ''forward'' engineering approach.[20] In the ''reverse'' engineering strategy, networks in a cell are perturbed at different nodes,[21] and the resulting changes in behavior provides insights into the role of different genes and proteins. The complementary ''forward'' engineering approach enables scientists to hypothesize and test different design criteria by programming artificial systems into a cell.[22] For instance, artificial feedback loops were generated to study the topology involved in controlling the *Bacillus subtilus* competence network dynamics,[23] and synthetic ''secrete and sense'' circuits were developed to investigate the onset of social or asocial behaviors in yeast.[24]

Toward commercial applications of synthetic biology

In addition to elucidating fundamental design principles, synthetic biology has sought to apply the principles of engineering cellular behavior towards major challenges in areas such as health and the environment. In some cases, synthetic biology has even been able to play a major role in commercial product development. Yeast and bacteria are attractive factories to manufacture chemicals that can be otherwise expensive to produce. These organisms grow quickly, can be scaled towards large-scale production, and—most importantly for synthetic biology—are relatively easy to engineer.[25,26] Yeast have been modified through genetic engineering to produce the immediate precursor to the potent antimalarial drug artemisinin, providing more stability to the production of a drug that was previously reliant on extraction from the wormwood plant *Artemisia annua* and thus subject to plant conditions.[27,28] The pharmaceutical company Sanofi has licensed the engineered yeast strain to produce more than 39 tonnes of the drug, which can be used for more than 40 million treatments. Similar strategies to program synthetic pathways in microbes have been developed to produce opioids in yeast[29] and biofuels in microorganisms.[30–33]

In addition to metabolite production, synthetic approaches have been critical in the development of cancer therapeutics and disease diagnostics. The engineering of T cells using synthetic cancer-targeting receptors has been hailed as a breakthrough in cancer therapy due to its unprecedented efficacy against leukemia.[34] Genetic Boolean logic circuits have been expressed in *Escherichia coli* to detect glycosuria in urine from diabetic patients,[35] and synthetic circuits have been adopted in a cell-free and paper-based format for the rapid and low cost detection of various chemicals and Ebola viral RNA.[36]

Learning from the synthetic biology process

Synthetic biology is a vibrant field, and many reviews have provided surveys of the fundamentals and applications of the field.[20,25,37,38] The goal of this review will instead be to highlight synthetic biology as a framework to bridge fundamental studies of biology with applications. We will illustrate this viewpoint by summarizing the developments of the chimeric antigen receptor, synthetic gene circuits, and genome editing tools, which each reflect developments in the three major categories of synthetic systems described earlier (sensors, circuits, and chassis).

The principles of synthetic biology have taken shape in these areas through examinations on the modularity of biology (chimeric antigen receptors), the use of iterative, computationally-driven design (synthetic oscillators), and the ability to develop tools for widespread use (genome editing). These characteristics are not mutually exclusive, and themes that describe one of these areas of research may be visible in the others. These works also reflect the fundamental requirement and challenge of synthetic biology: implementation of an engineered system with an environment that is both complex and not fully understood. The challenges that have arisen in these three areas have highlighted gaps in our knowledge and provided further insight into the relevant parameters for these technologies. These new approaches to biology are accompanied by serious questions regarding their safety and practicality. In assessing the scientific, economic, and ethical considerations that are part of the synthetic biology process, we can gain a deeper understanding of how to build genetic technologies that are applicable to real world challenges.

Chimeric antigen receptors

Chimeric receptors to understand T cell signaling

Our adaptive immune system regularly performs complex calculations with tremendous consequences for our health. T cells are vital to this system, driving the detection, elimination, and memory of pathogens through processes that are largely directed by the T cell receptor (TCR). The TCR assesses other cells for signs of pathogen invasion by analyzing epitopes—short peptide sequences excised from proteins in the antigen-presenting cell (APC)—positioned on the cell surface by the major histocompatibility (MHC) complex. Activation through the TCR is MHC-restricted, meaning that the receptor must bind to an MHC-peptide complex to trigger the T cell.

In the 1980s, the first chimeric receptor to trigger T cell activation was developed and expressed[39] (Fig. 1A). Connecting the variable regions of an antibody to the variable chains of the TCR, these chimeric receptors were able to detect antigens and activate the T cell upon target-binding (Fig. 1B). This response was dictated by the binding of the variable antibody region to the target antigen, indicating that this chimeric receptor-mediated T cell activation was not MHC-restricted. The clinical potential of this receptor to treat cancer was evident and noted in the paper. However, long before the appearance of promising clinical data, chimeric receptors became a powerful research tool to study the proteins involved in T cell signaling.

The TCR contains variable α and β chains in complex with several invariant proteins. One of the early goals in TCR research was to decipher the roles of these invariant proteins activating the T cell. In the 1980s, the variable chains of the TCR were known to complex with CD3 chains,[40–44] and further studies illustrated that the CD3ζ chain in particular was coupled to molecules engaged in signal transduction.[45,46] However, while the association between the TCR α and β chains with the CD3ζ chains was clear, the specific role of CD3ζ in T cell activation was not well understood.

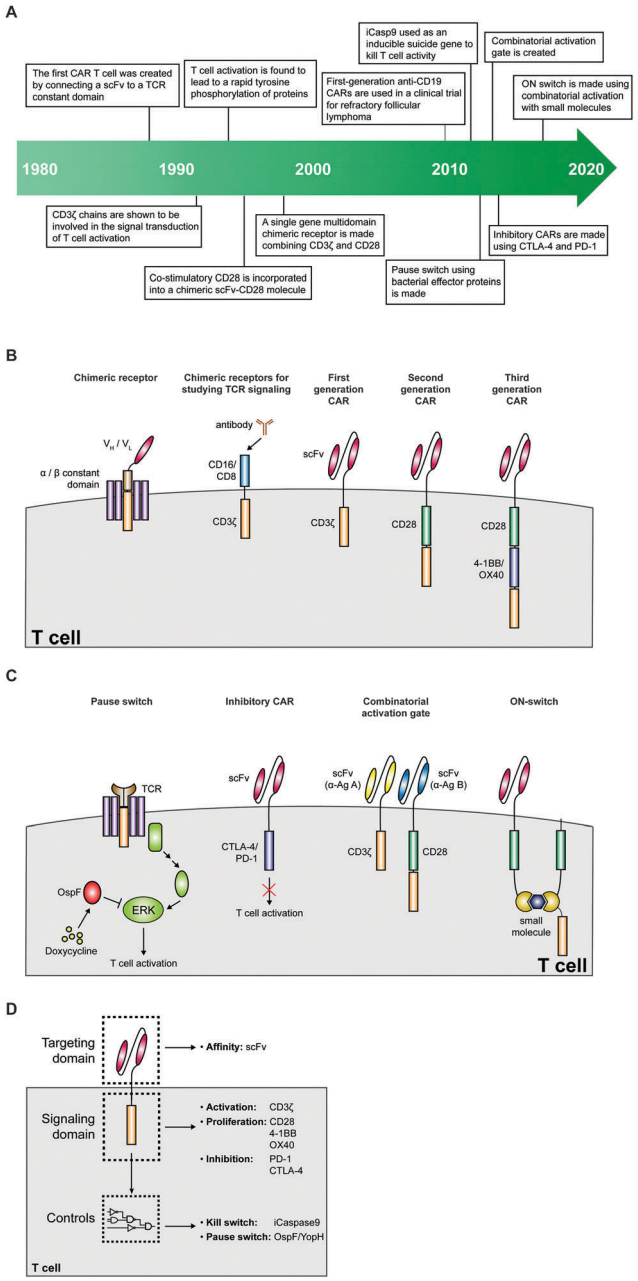

Fig. 1 Synthetic biology approaches for engineered receptors and activation controls. (A) Timeline of engineered receptors. (B) Chimeric receptors were created to help study T cell activation. Further development on their design has led to their use in therapeutic applications, for example the use of chimeric antigen receptors. (C) Knowledge of how T cells activate through receptors and downstream pathways has allowed for the development of various receptor domains and genetic switches to control T cell activity. Examples of this are the pause switch using bacterial effector proteins YopH and OspF; CAR using CTLA-4 and PD-1 inhibitory domains; split CAR to target multiple antigens; and an ON-switch using small molecule activation. (D) Chimeric antigen receptors are a modular system for engineering T cell control. Individual components can be modified to change the overall behavior of the receptor. The main modules of the CAR consist of a targeting domain, signaling domain, and downstream controls.

The chimeric receptors developed to study T cell signaling domains were constructed with different domains from the original chimeric receptor. In place of antibody regions, the extracellular domain of these chimeric receptors contained regions of proteins such as CD16[47] or CD8[48,49] that have known targets for antibody-binding. Meanwhile, the intracellular domain of the receptor was comprised of the CD3ζ chain (or in some studies, fragments of the CD3ζ chain) (Fig. 1B). In isolating this chain from the TCR complex, these chimeric receptors enabled studies on the involvement of CD3ζ in activation upon binding of an antibody to the extracellular domain.

These chimeric receptors provided significant insights into TCR design, demonstrating that the fusion of the intracellular region of the CD3ζ chain to the extracellular binding domain was sufficient to trigger activation.[49] Similar experiments using fragments of the CD3ζ in place of the full domain identified the 18 residue active site of the chain.[47,48] These efforts reflect the ability to use synthetic molecules to identify key characteristics of relevant molecules. The ability to turn these early chimeric receptors against a therapeutic target was apparent when a chimeric receptor specific for HIV envelope protein was able to direct cytolytic activity against target cells.[50]

Therapeutic applications of synthetic receptors

The knowledge obtained from chimeric receptor studies fed back into the development of chimeric antigen receptors (CARs) for cancer therapy. The performance of CD3ζ in the chimeric receptors for TCRs supported its potential for use as part of the therapeutic design,[51,52] and the first generation of CARs were a fusion of the CD3ζ domain to the single chain variable fragment (scFv) from an antibody (Fig. 1B). However, initial clinical results indicated that the first-generation CARs were unable to persist in patients long enough to trigger a therapeutic response[53,54]

This initial CAR design reflected a focus on the antigen-dependent aspect of T cell activation. However, T cell activation requires several signals. The binding of the receptor to a target antigen is one signal, but this step is supplemented by a co-stimulatory trigger that is generated by the interactions of surface molecules expressed on both the T cell and the APC. Previous studies demonstrated that chimeric receptors could be designed to trigger co-stimulation. In 1996, a synthetic receptor was designed to make CD28—a costimulatory glycoprotein expressed on the surface of T cells—antigen-specific through the construction of a scFv-CD28 molecule.[55] This chimeric receptor elicited a co-stimulatory response with the endogenous TCR complex that was comparable to wild type CD28. Furthermore, when the chimeric receptor was co-transfected with another scFv-CD3ζ receptor, the signals from these two chimeric receptors were able to mediate a T cell response, indicating that the CD28 and CD3ζ domains could be engineered together to drive optimal T cell response. The intracellular domain of CD28 was integrated with CD3ζ in 1998 to produce a multi-domain chimeric receptor.[56] Today, this CAR is considered a ''Second Generation'' CAR due to its integration of a single co-stimulatory domain with the activating CD3ζ domain (Fig. 1B). The repertoire of these second generation CARs has grown to include other co-stimulatory domains, including 4-1BB[57] and OX40.[58]

This structure has been further expanded in ''Third Generation CARs'' to include multiple co-stimulatory domains in one receptor[57–59] (Fig. 1B).

Synthetic approaches to advancing therapy

These advancements reflect a modular approach to receptor construction that has brought chimeric antigen receptors (CARs) to the forefront of cancer research. T cells are uniquely equipped with the ability to kill other cells, and CARs can effectively ''teach'' a patient's T cells to detect and eliminate cancer cells. Despite the poor clinical performance of first-generation CARs, the improvements made in second-generation CARs drove such strong responses against B cell malignancies that several clinical trials have elicited up to a 90% complete response rate in patients.[60–62] However, the onset of toxicities including cytokine storms and fatal off-tumor responses exposes the limitations of this therapy as well.[61,63]

Underlying these toxicities is a fundamental question of cell-based therapies in their current state: once the cells have been transfused back into the patient, how can a desired behavior be ensured? Current approaches to cytokine storms require the use of steroids to weaken the immune system and reduce the onset of cytokine release syndrome (CRS)-related symptoms.[60] However, compared to the relative simplicity of chemical drugs, cells require more powerful safety strategies to match their own complexity and provide the most effective treatment for an individual patient.

The synthetic approach underlying the development of CARs has played a prominent role in the development of more powerful controls over adoptive T cell therapy, creating an attractive arena for synthetic biologists and immunologists to collaborate. For example, the bacterial virulence proteins OspF (outer *Shigella* protein F) and YopH (*Yersinia* outer membrane) have been introduced into T cells to create a drug-inducible ''pause switch'' due to their ability to inhibit kinases involved in TCR signaling[64] (Fig. 1C). Synthetic approaches can additionally be implemented to control the growth or viability of T cells, such as a ribozyme switch to regulate expression of interleukin (IL) 2 or IL 15,[65] or the inducible kill switch iCaspase9, which has been implemented to treat graft-*versus*-host disease (GvHD) in stem cell transplant patients.[66]

The underlying promise of modularity that has made CARs successful has continued to drive the design of novel receptors with additional controls (Fig. 1D). By altering the signaling domains of the chimeric receptors, responses other than activation can be programmed into the cell for a given target. For example, instead of utilizing signaling activation and co-stimulatory domains, chimeric receptors assembled with inhibitory domains like CTLA-4 or PD-1 block T cell activation upon antigen-binding[67] (Fig. 1C). These inhibitory CARs (iCARs) have the potential to increase specificity in immunotherapy by blocking the T cell from attacking certain tissue without completely shutting down the treatment.

While we have largely discussed modularity in the context of adding domains together in a direct fusion, this property can also take effect when considering the splitting of the chimeric receptors into multiple components. For example, while the scFv-CD28 and scFv-CD3ζ experiments originally demonstrated the co-stimulatory role of CD28, these results also indicated that multiple chimeric receptors containing different signaling domains can synergistically drive activation. By choosing scFv's for this split receptor design that target different antigens, full activation of the T cell requires both the scFv-CD3ζ CAR and the scFv-CD28 chimeric co-stimulatory receptor to detect their respective antigens[68] (Fig. 1C). This combinatorial activation system can thus increase T cell specificity towards a tumor.

CARs can also be split into two halves to allow greater control over when the T cell will activate, as implemented in the ON-switch CAR. Instead of dividing the CAR into two separate chimeric receptors, the ON-switch CAR splits the CAR into two separate domains—antigen-binding and intracellular signaling domains—that attach in the presence of a drug[69] (Fig. 1C). Even if the antigen-binding domain binds to the target antigen, it will not activate the T cell until the drug has been added and the intracellular signaling domain has attached. Further efforts to improve CAR-based therapy with synthetic biology are ongoing, and as researchers from both sides work to develop technologies that will make this treatment safe and viable for many patients, the melding of these approaches may provide a greater understanding of both the immune system and the design of receptors.

Genetic circuits

Applications of genetic circuits towards medicine

Gene networks are vital for the regulation of cellular behavior. The topology of these networks can have important implications for processes that take place when a cell is establishing its identity, all the way through its death. One of the natural goals of synthetic biology—a field built out of genetic engineering—is to apply the principles underlying the behavior of these circuits towards the design of novel systems. Novel gene circuits have turned organisms into pharmaceutical and biofuel factories, as described earlier, as well as playing a role in the advancement of therapeutics. The drug-inducible ''pause switch'' described in the discussion of CAR therapies illustrates the potential for genetic circuits to enable bedside control of cell-based therapies, as does the riboswitch designed to provide control over T cell proliferation.[65] Synthetic gene circuits have also formed the basis of multiple strategies to treat diabetes. In one approach, the expression of hormone that mediates blood glucose homeostasis in type 2 diabetes is activated by a signaling cascade triggered by blue light, which was able to provide optogenetic improvement of blood-glucose homeostasis in a type 2 diabetic mouse model.[70] Another strategy that has been tested in mice for mediation of blood glucose levels is through circuit that produces insulin in response to radio-waves.[71] Synthetic circuits have also been considered as a tool to treat cancer, such as the microRNA-based cancer cell classifier that uses microRNA expression to identify cancer cells and trigger apoptosis.[72] And while these circuits have all been implemented within a cell, they may also have great

power when applied in other mediums. For example, the ability to program toehold switch mRNA sensors onto paper has opened up the potential to quickly produce sequence-based sensors at a low cost.[36]

Taking inspiration from designs in nature

These systems all reflect an ability to construct genetic circuits towards an application. However, synthetic biology often involves the iterative process of constructing and refining the design of a system through many different systems and components. This process often takes place within a lab, with the published work not reflecting the many designs that were attempted and discarded on the route to the final product. The synthetic oscillator—a foundational construct of the field[7]—enables a macroscopic view of improving on a design over time, as multiple works have been published over several decades to improve the performance of the circuit and create various implementations, ultimately opening up its potential use for application.

Oscillations are a common network in nature, causing the appearance of a particular output to be cyclicle. Examples range from chemical, such as the Belousov–Zharbotinsky reaction, to much larger systems, including the mathematically well-characterized predator–prey ecosystem model.[73] Within cells, many signaling pathways contain components exhibiting oscillatory behavior, such as the tumor suppressor p53,[74,75] NF-kB,[76] and cAMP,[77] and the Cdk1–APC system controlling cell division.[78,79] Periodic gene expression is also observed in budding yeast growing under nutrient-limited conditions.[80] Circadian rhythms in many organisms are controlled by oscillating genes, such as the KaiABC gene cluster in cyanobacteria,[81] the *per* and *tim* gene in Drosophila,[82,83] and the *frq* gene in Neurospora.[84] Additionally, oscillations can drive synchronous behavior between organisms, such as the rhythmic flashing of fireflies[85] and the acetaldehyde-driven glycolytic oscillations in yeast.[86]

The prevalence of oscillators correlates to their significance in biology. Plants rely on circadian dynamics to coordinate expression of certain genes to the light–dark cycle.[87] In addition, the frequency or amplitude of oscillations can encode temporal information, as is observed in the transient signaling of calcium.[88,89] While calcium ions are a common signaling molecule, they are toxic at high, sustained concentrations. Encoding information on the amplitude (amplitude modulation, AM) or frequency (frequency modulation, FM) of calcium oscillations can potentially enable the cell to decipher information from the transient presence of the ion. For example, B cells rely on AM signaling to decide between several different pathways of activation.[90]

Designing synthetic oscillators in cells

The primary design requirement to drive oscillations is the presence of a negative feedback loop with some delay.[91] However, the inclusion of this motif does not guarantee optimal performance of the overall circuit, particularly in cells where factors such as cell division and stochastic gene expression can affect the network.

Consisting of three transcriptional repressor (TetR, LacI, and λCI) arranged in a cyclic negative feedback loop, the repressilator was the first synthetic cellular oscillator[7] (Fig. 2A and B). With this system, 40% of cells showed oscillations of GFP in *E. coli*. However, performance of the repressilator was not optimal; noise in gene expression resulted in large variability in the amplitude and period of oscillations, and the circuit was unable to produce oscillations at the stationary phase of cell growth.

To produce a more robust oscillator in *E. coli*, a positive feedback loop was introduced in the general structure of the activator/inhibitor relaxation oscillator[92] (Fig. 2B). Containing both activator (NRI) and repressor (LacI) modules, the oscillation period was tunable by altering the nutrients in the medium. More importantly, the system was robust to noise and able to function in cells growing at the stationary phase. The tunable relaxation oscillator added a second autonegative feedback loop to the structure, driving oscillations in approximately 99% of the cells in the population[93] (Fig. 2B). These cycles were also preserved after cell division and tunable, this time by varying temperature and inducer concentration. However, synchronization between the cells was limited.

In nature, the behavior between cells in a population can be coordinated through quorum sensing. Relying on communication to synchronize oscillation dynamics between the cells, this mechanism provided inspiration for several synthetic designs to reduce population variability. In a micro-chemostat system designed to maintain *E. coli* density, the LuxI/LuxR system from *Vibrio fisheri* enabled cells to communicate with each other *via* acyl-homoserine lactone (AHL) accumulation in the media, which triggered the expression of a kill gene in receiving cells.[94,95] This AHL-mediated quorum sensing also provided negative feedback in a predator–prey ecosystem analog in bacteria. Utilizing LuxI/LuxR and LasI/LasR (derived from *Pseudomonas aeruginosa*), prey cells produced a signaling molecule that rescued predators[96](Fig. 2C). Meanwhile, predator cells produced a signaling molecule that killed prey cells. Quorum sensing has appeared in other designs as well due its ability to synchronize the behavior of cells[97,98] (Fig. 2C).

Oscillator circuits have also been designed in *E. coli* by integrating metabolic and genetic parts (Fig. 2B). This "metabolator" contains transcriptional elements whose activities are regulated by outputs from the acetate pathway, producing oscillations that were observed in approximately 60% of the cells and unaffected by cell division.[99] However, this circuit was subject to variability due to stochasticity in gene expression. Oscillators were also implemented in mammalian cells using the tetracycline- and pristinamycin-controlled transcription systems, though this system was also subject to variability[100] (Fig. 2B). As synthetic biology makes advances in engineering new systems and organisms, the same spirit underlying this process that produced more robust oscillators may help to elucidate many other circuit designs.

Advancing oscillator design towards an application

Due to their natural role in relaying environmental cues, oscillators hold promise as integral components in biosensors.

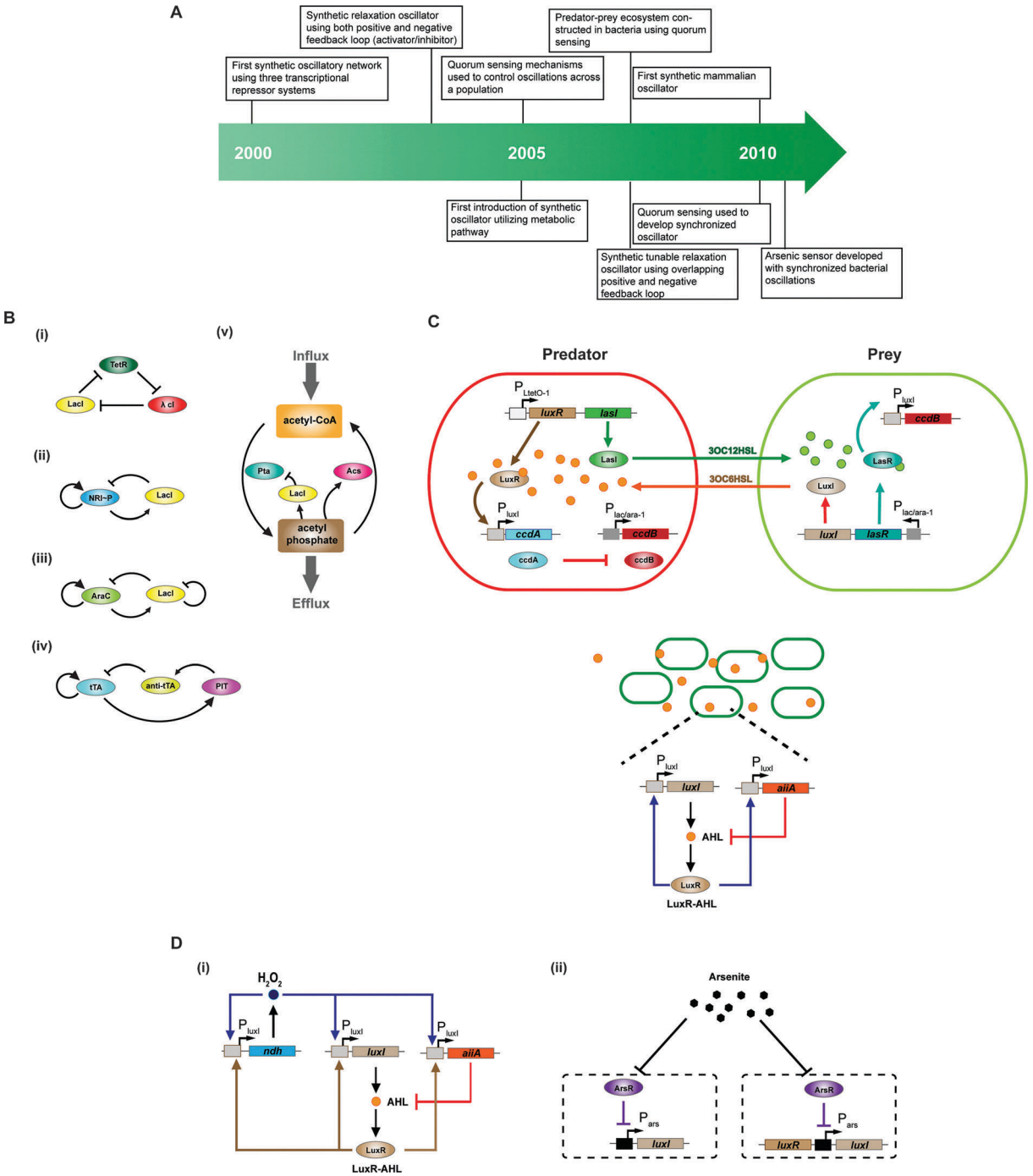

Fig. 2 Topologies, population based and application of the oscillators. (A) Timeline of synthetic oscillators. (B) Topologies of different oscillators. (i) Repressilator. Three repressor genes were used to repress other repressors. TetR represses λcI, and λcI represses LacI. LacI represses TetR. (ii) Activator/inhibitor relaxation oscillator. This design contains both activator (NRI) and inhibitor (LacI) modules. This design utilizes a positive autoregulatory circuit. (iii) Tunable relaxation oscillator. The second autonegative feedback was introduced to increases robustness of oscillator. (iv) Mammalian oscillator. Used both positive and delayed negative feedback. (v) Metabolator. This design utilize both transcriptional regulation and metabolic flux. (C) Population based oscillators. (top) Synthetic prey–predator system using bacterial quorum sensing signals. In prey cells, first QS molecule, 3OC6HSL (colored in yellow circle), is synthesized by LuxI and binds to transcriptional regulator LuxR to increase antidote gene expression (ccdA). In predator cells, second QS molecule, 3OC12HSL (colored in green circle), is synthesized by LasI and binds to LasR to activate kill gene (ccdB). (bottom) Synchronized oscillators. AHL QS molecule is synthesized by LuxI, which then binds to transcriptional regulator LuxR to regulate expression of LuxI and aiiA. aiiA gene degrades AHL and acts as negative regulator. (D) Arsenic sensing genetic biosensor. (i) Network diagram of sensing array. *luxI* synthesize QS molecule, AHL, that regulate gene expression of *luxI*, *aiiA*, and *ndh*. *ndh* gene synthesizes enzyme that generates H_2O_2 which acts as second QS molecule that activates expression of *luxI*, *aiiA*, and *ndh*. (ii) Network design of arsenic sensor. In thresholding (left), sensor reports the presence of arsenite above certain concentration. Here, *luxR* gene is removed from sensing array network, and it is now controlled by arsenite-responsive promoter that is repressed by ArsR in absence of arsenite. In period modulation (right), sensor is able to report the concentration of arsenite through frequency of oscillation. Here, *luxI* is under arsenite responsive promoter that affects oscillation period.

The work described to implement and refine synthetic oscillators has made it possible to apply them towards this purpose. One example is the development of an arsenic sensor using *E. coli*,[101] which relies on quorum sensing to synchronize the output of thousands of bacterial "biopixel" colonies (Fig. 2D). Two arsenic-sensing modules were designed with this capability: one that can report the presence of arsenic above a certain concentration, and another that is able to report the concentration of arsenic through the frequency of GFP reporter oscillations (Fig. 2D).

The designs of new oscillators that build upon previously established systems are emblematic of the iterative process that underlies all engineering. The primary focus of these efforts over time was to increase the robustness of the circuit towards stochastic fluctuations and growth dynamics, as well as to reduce cell-to-cell variability. With each new design, the addition of new components and connections helped to improve the behavior of the overall circuit. The oscillator is thus an excellent case study in the exploration of new design rules over time to combat some of the challenges posed by biological noise.

Genome editing and control

Bacteriophage and bacteria for genome editing technologies

The interaction between invasive organisms and their attempted hosts can often turn combative. Our own bodies have evolved a complex machinery of defenses against pathogens, executing innate and adaptive commands to both detect and eliminate threats. Bacteria may not have the same capacity to develop this extensive immune system, but they also mount their own strategies against bacteriophage. For these organisms, the genome is the battlefield. Bacteriophage attempt to integrate their genetic material into the target cell, and the infected bacteria respond by cutting these sequences out. Both of these processes require enzymes that can target and cut specific DNA sequences, and investigations into the interactions between bacteriophage and bacteria have driven the development of several tools for genome editing and control (Fig. 3A and B). Bacteriophage-derived enzymes such as the lambda tyrosine integrase,[102] Cre recombinase,[103–105] PhiC31,[106,107] and Bxb1[108] have allowed for site-specific control over inversion, excision, and translocation of DNA (Fig. 3C). The ability to use these enzymes *in vitro*[109] and in other organisms, including yeast and mammalian cells[110,111] have made them useful tools in engineering downstream applications, such as disease models based on cell-specific expression of a particular gene.[112,113]

While recombinases are powerful, their specificity is already programmed. Two systems for targeted genome editing have been developed with the bacterial nuclease Fok1, which dimerizes to cleave DNA (Fig. 3D). One system utilizes transcription activator-like effectors (TALEs), a bacterial protein involved in infecting different plants.[114] By altering two residues in the TALE and then fusing the modified protein to the Fok1 nuclease catalytic domain, DNA cleavage can be targeted towards a desired sequence with this transcription activator-like effector nuclease (TALEN). A similar strategy has been devised with zinc fingers,

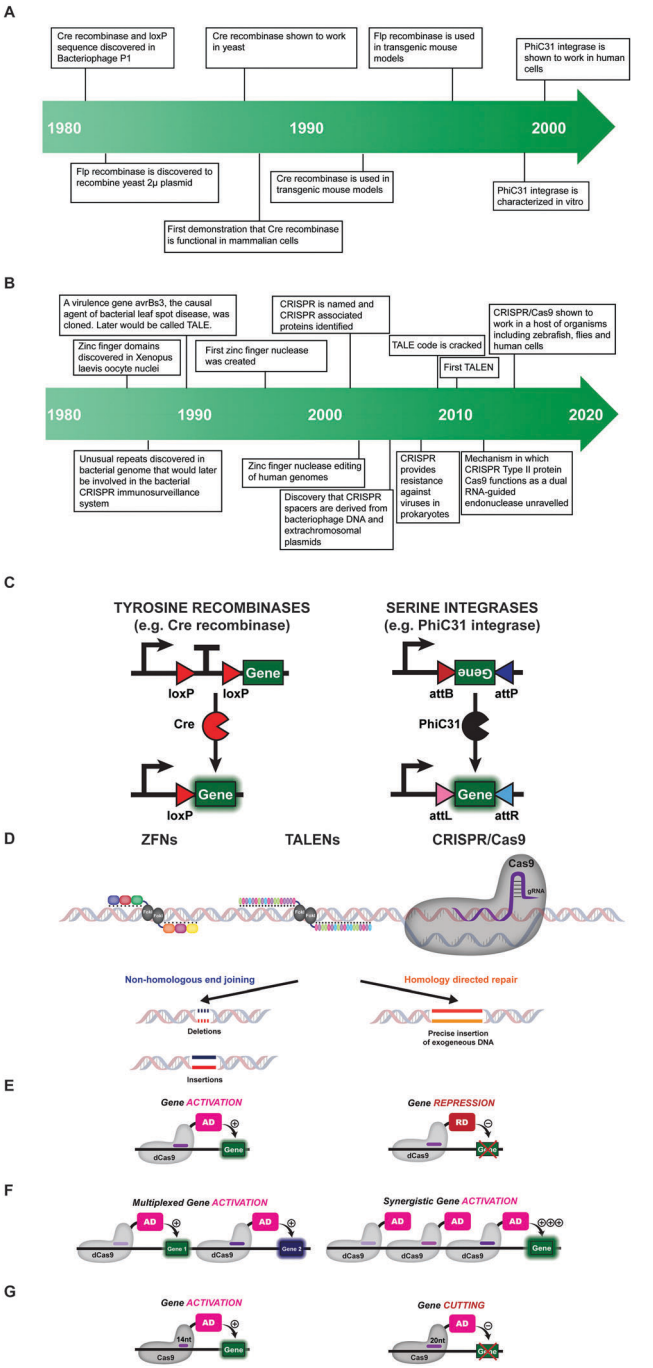

Fig. 3 Genetic tools for genome engineering. (A) Timeline of recombinases and integrases. (B) Timeline of genome editing proteins. (C) Site-specific recombinases are commonly used to delete or invert DNA sequences through two often used classes. (D) Site-specific nucleases, such as ZFN, TALEN, and CRISPR/Cas9 use different DNA binding elements to achieve imprecise DNA deletions or insertions through non-homologous end joining or precise DNA addition through homology directed repair. (E) Catalytically dead Cas9 (dCas9) can be fused to transcription activation (AD) or repression (RD) domains to enable activation or repression of genes, respectively. (F) Multiple guide RNAs can be used for multiplexed gene activation or repression. Multiple guide RNAs can also be designed to target a promoter to achieve higher levels of gene activation in a synergistic fashion. (G) Using a shorter gRNA target sequence (14nt) abolishes Cas9-mediated nuclease activity yet permits DNA-binding and activation of genes through fusion of a transcription activator.

which contain a Cys2–His2 zinc finger DNA-binding motif, in place of TALEs. This design is advantageous because zinc finger nucleases are small and can be easily engineered to target a desired sequence when connected to the Fok1 catalytic domain.[115] In addition to genome cutting, both TALEs and zinc fingers have been engineered with other effector domains to trigger targeted gene activation and repression.[115–118]

Recombinases and Fok1 are both derived from the bacterial "innate" defenses. However, a broader picture of a bacterial adaptive immune system has been illustrated following the observation of repeats within the bacterial genome termed Short Regularly Spaced Repeats (SRSRs).[119,120] More than a decade after the discovery of these repeats, proteins associated with the SRSRs were uncovered, and the observed system was labelled as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR).[121] Eventually, the origins of the CRISPR spacers were traced to bacteriophage and extrachromosomal DNA,[122–124] supporting the hypothesis that CRISPR is a system of acquired resistance against viruses and plasmids that allows bacteria to "memorize" sequences associated with threats.[125,126]

The naturally occurring CRISPR requires two RNA molecules: a mature crRNA that is complementary to the target sequence, and a tracrRNA that activates Cas9 cleavage of DNA.[127–129] The only requirement for these RNA molecules is that the target sequence for the crRNA be adjacent to the three base-pair NGG sequence, called the PAM sequence. Cas9-mediated cleavage of a sequence triggers repair mechanisms that can effectively knock out expression of a gene. With the ability to easily target genes for knockout, Cas9 contains many appealing traits as a genome editing tool, but further engineering of the enzyme has been undertaken to make it more widely used.

Developing CRISPR into an easy-to-use tool

One of the first steps to making an accessible Cas9-based system was to reduce the number of components required for its use. In place of separated tracrRNA and crRNAs, these two strands were combined into one synthetic single guide RNA (sgRNA).[129] Due to the simple PAM-adjacent requirement for a target sequence, the identification and verification of a sgRNA targeted towards a particular gene is easy, especially when compared to zinc finger nucleases and TALENs. This chimeric sgRNA design turned a compelling study of bacterial adaptive immunity into an easy-to-use tool for genome modification, making CRISPR currently one of the most promising tools of synthetic biology and setting a precedent for further modifications of the system.

Zinc fingers and TALEs took on new functions through the addition of different effector domains that could trigger nuclease activity, gene activation, or gene repression. To turn Cas9 into an enzyme of similar potential, a catalytically inactive dCas9 was generated.[19] The dCas9 enzyme can still be guided towards a target sequence using a sgRNA, and while in this catalytically dead form, the dCas9–sgRNA complex can interfere with transcription and repress gene expression. Similar to zinc fingers and TALEs, dCas9 can regulate gene expression by connecting the enzyme to a transcriptional activator (such as VP64) or repressor domain (such as the KRAB domain), and then guiding the dCas9 towards a desired promoter (Fig. 3E).[18,130,131] By expressing several sgRNA targeted towards different promoters, this system can activate or repress several promoters in a multiplexed fashion (Fig. 3F).[132] The field of synthetic biology has a vested interest in improving CRISPR in these various functions, and the exploration of new domains such as the SunTag (a peptide array that can recruit antibodies fused to VP64)[133] and VPR (a fusion of VP64, p65, and Rta)[134] have helped improve the gene activation capability of dCas9. The construction of split-Cas9s that allows for chemical- or light-inducible control of the enzyme may also aid further implementation of CRISPR to control gene expression.[135]

One of the major concerns with any genome editing technology is off-target activity, which could both confound scientific results and drive toxicities in therapeutic applications. Investigations into the specificity of CRISPR have provided mixed results, with suggestions that sgRNA size and concentration can affect the probability of off-target events,[136–138] and a computational model has been developed for optimal sgRNA design based on these results.[139] Another strategy to improve CRISPR-based nuclease specificity does not use the native Cas9. Instead, the catalytically inactive dCas9 is fused to the Fok1 nuclease, which requires the dimeric binding of two sgRNAs to trigger cleavage.[140,141] This dimeric binding requirement thus improves the specificity of the overall system.

The performance of dCas9–Fok1 is a prime example of how the native CRISPR system can be improved to suit our engineering purposes. As synthetic biologists continue to modify and experiment with different applications of Cas9, it is entirely possible that the current iterations of CRISPR for genome control will not be the forms used in the future. A new CRISPR enzyme Cpf1 has been uncovered with tremendous promise due to its smaller size and potential for more accurate genome editing.[142] Furthermore, our understanding of how to design technologies around Cas9 is evolving as we gain further insight into the effect of sgRNA length. While shorter sgRNAs cannot mediate DNA cleavage, they are able to mediate Cas9–VPR activation of gene expression[143,144] (Fig. 3G). And yet Cas9–VPR still contains the catalytic nuclease activity of the original Cas9 enzyme and can be applied as a nuclease when targeted towards a sequence with larger sgRNAs. These results demonstrate that one enzyme can be programmed towards different purposes based on the size of the sgRNA and can even carry out orthogonal knockout and activation of genes in the same cell.[143] Whether there are other strategies to manipulate Cas9 behavior remains to be seen.

CRISPR has been more widely adopted than other genome editing technologies because of its efficiency and ease-of-use. This is a case where synthetic biology strategies have played an important role in simplifying the CRISPR workflow, and thus helped democratize a technology to make cellular engineering a widely used tool. The ultimate contribution from CRISPR may not be one specific tool, but instead a toolbox that provides scientists with a wide range of options that are appropriate for different systems and questions. Already, the methodologies underlined by CRISPR have helped in the rapid generation of

complex animal disease models[145–149] and is being further explored for use in gene therapies.[150,151] As the arsenal of genome editing tools expands, the scope of scientific questions and engineering challenges that can be addressed will also expand.

Broader lessons

Every engineering field strives to produce technologies that can address real world challenges. This goal requires careful understanding of the rules governing the materials used, a task that can be particularly challenging when trying to develop tools based on cellular substrates with ill-defined properties. Synthetic biology represents a new frontier of technology, and understanding its ability to solve problems in the world is an ongoing undertaking. The work described in this review highlights the potential of building novel systems with biological components. Their success can provide insight into strategies that will make synthetic biology more widely applicable.

Designing better biological tools

To design structural buildings and transportation that fulfill varying requirements, engineers have relied on a careful understanding of the world they are constructing in. Constructing within a cell thus poses a distinct challenge because of the many components and processes that are not fully understood. These unknown factors can wreak havoc on otherwise carefully planned systems.

The most direct approach to address this challenge is to extensively characterize the known components and interactions of the engineered system. For example, varied results between clinical trials with CARs that use different co-stimulatory domains have emphasized that to fully take advantage of this system's modularity, we must understand the parts used. CARs with CD28 chains have exhibited faster action and shorter persistence when compared to 4-1BB CARs, affecting the onset of cytokine release syndrome and B cell aplasia in patients.[60–62] Further *in vitro* and *in vivo* characterization of CARs containing these domains have revealed that—dependent on scFv and expression level—CD28 can mediate constitutive proliferation of T cells prior to target binding, a result that may have implications for its use in therapy.[152] Another comparison of CD28 and 4-1BB CARs have suggested that CD28 CARs may provide better tumor control at low T cell doses compared to 4-1BB.[153] This study also tested the performance of a newer design that—instead of linking the co-stimulatory domain directly to the receptor—co-expresses 4-1BBL (the ligand that binds to 4-1BB) with the first generation receptor. This receptor/4-1BBL co-expression model exhibited the greatest control of tumors at low T cell dosages, expanding the space of CAR design.

Further characterization of the scFv of the CAR can provide additional insights into the development of safe and effective therapies. Choosing a lower affinity CAR has the potential to increase T cell selectivity of the tumors,[154] implicating the scFv affinity as a possible parameter to tune the safety of the therapy. As noted in the study of constitutive proliferation associated with the CD28 domain, this continuous proliferation was only observed with certain scFvs, though the exact rules describing this effect are still not understood.[152]

Indeed, there are a number of variables that can affect therapeutic outcomes, including receptor expression level and transduction method. These effects may have predictable consequences, but there will likely be responses that require further investigation and characterization. CAR-based therapy reflects a need to understand not just how engineered parts affect the cell, but also how engineered cells can affect the body. Complexity does not only originate from the cell, but also from its surroundings, especially when the environment contains the intersection of physiological systems that affect toxicity, immunogenicity, and functionality. These considerations have long been important considerations in medicine, and as synthetic biology and adoptive immunotherapy seek greater relevance, these fields will need to learn how to take the whole body into account as a design constraint.

Computational models can also play a powerful role in characterizing the interactions that affect these synthetic systems, as evidenced in construction of increasingly robust oscillators. The iterations of synthetic oscillators relied heavily on theoretical models to determine how tuning different parameters would affect experimental performance. For example, the repressilator model illustrated the roles of protein synthesis and protein decay rates, which was instituted experimentally with promoters to vary synthesis rate and a carboxy-terminal tag to control protein decay rate.[7] Models also helped inform the development of stable oscillations in the activator/inhibitor relaxation oscillator[92] and the relationship between glycolytic flux and oscillations in the metabolator.[99] Beyond just informing parameter constraints, these models also provide information on network structures that improve circuit behavior,[155] such as the importance of the delayed negative feedback loop and positive feedback loop towards robustness and tunability in the tunable relaxation oscillator.[93] Computational models have played a role in developing CRISPR into a more powerful tool as well, allowing scientists to design sgRNA to more intelligently target endogenous genes.[139] These computational tools are widely available and enable the design of guide RNAs of optimal length and specificity to avoid off-target effects.

Distilling biological results into mathematical terminology can often pose a challenge due to the wide range of potential parameters, but their use underscores a deeper understanding of the system being designed. As the field seeks to design robust systems for wider applications, computational models will continue to play a vital role in improving and characterizing engineered tools.

Assessing broader world views of synthetic biology

While synthetic biology has been able to reduce the cost of drug production, some global challenges remain to be addressed for this approach to be viable towards new technologies. The projects in this review also illustrate some of the global challenges that synthetic biology must address to establish itself as a true player in developing technologies. One of the

major challenges is the cost of many of these technologies, especially those being applied towards therapy. The requirements associated with modifying a patient's own cells have resulted in an enormous cost attached to CAR-based therapies (http://www.wsj.com/articles/new-costly-cancer-treatments-face-hurdles-getting-to-patients-1412627150). While CARs have tremendous potential, this cost could prove prohibitive and prevent its widespread adoption. However, synthetic biology approaches may also help to lower the cost of CAR therapies, such as the development of an "off-the-shelf T cell" through gene editing.[156] The factors affecting the cost of CARs may also apply to future cell-based therapies, and considering both the potential challenges and solutions will aid the development of accessible, powerful therapies.

There are also many considerations to be taken into account when assessing the effect these technologies may have on the surrounding world. For example, the use of genome editing technologies on mosquitoes[157] to eliminate malaria has been controversial due to the potential unforeseen effects these modifications may have on the environment,[158] driving investigations into strategies for increasing the safety of these techniques.[159] Similar controversies exist when considering the potential that genetically modified crops may have on the environment. Outside of environmental concerns, ethical concerns have arisen regarding the use of CRISPR to modify human embryos.[160]

These questions are part of an ongoing conversation about not just what is feasible with synthetic biology, but how scientists in the field should decide and communicate their priorities. The battles that have taken place in the legal arena over genetically engineered seeds and patents have brought synthetic biology technologies to a broader audience, leading to greater scrutiny over these works and how they are done. Many of the works being undertaken in synthetic biology labs were just the realm of science fiction not too long ago, and it should not be surprising that as the public becomes more aware of these discoveries, questions are asked about ethics, safety, and practicality. It is important for synthetic biologists to enter these conversations with as much intention to learn as they have to educate. Nature has shaped biology to tremendous effect, and as we learn to shape biology ourselves, the broader impacts of these works will feed back into the types of research undertaken and how these technologies are received.

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