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Synthetic Biology: Not Just Novel Therapies

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Abstract

Synthetic biology, an electrical engineering, circuit-driven approach to biology, has developed whole new classes of therapeutics. Unfortunately, these advances have thus far been undercapitalized upon by basic researchers. As discussed herein, using synthetic circuits, one can undertake exhaustive investigations of the endogenous circuitry found in nature, develop novel detectors and better temporally and spatially controlled inducers. One could detect changes in DNA, RNA, protein or even transient signaling events, in cell-based systems, in live mice, or even in humans. Synthetic biology has also developed inducible systems that can be induced chemically, optically or by using radio waves. This induction has been re-wired to lead to gene expression changes, RNA stability and splicing changes, protein stability and splicing changes, and even signaling via endogenous pathways. Beyond simple detectors and inducible systems, one can combine these modalities and develop novel signal integration circuits that can react to a very precise pre-programmed set of conditions or even to multiple sets of precise conditions. In this review, we highlight some tools that were developed in which these circuits were combined such that the detection of a particular event automatically triggered a specific output. Furthermore, using novel circuit-design strategies, circuits have been developed that can integrate multiple inputs together in Boolean logic gates composed of up to 6 inputs. We also highlight the tools available and what has been developed thus far. Most of the systems that are presented can be integrated together; and the possibilities far exceed the number of currently developed strategies.

Insight Box

Synthetic biology strives to integrate components of electrical engineering and circuit design with physiology. This integration occurs in two distinct but interrelated ways. Synthetic biology has allowed for the development of model systems in which variables can be carefully permuted and measured. This allows for the development of much better quantitative models that may eventually replace expensive *in vivo* studies with *in silico* investigations. In addition, synthetic biology has developed a number of tools that integrate engineering circuit-design approaches to biology and that

can be used to sense the state of a system or to perturb a system in its native contexts and to then study the effects.

Introduction

Humanity has long striven to control Mother Nature for our betterment. From domesticating animals to modifying food, our desire to mould the world around us can be traced far back. Ultimately, synthetic biology is a rational approach to modifying biological systems, giving us previously unimaginable power over them. Through a combination of biotechnology, molecular biology and electrical engineering, synthetic biology is allowing us to rebuild biological systems from the ground up, redefining what DNA is(1), what genes an organism has(2), how those genes control each other(3), how the proteins that these genes for code interact(4) with each other and form cells and whole organisms(5). Scientists have already taken advantage of these new-found powers to build bio-production systems for previously impossible tasks to produce fine chemicals(6) and to create life-saving theranostics(7, 8). Synthetic biology is also providing us new and more powerful tools to interrogate life and to learn from its billions years of experience. While novel synthetic biology-enabled therapeutics readily attract attention(9), the biological insights gained during the development of these novel therapeutics and the biological insights made possible by the same advances that produced these therapeutics are not always as obvious. This review will attempt to illuminate some of the aforementioned advances and some of the new tools that synthetic biology has produced, primarily focusing on complex and medically relevant systems. Many synthetic biology firsts are developed in prokaryotes due to their simpler nature compared with eukaryotes and mammals. While this review will not cover prokaryotic synthetic biology, one may be interested in consulting recent reviews on this topic to obtain insight into what is yet to come from synthetic biology(10).

As has been discussed extensively elsewhere (7, 11), synthetic biology has prompted a number of fruitful advances in medicine. In some ways, building advanced medical therapies and developing advanced biological tools are very similar goals; however, there are some notable differences. One key difference is that for medical therapies, delivery and toxicity are major concerns. Those issues exist for biological model systems, but their challenges and solutions are different, and some of the solutions chosen in the discussed articles will not be ideal for applying the advances towards non-patient-oriented modeling systems. As a recent review discusses, a major concern with top-of-the-line synthetic medical devices is how to insert these devices into patients safely(12). With biological systems in a laboratory, making organism-wide changes such as germ-line modifications in mice is a viable strategy that side steps a number of these issues. While the review will touch upon some of the limitations and benefits of the different circuit insertion strategies, the ideal application of these tools will depend on the specific process being studied.

This review will highlight the key areas in which synthetic biology has provided us with biological insights and some of the tools that will be responsible for major insights in years to come. One of the clearest applications of synthetic biology is in making models of systems and then refining the models using artificial circuits. Taking a broader view, using synthetic biology, one can re-wire components of a cell to detect specific intracellular or extracellular events. This approach has been extensively used for cell-based screening strategies. Similarly, one can modify endogenous systems to connect them with externally applied stimuli such as light(13), or small molecule(14) inducers to induce specific conditions in the cells of interest to control the cellular environment in a spatially and temporally controlled manner(15). Combining the ability to both detect the state of a system and to

modify it using synthetic biology has allowed for the creation of a therapeutic approach – theranostics(7). Theranostic therapies are those that detect and dynamically cure a patient's pathology. This autonomous approach is also broadly applicable to the design of novel model systems for studying processes in complex organisms such as mice. Finally the review will conclude with an overview of some of the considerations one must keep in mind to ensure that the developed models are productive.

Towards a more Quantitative Understanding

As more evidence is collected on a process, and as our understanding of it increases, it is often temping to propose a model of how the system works and how it is regulated(16). While these models are useful, it is often difficult to verify them directly with conventional biology as one cannot be sure that their perturbation is not having an indirect effect on the system being modeled. However, one of the strengths of synthetic biology is that it allows one to build example systems outside an existing structure and then freely examine how the system operates, and the various variables that can affect its function. This method has been applied in a number of test systems and has yielded information on how transcription factors are organized on a promoter and how signaling pathways are activated; it also has allowed researchers to confirm which components are necessary and sufficient to observe a physiological affect.

Biological Insights in Genomic Element Design

Given its importance in almost all biological systems and its relevance towards designing regulated circuits, the various facets and kinetics of the central dogma have been studied in detail. In recent papers, synthetic biology has been applied to increase our understanding of multi-transcription factor synergy(17), the kinetics of heterochromatin development(18) and the spatially dependent effects of transcription factor binding(19).

It is known that multiple transcription factors binding to the same promoter increase transcription in a synergistic manner. While the requirement of promoter proximity was known (20), the nature by which the spacing between the two transcription factors affects their synergistic activation was well understood. To exhaustively test how the spacing between multiple transcription factors affects their activation, Huang et al developed a synthetic system to test this effect. They tethered a well-known viral transcription factor, VP16, to GAL4(21) and attached two GAL4 binding sites separated by a variable spacing region to a minimal promoter coupled to a luciferase output, as illustrated in figure 1A. Using their synthetic model they were able to show that in contrast to what was commonly believed at the time, the transcription factors must bind on opposite sides of the DNA molecule to cause maximal activation. Because this is an artificial system, one must be careful to ensure that this is representative of an endogenous process; these authors checked this by using another transcription factor in their model.

Along the same lines, Farzadfard et al were among the first to develop a CRISPR/dCas9-targeted transcription factor, and they used this transcription factor to activate or inhibit a measurable gene (GFP)(19). They fused VP64, which contains four copies of the VP16 transcription activator to a catalytically inactivate Cas9 (dCas9) and showed that depending on where it bound on the promoter, it could either activate or repress genes. Binding upstream of the TATA box allowed for promoter activation, while binding on or 3' of the TATA box inhibited protein expression, especially when multiple transcription factors were bound. Building on their work and others', Konermann et al used a CRISPR/dCas9 activation complex to activate a library of 70,290 guides, targeting 200 bp upstream of every known human coding isoform(22). Using this synthetic tool, they

were able to activate up to 10 genes at once and to screen their library for a combination of genes that allowed for resistance to a specific inhibitor.

Once a protein is transcribed in eukaryotic systems, it often undergoes splicing, during which certain regions called introns are removed to form the RNA that will be translated. While the exact 'splicing code' is not yet known, Culler et al investigated how the binding of proteins to various regions along the intron could affect splicing(23). Into a two-intron, three-exon transcript containing GFP, they inserted a sequence bound by the bacteriophage coat protein MS2 (figure 1B). They also developed a chimeric protein of a fluorescent protein coupled to the protein MS2 that would bind to this pre-mRNA. They investigated the effect on splicing of the protein binding to 12 different regions inside the introns and observed that some regions caused increased rates of splicing, while other regions caused almost no splicing(23). They observed that protein binding immediately 5' of the variably-spliced exon promotes the occurrence of exon inclusion, while protein binding closer to the middle of the intron promoted exon exclusion (23).

In another study that illustrated the power of synthetic models, Hathaway et al studied the kinetics of heterochromatin spread(18). They modified a commonly repressed promoter to add two different sets of targetable binding sites and introduced an output system into exon 1 of the gene (figure 1C). They expressed two peptides. Upon the addition of an inducer, these peptides hybridize to recruit Heterochromatin Protein 1 (HP1) to the DNA. Using flow cytometry and ChIP, they were able to watch how the heterochromatin spread from their targeted region and examined the kinetics of its maintenance. They further tried to model gene activation by recruiting a transcriptional activator (VP16) to the gene using another inducer and examined heterochromatin persistence. Further work can now build upon their model to test other potential chromatin regulators to examine their effects.

If instead of inducing the binding of synthetic transcription factors, one is interested in studying the binding of endogenous transcription factors, one can apply a method developed by Schlabach et al(24). To determine the best promoter to use, they created 52,000 synthetic promoters, composed of 10 repeats of all possible 10-mer DNA sequences. Using a synthetic expression system, they inserted their enhancer library and screened for GFP fluorescence. Using only the two strongest 10-mer repeats, they were able to generate promoters that were stronger than the commonly used constitutive CMV promoter. They observed variations among cell lines, which may be indicative of the different transcription factors that are active in different cell lines. Similarly to the examples above, their approach provided them with important engineering information as well as interesting biological insights into the nature of active transcription factor binding sites that could be investigated further.

Kinetics

As is becoming clearer in part as a result of synthetic biology, the kinetics of an interaction between a receptor and its ligand can affect the nature of receptor signaling. Studies have shown that the receptors have different signaling characteristics depending on whether their activation is continuous or intermittent and on how strongly their ligands bind (25, 26). Therefore, neither adding a continuous chemical agonist nor tracking the binary activation status alone is sufficient to obtain a thorough understanding of receptor function. Synthetic biology, with the development of light-activatable receptors such as optoXR GPCRs(27), and its advanced sensors(28, 29) offers solutions to both of these problems. To allow for the temporally and spatially precise control of receptor activation, Airan et al developed a chimeric GPCR that integrated the Rhodopsin extracellular and membrane domains with intracellular domains of interest. Using their system, they were able to

selectively trigger β_2 and α_1 adrenergic receptors in live mouse neurons via fiber optics. While their work did not assess the activation kinetics or how they affect downstream signalling, they were able to modify the behaviour of mice; thanks to the development of their system, kinetics studies became possible.

To study the other end of the signalling pathway, Bloom et al developed miRNA circuits that allowed for the minimally invasive, sensitive detection of β-catenin nuclear entry(28). Bloom et al developed a well-regulated miRNA circuit that constitutively expressed miRNA targeting a reporter gene. When a protein of interest, such as β-catenin, bound to the protein-responsive miRNA, the miRNA was inhibited, allowing for production of the reporter. They developed a multi-parameter quantitative model that was representative of their circuit's function and allowed them to fine tune it such that reporter production correlated to the quantitative β-catenin levels in the nucleus, as verified by Western blotting. Notably, because their work directly detected nuclear β-catenin, it should be less prone to the side effects of more indirect measurement methods. Furthermore, because their sensor does not require lysing the cells for Western blotting, it allows for multiple readings from the same cell, providing unique biological insights into how various Wnt pathway members trigger β-catenin activation. The ability to develop systems that can then be modeled and explored *in silico* is another important biological insight that synthetic biology allows.

Modeling and Verifying Nature

Synthetic biology also provides is the possibility of building mock whole systems and perturbing them to see how the various pieces function together. A number of circuits have been developed in prokaryotic systems, and a significant portion of these circuits have been ported to mammalian cells. Thus far, toggle switches(30), time delay devices(31), oscillators(32, 33), logic gates(34), band-pass systems(35), and cell-cell communication devices(36) have been recapitulated in synthetic systems. Although these systems are synthetic, they provide insights into the nature of real biological circuits. These systems also serve to check that our current models of biological processes are correct.

As an example, it is known that the persistence of signalling can regulate the output of genes such as NF-kB(26). Practically, this is managed by incorporating a time delay system into the signalling pathway and checking whether both signals remain engaged. Time delay circuits are also known to dampen noise and make biological systems more robust. To examine which variables are involved in the development of time delays, Weber et al first modeled and then built a synthetic time-delay device(31, 37). Their device was composed of two sequentially activated genes. The first gene in their circuit, which encodes a transcription factor acts as a buffer, which must reach a certain concentration before activating the last gene, a reporter. Using their *in silico* model, they were able to predict how modifying the stability of the transcription factor would impact its buffering capability and to then verify their predictions using their *in vivo* model. Another design for a time-delay circuit which allows for small-molecule regulated time delay is illustrated in figure 2A(37).

Similarly, when Kramer et al built a toggle switch in mammalian cells, it provided multiple insights into biology: as it (i) allowed the creation and verification of an *in silico* model; (ii) allowed one to determine whether all the required parts were known; (iii) provided novel tools that could be useful in non-quantitative experiments(30, 38); and provided biological insights into key variables of the process. This was performed through two novel circuit designs. In one case, a repressor represses a specific gene until an inducer is added, which inhibits the repressor. This allows for the production of the normally repressed gene, which acts as its own transcriptional activator, promoting its own continued activation in a positive feedback loop(30). Their second method used two transcription

factors that each repressed the other and could further be inhibited by an external inducer (figure 2B). Their semi-quantitative model will not be discussed here, but they were able to observe the importance of balanced transcription factor expression, as when it was skewed towards one transcription factor, it led to a dose-responsive signalling system rather than to a switch.

These models are particularly useful when the system is complex, and while simpler models in eukaryotes provide useful information on the system, they do not always recapitulate all the features that are present in more complex systems such as humans. As an example, a recent success in the synthetic biology field was the building of a tunable mammalian oscillator by two different groups (example in figure 2C). One group, in a paper by Tigges et al, examined the importance of gene concentration on the oscillation frequency and robustness(32). Another group studied the effect of intron length on the oscillation frequency(39). Both of these papers examined features that would significantly differ from a prokaryotic model and highlight the importance of modeling in mammalian cells. Another key benefit of modeling is that it allows one to confirm whether all the key features of the system are known. Indeed, in this case, there is good evidence that key features remain unknown, as thus far, synthetic models have not been able to reproduce the natural rhythms observed in oscillating cells(32).

It has long been desired to model the human organism *in silico*, and with synthetic biology, it is finally becoming possible. As discussed above, synthetic biology provided unique tools for the exhaustive determination of a system's operational parameters, incorporating those parameters into a mathematical model, and then verifying that this model recapitulates the natural system.

Detecting Intra- and Extra-Cellular States

Unfortunately, a system is sometimes too complex or too poorly understood to be modelable. However, even in such cases, synthetic biology has provided novel tools to interrogate the system of interest in cells or even in whole animals. Synthetic biology has been used to develop precise sensors for several different intracellular markers that allow for the detection of specific miRNA, protein, and signalling conditions in living organisms without sacrificing them. Furthermore, as will be discussed in more detail below, synthetic biology allows not only for the detection of specific conditions but also for selective action based on those inputs.

One of the earliest methods of controlling gene expression in knock-in models was the use of endogenous promoters(40). However, possibly as a result of constant whole-genome low-level transcription(41), there was often leaky expression beyond the tissues or areas of interest(42). Furthermore, it is often difficult to determine the full extent of a promoter, including all applicable distal enhancer sites, which may be key in the model being developed. miRNA levels, which are easier to detect, are increasingly being shown to be key to controlling the cell state(43), regulating the immune response(44) and even detecting cancers(45). It is therefore not surprising that synthetic biology has been applied to build tools that allow for the detection and integration of miRNA. Using a synthetic integrating vector that allowed for the transcription of two different mRNAs, one of which possessed a perfect binding site to the miRNA of interest, Brown et al were able to test whether one can use miRNA inhibition to selectively express transgenes of interest. They were able to make sophisticated expression profiles restrictions, such as restricting their gene of interest to all hematopoietic cells except mature dendritic cells or exclusively to lymphoid cells. By adding multiple binding sites, they were able to increase the specificity of their system, setting up 'OR' gates (see

figure 3B for an example). Importantly, Brown et al also showed that their system was not interfering with endogenous miRNA regulation systems(42).

In addition to detecting whether miRNA is present, one may also want to detect a specific protein or its specific localisation. Without modifying the endogenous protein, which can affect its function, there are few options to detect protein levels without lysing the cell and performing a Western blot or another invasive technique. Synthetic biology has solutions for this concern, as well. One solution, proposed by Kashida et al, involves the generation of specially designed shRNA such that the protein of interest binds it and, through its binding, prevents the binding of Dicer to the shRNA via steric hindrance. As a result of Dicer not binding, the shRNA can no longer inhibit a reporter (46). They tested two different constructs of their system with two different proteins of interest, thereby identifying two potential methods of reacting to protein. In the process of developing this system, Kashida et al also highlighted and advanced the utility of *in silico* RNA design, as their system was initially designed in 3D *in silico* and then verified in cells. Another option, also based on the interaction of an RNA aptamer with a protein of interest was proposed by Beisel et al. This system used miRNA that was not processed by Drosha upon ligand binding; this miRNA could be used to detect protein binding, if it, when uninhibited, repressed the output of a reporter protein(47). This work was then further developed into a quantitative model, as discussed above, which allowed the calculation of the protein concentration based on reporter output(28). Finally, a third option involved using an aptamer that could bind the protein of interest and inserting this aptamer into an intron. This method provided interesting insights into how splicing was affected and was discussed above(23). While this method is not as quantitative as the miRNA/shRNA methods, it may be more useful in cases in which the use of RNAi is not desired. All of these options allow for the continuous, very minimally invasive detection of protein in live cells.

While the options described above can detect an increase in protein concentration, it is not always known how the signal of a particular receptor is interpreted in terms of proteomic level changes. For example, there are over 800 known G-protein coupled receptors (GPCRs), of which 350 may be involved in disease; however, 100-150 of these receptors do not have a known function or ligand(25). As GPCRs have been shown to be excellent targets for therapeutics, there is significant interest in identifying the ligands, functions and the signalling kinetics of these ‘orphan’ GPCRs(25). Recently, synthetic biology has proposed two approaches to detect protein signalling, one of which directly helps to detect GPCR signalling.

After GPCRs are activated, they are phosphorylated, which in turn recruits arrestin to them to inhibit their subsequent activation. This knowledge was taken advantage of by Barnea et al to generate a ‘Tango’ assay that could be used to detect the activation of a specific GPCR(29). The cells of interest are modified with the insertion of three systems. One system constitutively expresses the GPCR of interest with a protease-cleavable domain and bound to a transcription factor. Another system encodes an Arrestin protein with a sequence-specific protease attached, and a final system encodes a transcription factor-driven output. Upon GPCR stimulation, the endogenous cell machinery will recruit the Arrestin/protease protein to the GPCR and trigger proteolysis of the linker between the GPCR and the transcription factor, releasing it to act upon system 3, thereby inducing the production of a reporter(29). This system allows for the prolonged signalling of a reporter based on a single transient activation. Furthermore, because this method is entirely exogenous to the cell’s signalling pathways, it will not be confounded by any other signalling pathways. This is a very powerful method and could be useful for detecting natural ligands for orphan GCPRs or even for screening pharmaceutically useful agonists/antagonists. Wehr et al provide an excellent review of the process of developing a similar receptor activation system for any GPCR (48). A recent paper

from Zhao et al presented a modified approach to produce β-lactamase instead of a transcription factor which allowed them to obtain a more direct readout (49). In their work, they showed that their system provided them with a graded response, which may be useful in determining the strength of signalling following treatment with various ligands.

Many signalling pathways involve the use of second messengers, which can also be detected using synthetic biology. This approach does not require the modification of a receptor and therefore may be better suited if the receptor loses function upon modification or is otherwise difficult to modify. One such system, developed by Kim et al, detected intracellular cGMP levels and produced an easily-discernable output upon detection(50). To detect the cGMP surge following signalling, they took advantage of a natural non-human cGMP sensor and rewired it to act as an inducible transcription factor(50). They showed that the system specifically reacted to cGMP in a graded manner. cGMP is normally degraded by phosphodiesterases (PDEs), and the inhibition of PDEs to prolong cGMP signalling is a clinically applied therapy. Kim et al showed that their system was sufficient to detect the prolonged signalling as a result of the commonly used therapeutic agent Sildenafil (Viagra). Further studies could apply this system to screen for novel PDE inhibitors or to measuring signal strength.

If one is not interested in the specific pathway that is being triggered and instead seeks to have a broader-scale overview of which extracellular signalling molecules are present in a whole organism, one can take advantage of two new approaches recently presented by Rossger et al(51), and Auslander et al(52). Rossger et al rewired the human dopamine receptor D1 to signal via $G_{s\alpha}$, causing the conversion of ATP to the second messenger cAMP, which could then be detected by PKA, which in turn could induce signalling. Because dopamine is a neurotransmitter that is released in response to reward-triggered stimuli, using this system, they were able to detect the concentration of dopamine in the blood of live mice, which could then be correlated with the triggering of reward pathways such as those triggered by glucose or female company (in male mice). Interestingly, their system was sensitive enough to differentiate between different concentrations of glucose in the water provided. Such a modification cannot be performed in cells that are part of the organism being studied, as this process may affect endogenous pathway signalling. Therefore Rossger et al encapsulated engineered cells in a porous material and injected the cells intraperitoneally(51). They used a stable protein that does not react with mouse tissues as a detectable output. One of the features of synthetic biology is that the systems that are built with it are generally modular allowing one to easily modify the system to release a different output such as luciferase or, as Rossger et al did, to release a therapeutic peptide. These closed loops systems will be discussed in detail below (51).

Auslander et al instead designed a system for use in detecting human signalling molecules to detect allergies in a uniquely non-intrusive way(52). Similar to the system above, the G-protein coupled histamine receptor HRH2 is re-wired to trigger the activation of adenylcyclase and the production of cAMP, which in turn transmits the signal to a transcription factor, which activates the production of a detectable maker(52). Their system shows a high dynamic range (1-10,000 nM) and a high sensitivity (2-4 nM). Their system could theoretically be used in a similar format to the dopamine system described above; however, Auslander et al wanted to develop an ex vivo allergy testing system that would allow for the rapid and non-invasive detection of histamine release from a patient's blood upon ex vivo allergen exposure(52). They proposed that their system could be used in lieu of the current standard of care in which potentially allergic patients are directly exposed to the allergen they are being tested for (figure 3A).

Cell-Based screening systems

Another key application of the system developed by Auslander et al(52) is that it could be used to rapidly screen potential anti-inflammatory and/or anti-allergic drugs. Because their system operates using the endogenous allergy-detection machinery of the body, any success in inhibiting an allergic response can be detected. They verified the suitability of their system by showing that when a drug known to inhibit an upstream component of the histamine-release pathway was added to their test, their system did not detect any histamine beyond the 'negative' baseline(52). One could further apply such a system to screen for which drug would function best in a given patient using a small sample of their blood without any unnecessary side effects. Such screening strategies are extremely valuable from a therapeutic perspective, and novel drugs developed from such systems are in the pre-clinical pipeline.

Tuberculosis infection is a world-wide health concern, especially because a significant number of patients are now becoming infected with multi-drug-resistant tuberculosis, which is resistant to most common antibiotic agents. Extremely Drug Resistant TB (XDR-TB) is even more drug resistant, making it extremely difficult to treat(53). One novel approach that has been facilitated by synthetic biology is the use of pathogen-free cell-based screening systems. It has been noted that a common drug (Ethionamide) must be activated by a pathogenic enzyme prior to functioning. In resistant tuberculosis, this enzyme, EthR, is typically repressed at the transcriptional level, thereby preventing the action of Ethionamide. Scientists have attempted to identify repressor inhibitors using structural biology; however, the identified compound was too hydrophobic to enter the cell(54). If one could inhibit the binding of this repressor to the gene, then it may be possible to induce Ethionamide sensitivity. Weber et al proposed a system in which EthR is re-wired to act as a eukaryotic transcriptional activator that activates a readily detectable synthetic output (figure 3B). In this manner, they were able to set up a small-molecule screen for three key factors simultaneously: (i) the inhibition of EthR binding; (ii) the ability to pass through a cell membrane and act inside the cell; and (iii) the lack of cytotoxicity. Their work identified several compounds that are proceeding towards clinical licensure.

Another cell-based screening strategy involved the use of light-inducible systems to screen for sunscreen efficacy. Upon UV light exposure, the cryptochrome CRY2 and the C1BN domain undergo dimerization. Wieland et al took advantage of this process, rewiring it to induce the production of a reporter(55) to detect the ability of various sunscreens to block harmful UV light. In the absence of light, both of the domains are expressed on the cell membrane. Upon light stimulation, the domains dimerize, which allows for a selective protease to cleave at its site on the other protein, thereby resulting in the release of a transcription factor. Any of the fine sensor tools described above could be conscripted using synthetic biology to create screens such as these to obtain a readout in a direct manner, as in the sunscreen example, or in an inverted manner, as in the tuberculosis compound approach.

Beyond Detection – Fine-Tuned Perturbation

Thus far, a number of systems have been discussed that allow one to detect certain endogenous conditions and then output a readily detectable marker that signals that the condition of interest has been met. Often, one also desires to do the opposite – to create a certain condition either in a cell or in an entire organism using a controllable inducer. Three main inducible triggers have been developed for this purpose: chemical, light and radio wave triggered systems. These three triggers have been used to control DNA transcription, chromatin regulation, RNA stability, and protein and RNA splicing, as well as to trigger endogenous pathways. Synthetic biology strives to

design circuit components that can be used interchangeably, which allows one to re-wire almost any inducer with any target (figure 4A).

Chemical Options for Control

A powerful tool in biology has been the examination of mutants that are defective in a certain process, which allows one to identify the responsible gene. However, as some genes were essential, their null mutants could never be identified; instead, one had to search for temperature-sensitive mutants. This process has been very fruitful and has allowed for the identification of key mitosis-related cell-cycle control genes(56). However, this process depends on the random creation and identification of temperature-sensitive mutants, which may not exist for all the proteins involved in the process. Another option is to use the conditional knock-out mouse, where Cre recombinase is expressed under an endogenous promoter and excises a gene of interest only in specific cells. However, there are a number of problems with using endogenous promoters, including evidence that they are continuously expressed at a very low level in all cells (41)**.**

One of the earliest developments of what has now grown into synthetic biology was the development of the tetracycline-inducible repressor system in 1992(14). It was later adapted to also act as an inducible activation system, allowing for the temporal control of gene activation(57). Using these systems, it became possible to interfere with the expression of certain genes in a very temporally controlled manner. This could be combined with an endogenous promoter system to obtain even higher specificity. By some reports, more than 18,000 studies have been published that used the tetracycline system(58). The Tet-inducible system is a very powerful tool, and since then, synthetic biology has developed a number of other tools that have unique advantages. Three main strategies have been employed in the process of making chemically inducible systems.

One strategy that has been very successful is the use of bacterial transcription factors(59). These are usually conditional repressors in their native environment, where they lose their binding affinity for DNA upon the addition of a certain ligand, and they thus no longer block the appropriate promoter. A prototypical example is the lactose system, in which upon lactose binding to lacI, the repressor can no longer bind to its repression site, allowing for lac operon expression(60). Because a mammalian promoter is organized differently than a bacterial one, these systems require some modifications prior to their use(59) and are often coupled to viral transcriptional activators(57) or repressors(61). The systems developed since the initial tetracycline strategy have multiple advantages, depending on the desired use. The vanilic acid system, developed by Gitzinger et al, allows for the use of a food additive that is commonly consumed by humans with no known side effects at the necessary doses to be used as an inducer(62). A system published earlier this year by Wang et al allowed for the transdermal control of gene expression in whole mice with paraben, a chemical family used in cosmetics for over 60 years(63). Both of these systems can be used in their ‘ON’ configuration, where the addition of the regulator activated gene expression, or in the ‘OFF’ configuration, where the addition of a regulator repressed gene expression. These systems show good specificity and show a 50-fold increased dynamic range(62). To further increase the number of options available, there is active interest in gaining the ability to engineer these systems *in silico(64)*.

Another option for inducing chemically driven change is to use RNA aptamers that are sensitive to ligand binding and can either stabilize or destabilize a hammerhead ribozyme, thereby leading to or inhibiting ribozyme-mediated self-cleavage of the mRNA strand. Once the mRNA strand is cleaved in eukaryotes, it is rapidly degraded. Similar to the chemically inducible systems described above, the inspiration for this system comes from prokaryotes, where mRNA cleavage exposes a ribosomal binding site, allowing transcription to occur. These systems have been extensively studied,

and a recent report by Chen et al proposed how this system could be used to induce a controllable T-cell proliferation system(65). A system was developed that encoded IL2, which was degraded by a cis-acting ribozyme. In the presence of a drug (theophylline), the ribozyme was inactivated, which allowed for IL2 translation and production, thereby inducing T-cell proliferation(65)**Error! Bookmark not defined.**. A recent methods report by Wieland et al provides simple-to-follow instructions on how one could develop a ribozyme-mediated control system for a gene of interest using artificial selection in *E. coli(66)).*

Finally, a third option is to take advantage of natural ligand-binding proteins that induce hybridization (figure 4A,ii). The majority of these systems are built upon FK506 binding protein (FKBP) and FKBP rapamycin binding protein (FRB), which dimerize upon the addition of rapamycin. This system allows for induced protein-protein interactions, which could be used to generate selectively functional proteases (figure 4A,vii)(67) or transcription factors(figure 4A,vi)(18). This concept involves the use of endogenous rapamycin-binding domains, which, while having the benefit of being non-immunogenic, can be found in other tissues and can therefore make interpreting results after rapamycin addition difficult. For example, rapamycin is widely known to inhibit the immune response(68), and this may interfere with the effect being studied. To solve this problem, an alternative small molecule inducer was generated that could bind to a modified form of KFBP but not to the wild-type form(69). This allows for a much simpler experiment, where only one interaction is specifically controlled. Confirming the safety and lack of general off-target effects, this device was proposed(70) and used as a safety switch in adoptive T-cell therapy to induce apoptosis (figure 4A,x) (71) or to augment the strength of signalling in engineered T cells(72). Similar inducible protein-protein interaction systems, based on endogenous dimerizing proteins have also been developed for the estrogen receptor(67).

In the specific case in which one desires to trigger GPCR signalling in a highly specific and controlled manner, there is a fourth option based on small chemical inducers (figure 4B). This technique is based on modifying the GPCR to respond to an alternative ligand that is also screened to be inert in other contexts. Dong et al developed a protocol to generate these 'designer receptors exclusively activated by designer drugs' (DREADDs)(73). This would allow one to selectively activate a specific receptor and then examine the effect of this activation in a live organism such as a mouse. Alternatively, one can combine the Arrestin-based GPCR detection system to detect the activation of these synthetic GPCRs (figure 4A,i).

Light Options for Control

Chemical ligands are useful because they can be temporally induced; however, it may be of interest to also be able to spatially induce these ligands to induce an effect either in a specific cell or in a specific region of a cell. This option is available with optogenetics. There are three main approaches that have been developed using optogenetics.

One approach involves the insertion of a GPCR that is sensitive to light and allowing it to participate in signalling. Kim et al showed that Rhodopsin and the β_2-Adrenergic Receptor were sufficiently similar to allow them to be made into a chimera composed of the light-sensitive domain of Rhodopsin and downstream signalling circuits of β_2-adrenergic receptor (74). Airan et al expanded upon this work to develop a set of chimeric GPCRs, which they termed optoXRs. These are proteins that contain a light-sensitive region from rhodopsin and a signalling region from a GPCR of interest (27). When blue light was shone on the rhodopsin domain, it transmitted a signal to the intracellular region of the protein, which released a signal – either adenylyl cyclase activation in the case of β_2AR or phospholipase C activation in the case of α_1AR. They were able to incorporate these receptors into

a mouse model and were able to manipulate mouse behaviour using a fiber optic cable that transmitted blue light directly onto the appropriate neurons in the mouse brain. One could use this approach to also investigate how different signalling kinetics are able to cause different downstream signalling events. Other approaches involved directly transducing cells of interest with a naturally light-sensitive GPCR and detecting its endogenous signalling. Bruegmann et al made a stably transfected ES cell line expressing ChannelRhodopsin2 (ChR2). They were able to modulate beating using blue light in embryonic stem cell-derived cardiomyocytes as well as in whole mice(75). In both of the above cases, the approaches depended on the reaction of the endogenous cell to voltage changes across its membrane, as induced by a light stimulus.

Ye et al generalized this approach by developing a novel synthetic circuit that detected light-stimulated calcium influx to drive the expression of any gene of interest(76). A similar generalized system was developed by Folcher et al in a system where near-infrared light was used to induce c-di-GMP formation by a protein from *Rhodobacter sphaeroides*. Upon light stimulation, c-di-GMP is formed, which is detected by the "stimulator of interferon genes" (STING) sensor, which then triggers interferon response genes(77). By re-wiring the target of Interferon Response Factor 3 (IRF3), the authors were able to activate a detectable marker. In the same report, Folcher et al also advanced the state-of-the-art of light-responsive mouse models by developing a novel wireless-powered optogenetic device that could produce light directly inside the mouse to stimulate the cells of interest, as opposed to requiring the placement of a fiber optic cable into the mouse(77).

Another option for engineering light-sensitive systems was used by Chen et al, who took advantage of UVR8, a plant photoreceptor(78). UVR8 forms heterodimers in the absence of light. However, when irradiated with UV-B, it separated into monomers. Taking advantage of this, Chen et al were able to generate a conditional cell-secretion system. Because their system was induced by UV light, it was compatible with most fluorescent proteins, and they took advantage of this to track how their protein traversed the Golgi apparatus and was secreted. Similarly, Zhou et al used a fluorescent protein, Dronpa that is found in an oligomerized state in the absence of light. Upon shining light at 390 nm on Dronpa, its domains dissociate (79). The authors used this to induce protein release from the cytosolic membrane and to 'uncage' a protease. Also taking advantage of photo-induced folding changes, We et al fused a photoreactive light oxygen voltage (LOV) domain to a constitutively active Rac1 protein, forming a photoactivatable Rac1(80). Under dark conditions, the LOV domain sterically interfered with Rac1 activity. Upon illumination with blue light, the steric inhibition was reduced, and Rac1 was able to function. Using this system, Wu et al examined the importance of Rac1 activation in protrusion and whether the process depends on myosin by shining light on a small segment of a membrane and examining how protrusions appear in it(80).

The third option for using light-sensitive fluorescent proteins involves the use of domains that naturally dimerize upon exposure to light (figure 4A,iii). The earliest such system used FKF1, a LOV protein, and GIGANTER (GI) which uses a derivative of riboflavin that is found in eukaryotic cells(81). Using this system, Yazawa et al were able to recruit Rac1 to the membrane and to examine how it can induce protrusions. Their system was able to induce translocation in 30% of cells, and the dimerization lasted at least 90 min after transient illumination. They further observed that at high transgene concentrations, dimerization occurred even in the dark(81). Using another set of dimerizing proteins, CIB1 and cryptochrome 2, Kennedy et al were able to generate a system with faster kinetics than the system proposed by Yazawa et al(82) that induced protein dimerization upon exposure to blue light. Strickland et al further improved upon the systems that were reported by Kennedy et al and Yazawa et al by developing a tunable light-control system using a LOV domain-containing protein as well as engineered variants of the Erbin PDZ domain(83). They chose to use the

PDZ domain because its interaction affinity is highly tunable and can be varied between 0.5 nM and >10 μM(83). This allowed them to tune the strength of the interaction upon illumination to obtain more control over the kinetics of the system. Finally, Müller et al developed a switch-like system, which allowed for light-inducible dimerization and subsequent light-inducible dissociation(15). Their system used the controllable interaction between Phytochrome B and phytochrome-interacting factor 6 (PIF6) and required the addition of an exogenous chromophore.

Radio wave Options for Control

In addition to systems using chemical or light inducers, a system that uses radio waves has been developed by Friedman et al(84). In one approach, they inserted a temperature-sensitive channel, TRPV1, into cells and used Fe-coated antibodies targeting the cell. When the cell was exposed to radio waves, the Fe-coated antibodies generated heat, triggering activation of the TRPV1 protein, which in turn triggered a Ca^{2+}-sensitive signalling pathway, allowing for gene transcription. In another approach, Stanley et al were able to use expressed Ferretin inside cells to force the cells to form iron particles on their own, which could subsequently be heated to trigger the same pathway(84).

Summary of Downstream Targets

There are a number of ways to trigger circuit activity, including the use of small molecules, light or radio waves. While the nature of the induction is different, the inducible targets can be very similar. As a result one can choose whichever induction scheme is more appropriate in a given setup.

A number of inducers are able to trigger actions on DNA, and in the presence or absence of a small molecule, bind to DNA. The vanilic acid(62) and paraben(63) systems are both composed of DNA binding domains that were generated by bacterial systems to control their own genes. These systems were modified via the coupling of a VP16 transcriptional activator(21) or a KRAB transcriptional repressor(61). Coupling other domains to this system, such as HP1, which was used in a chromatin kinetics study(18), and p300, which is an acetyltransferase(85), could allow this system to modify the chromatin state. Protein-protein interactions, which could be triggered by a number of methods such as light-driven dimerization, chemical-driven dimerization, or GPCR signalling-driven dimerization(29) could allow for the dimerization of a DNA-binding domain and an effector domain (figure 4A, vi)(18). Using TALEs(86), Zinc-fingers(66), or more recently, CRISPR/dCas9(19), one can obtain unprecedented sequence specificity, allowing the targeting of any endogenous sequence without having to insert any special promoter fragments into it. One could also target permanent genomic modifications using a system similar to one that was developed by Hirrlinger et al(87). Cre and Flp are commonly used enzymes for recombination that, upon activation, will excise DNA located between two lox or frp sites. Hirrlinger et al developed a 'Split-Cre' that contains Cre split into two components that both must be co-expressed for it to function (figure 4A, vii). Using one of the protein-protein interaction-inducing systems described above, one can develop a light- or chemically inducible Cre recombinase that permanently excises an unwanted region of the genome. Using the RNA aptamer designs discussed above, one could also promote the stabilization or degradation of mRNA(65) or miRNA(28), triggered by ligand binding.

Protein-protein interactions can also be used to induce protein splicing, where two inteins are brought together. Upon binding, the two inteins catalyze a change in the peptide bond, and they splice themselves out, leaving only 'exteins' in the peptide chain(figure 4A, iv). Using this process as a way of regulating protein activity was initially reported by Berrade et al, who used an ex vivo system composed of two inteins that could not normally interact. Upon light stimulation, a photolabile

hindering group is removed, and the inteins can interact, allowing for splicing and protein activity(88). Berrade et al looked at the nature and kinetics of this process using the *Ssp* DNE intein. Their work was taken into mammalian cells by Henning et al, who showed that this system could be used to induce protein splicing in response to a dimerization inducer, Rapamycin, using an intein from Saccharomyces cerevisiae, which was split into two components (89). Chemical-dependent intein splicing was also used by Davis et al, who modified a Cas9 protein such that under normal circumstances, it contained an intein that blocks it from functioning. Upon the addition of a chemical activator, 4-HT, the intein is activated and splices itself out of the Cas9 peptide chain, leaving a functional Cas9 protein(90). This system generates a more –persistent effect that will not immediately dissipate upon inducer removal.

Any of the systems described above that allow for protein-protein interactions could be used to trigger intein-driven splicing. As an illustration of the modular nature of synthetic biology, Slomovic and Collins recently published a protocol that combines DNA-binding domains (Zinc-finger domains) and inteins to generate a novel system that allows for the detection of and reaction to of DNA(91). They designed a pair of transcription factors that bound nearby DNA sequences in a target of interest. When the target was detected, the binding brought together two inteins, which allowed for the recapitulation of a protein of interest. Using this method, they were able to develop a DNA-triggered apoptosis signal as well as to detect an ongoing viral infection(91). Using this method, they could screen for the presence or, by inserting a 'NOT' gate, the absence of a sequence of interest. This could improve the common transduction and transfection protocols that currently are confounded by the presence of unmodified cells, by inducing apoptosis in unmodified cells.

Using recent developments in synthetic biology, it has now become possible to control protein stability. As reported by Banaszynski et al, one could use a protein termed Sheild-1/Shld1 to selectively protect proteins that would otherwise be degraded (92). They identified a 107-amino acid FKBP12 domain that, when coupled to another domain of interested, causes the entire protein to be destabilized and rapidly degraded. Upon the addition of a chemical termed Shld1, this protein was stabilized and thus no longer triggered its own degradation. To increase the utility of their system, Banaszynski et al developed a C-terminal domain as well as an N-terminal domain that could both destabilize a protein of interest. Later, the same group reported that they were able to apply this system toward the degradation of tumour-expressed IL2 and TNFα in live mice tumour model, with no effects on murine viability(93). Recently, the same group reported an alternative system based on Shld1, which induced IL2 and TNFα degradation upon the addition of shld1(94). In this system, FKBP normally bound and sequestered a 19-amino acid degradation-stimulating protein. Upon Shld1 binding, this 19-residue fragment was unbound and could therefore promote the degradation of the whole protein of interest. Another group recently applied a similar idea to modulating the stability of dCas9(95). In their study, the addition of TMP stabilized dCas9 and allowed it to act, while its absence promoted dCas9 degradation (figure 4C). Protein stability can also be controlled using auxin, a chemical inducer of degradation normally found in plants but that was adapted for use in non-plants by Nishimura et al. A small degron is bound to the protein of interest in an auxin-inducible manner, and upon binding, the protein will be ubiquitinated and degraded(95).

Inducing specific protein localization is also possible in response to the signals discussed earlier. One of the examples of light-directed control, in which UVR8 de-dimerization was induced via light, was one such system. Normally, in the dark, the two domains dimerize, and the protein is localized to the cell membrane. In response to light, it dissociates and can then be found in the cytosol (78). Systems dependent on dimerization have been built that allow for dimerization-induced mitochondrial-membrane localization(83), as well as nuclear localization(96). Niopek et al developed

a light-controllable nuclear dimerization system in which the protein of interest contains a weak nuclear export signal (NES) as well as a strong nuclear localization signal (NLS) (figure 4A, ix). Under dark conditions, the nuclear localization signal was hidden and could not act. Upon blue-light absorption, the NLS is exposed, which induces nuclear dimerization. The protein is maintained in the nucleus until the light is turned off, which allows for preferential NES activity, exporting the protein into the cytosol(96). Beyer et al designed a protein dimerization-controlled system for nuclear localization(97). Red light at 660 nm induced the binding of PIF3 to PhyB, which, as a result of the NLS that PIF3 contains, induced both proteins to be brought into the nucleus. Either spontaneous dissociation or induced dissociation at 740 nm allowed the nuclear export of PhyB. The binding of a protein of interest to PhyB would allow for its selective nuclear/cytosolic transport.

Finally, one could also use the inducers described above to trigger cell signalling. Restricted-function systems such as those that involve DREADDs(73) or light-driven GPCRs(74) were discussed above. These systems allow one to modify GPCRs, such that either a specific chemical inducer or light can trigger their activity. Another option is to take advantage of the ability of chemical ligands or light to induce protein-protein interactions such that one can induce caspase-9 activation(70). In the case of caspase-9 activation, the oligomerization of caspase 9 is believed to be the endogenous activation pathway, where is activates itself and then triggers apoptosis. Straathof et al used this system to develop a safety switch for T cells used in therapy(70), which was tested in patients(71).

Detection – Reaction Systems

Being able to detect specific cellular states and to induce them has significantly advanced our ability to interrogate biological systems. Recently, synthetic biology has started allowing the development of systems that not only can detect or induce a state but rather can do both. In essence, these systems detect an intra- or extra-cellular state, process it, and produce an output. Such systems have been built before, typically with a modifying protein driven by an endogenous promoter, however synthetic biology greatly advances the possibilities. There are now over 500 mouse models that drive Cre recombinase under different endogenous promoters, which allow for the cell-specific permanent excision of a targeted region of DNA(98). The systems create by synthetic biology have generally been designed for use in medical devices; however, there is a breadth of possibilities available for these devices in developing more advanced mouse models.

A recent system developed by Kemmer et al involved sensing luteinizing hormone, a hormone expressed when a cow is fertile, and in response, producing a cellulase, which degrades a cellulose-containing capsule,. This in turn allows for the release of bull sperm at the ideal time to ensure conception(99). Such a time-controlled system can either be directly applied to other systems in which estrous cycle timing is important, such as in the study of reproductive diseases(100, 101). Similar systems may also be developed to detect other triggers and to then release their payloads at an appropriate time.

Such detection-reaction circuits could also be used to simplify the induction of a specific condition in a mouse model. Rossger et al developed a hypertension-control system induced by increased dopamine in the blood as a result of the endogenous reward pathways in mice triggered by sugar-water consumption (figure 5A)(51). A similar system was developed by Ye et al that detected the presence of the licensed antihypertensive drug guanabenz and then produced a therapeutic hormone that worked in combination with the drug to improve outcomes in mice(102). Other systems have been developed to detect food consumption via increased fatty acid levels in the blood, and to release a natural appetite-controlling hormone (figure 5B). These works were aimed at

producing novel therapeutics, however, such systems could be used to develop novel models that use one chemical inducer to trigger a complex change in state.

A third application of a system that detects a state and then expresses an output is the development of 'closed-loop' systems. These systems detect a pathological condition and release a therapeutic agent in a self-sufficient manner. These systems further, automatically detect when the pathological state has ended and cease production of the therapeutic agent, ensuring fully automated treatment options (figure 5C)(103, 104). The current designs, which are aimed at the treatment of disease, strive to return the system to a normal physiological state. It is, however, possible to modify the system such that it attempts to induce a specific pathological state, either directly or by producing a known inducer of such a state.

More Integrative Systems

Beyond the single input/single set of outputs systems described above, synthetic biology also provides the user with many options that allow for more robust or more specific circuits. Synthetic biology has been used to develop various logic gates, including 'AND' and 'OR' logic gates, and has been used to develop 'band-pass' filters and more sensitive switches. Further developments have also enabled the development of orthogonally controlled systems, systems with memory and systems that can communicate between each other.

Increased Specificity

One of the methods by which synthetic biology can be used to increase specificity is the construction of 'AND' gates. These are circuits that require two inputs prior to their activation. The simplest organization of a two-component 'AND' gate involves two regulated promoters expressing factors that, when co-expressed, dimerize and exert their actions together.

One such system was proposed by Nissim and Bar-Ziv, who developed a tunable two-input system(105). One input produces a DNA-binding domain (from GAL4), while another produces an enhancer domain (e.g. from VP16). When these two proteins are co-expressed, they dimerize and drive the production of a downstream gene that can then be detected (figure 6A). They proposed their system for detecting certain types of cancerous cells(105). Alternatively, another option is to use two separate endogenous promoters to drive fragments of Cre recombinase(87). This system, proposed by Hirrlinger et al, allowed for the more specific selection of cells prior to Cre expression. The viability of this approach was verified in both cell lines and primary cells after viral transduction (87). Improving upon this system, Wang et al used an intein-mediated Cre(106). This device requires the activity of two endogenous promoters, which reconstitute Cre bound to inteins. The inteins interact and form a cohesive Cre recombinase. This method appears to provide more robust excision(106).

Selgrade et al developed a system that mediates splicing in trans(107). Three proteins are expressed, one containing an N-terminal fragment of luciferase, half an intein and an antiparallel coiled-coil such as LZa, another protein contains a C-terminal fragment of luciferase, the other half of an intein and another antiparallel coiled-coil, such as EE, and a third component contains the binding partners for both antiparallel coiled-coils, LZB and RR, linked together. When all three components are expressed together, the antiparallel coiled-coils interact and bring the inteins together, which allows them to splice. The intein splicing activity is calibrated to be insufficient without coiled-coil binding(107). If all three components are made to be inducible, one can obtain a three-way 'AND' gate, requiring the production of all three components prior to Cre reconstitution. Auslander et al report on a strategy that allows for an alternative organization of a 3-way 'AND' gate. It depends on

the production of three different orthogonally inducible components, one of which encodes for an intermediate (output mRNA), another that stabilizes the output to allow its translation, and a third that promotes translation of the intermediate into an output. Without all three components, the circuit does not function (108).

Xie et al recently proposed a system in which two different inputs can be integrated in an antagonist manner. The system would produce an output only if input A (benzoate) was present and input B (Vanilic acid) was absent (109). Using such a topology could be useful if one aims to trigger an output only in a certain lineage of cells and only in cells that have down-regulated a certain gene.

'OR' gates are a good tool to increase the breadth of expression of a gene if used with promoters. A simple 'OR' gate could be built if cells are transduced with two different vectors, each containing an endogenous promoter. The activation of either one would be sufficient to drive gene production. In miRNA, 'OR' gates are typically used in the form of NOT OR gates, for which the expression of either miRNA inhibits expression of the target (figure 6B) (110). Annoini et al were able to use such a system to highlight the importance of liver expression of an antigen for regulatory T cell development (111). To develop such a system, one would have to express two miRNA sites on the mRNA, such that if either miRNA binds, the system is inhibited, and no output is produced. Using these relatively simple designs, very complex circuits can be made. Leisner et al report on a design strategy for complex gates such as "(NOT(A) AND B AND C) OR (D AND NOT(E)) or (F)", which allows for the rational integration of 6 different inputs (figure 6C) (34). Similarly, Wroblewska et al report on the integration of 4 different inputs to generate an output(112). To simplify the development of complex circuits, Win and Smolke developed a scaffold for designing and optimizing multi-input RNA devices(113). Such systems could be useful as therapeutics for cancer detection or for the isolation of very rare cells(114).

More complex circuits have also been developed, which allow for more robust or even more selective expression. For example, Deans et al and Lapique et al both proposed systems that allow for the more robust regulation of circuits. Deans et al proposed a circuit that, in the 'OFF' state, produced miRNA against its output to ensure decreased leakiness (figure 6D)(115), while Lapique developed a time delay device to minimize expression of an output gene while the repression is being established in transiently transfected systems(116).

Higher Order Systems Through Orthogonal Control

Because a significant number of these systems use circuits that are composed of orthogonal components, one could induce specific circuits independently and in a spatially and temporally distinct manner. Such independent behaviour was illustrated in a vanilic acid responsive system designed by Gitzinger et al(62). Similarly, optogenetic systems are compatible with each other and can be used in a temporally regulated manner, as illustrated by Muller et al(117). Their system required the use of light in a specific order, as some of the shorter-wavelength light could somewhat induce systems calibrated towards longer-wavelength light.

Another option for inducing temporal control is to use systems that are targeted by the same inducer but that react in an opposable manner. Most of the inducible systems could be made into activators or inhibitors of gene expression, depending on the nature of the regulatory domain attached to them. For example, one could generate two different proteins, one of which is degraded in the presence of Shld1 while another one is maintained in the presence of Shld1. Such a system would allow for rapid switching between the two states upon the addition of Shld1(94).

Further illustrating the possibilities when using multiple systems together, Greber and Fussenegger proposed a band-pass network that was activated only when an intermediate concentration of inducer was detected and was turned off when the inducer was above or below a certain threshold(35). Using such a system, one could generate three different types of behaviours in one experiment, depending on the dosage of inducer added and the detection level of the cells. One system could be responsive only to high levels of inducer, another system could respond to an intermediate amount using the band-pass system, and a third system could respond negatively to anything other than a low concentration of inducer.

Memory

Cell-based memory, such that cells can remember exposure to a certain inducer and react to it generations later, is something that is being actively pursued. Memory devices that are meant for manual read-out have been developed and used extensively even before the extensive development of synthetic biology. Typically, this was performed by placing lacZ under the control of an endogenous promoter and adding an exogenous substrate, causing cells to be coloured blue. The presence of lacZ can be detected after cell division, thereby allowing for cell labelling (118). Synthetic biology has improved upon this process by allowing for the use of substrate-free dyes such as the non-ribosomal peptide indigoidine, which may be applicable in a larger number of situations(119).

Synthetic biology has also started to develop cells that can remember being exposed to certain stimuli; however, so far the results are not as robust as would be required for use in either medical applications or basic investigations. In a recent report by Burill et al, a memory device for DNA damage and hypoxia, and an inducer were developed; however, some daughter cells lost expression and did not 'remember' their exposure(120). One option could be the use of circuits that are can be inserted into bacteria in the gut. Such a system was proposed by Kotula et al, who developed a prokaryotic memory device and showed that bacteria that express their device could survive in the mammalian gut for an extended period of time. Perhaps by using some of the recent two-communication systems that have been developed, these prokaryotes could communicate with their mammalian host and inform their host that a certain event has been previously experienced(121).

Designing a Synthetic System

When developing synthetic biology tools to model a process of interest, one must also make decisions on how the system will be inserted into cells. Systems can be transiently transfected, transduced or integrated. Furthermore, if designing a mouse model, one must consider the type of modification that one desires to perform on the mouse. Mouse models can be developed using germ-line modifications, transductions, or encapsulated cell injections. The latter is currently predominantly used in synthetic biology models because it is close to how a synthetic biology therapy will be applied for therapeutic purposes, and it may still be a desirable option due to its faster iteration time compared to developing a novel genetic mouse model. Furthermore, unlike virally transduced models, encapsulated cells are protected from the endogenous immune response and can be used even in blood culture(122).

This review has illustrated the power of synthetic biology and how it can be used to make more intricate models that allow for carefully considered biological questions. As with any model, it is important to be aware of its limitations. Tetracycline is by far the most popular application of synthetic biology, used in thousands of models. However, as recently reported by Moullan et al, tetracycline causes mitochondrial protein imbalance and mitochondrial dysfunction, which could

confound results, especially if one is studying metabolic processes(58). Similarly, Rapamycin may be an appropriate inducer for some situations when mTOR is known to not be important for the process under investigation(123). In other situations, its replacement, AP1903, which binds to a modified FKBP protein, may be a better option (69). Similarly, UV light is known to cause DNA damage and thus may be not appropriate in a number of cases. However, as reported by Chen at al, UV light caused minimal toxicity in their system at the durations that they used(78). With the breadth of synthetic biology tools, there is almost certainly a tool that can allow for any investigation of interest; however, these tools must be used properly, with due consideration of their effects. Synthetic biology is an extremely powerful technique, and "with great power comes great responsibility".

Conclusions and Outlook

Synthetic biology is an enlightened approach to biology that seeks to standardize biological components and develop them into plug-and-play modules that can be used for medical and basic science purposes. On the medical front, synthetic biology is developing revolutionary new therapies that seek to treat chronic disease on a continuous basis, effectively curing the disease permanently. On the basic science front, synthetic biology has allowed for the development of quantifiable models that recapitulate a number of complex phenomena in nature such as oscillation and hysteric switches. Understanding these model systems has allowed us to better understand how similar processes, such as circadian rhythms and developmental switches, are regulated. Synthetic biology also provides us a wide assortment of tools that can be modularly recombined to detect a trigger of interest and to report on it, to induce a condition of interest, or to combine the detection of a trigger and the induction of a response in well-designed comprehensive models.

With the rapid development of CRISPR/Cas9 technology, the future in synthetic biology is looking even more promising. As there are many excellent reviews of CRISPR/Cas9 technology that have recently been written, this review did not extensively discuss CRISPR/Cas9; the curious reader is invited to refer to a recent review by Sternberg and Doudna(124). CRISPR/Cas9 is especially promising when applied to synthetic biology because with its extensible nature, it may allow for the development of tools such as multi-state memory devices, counting circuits, and extensible signal transduction pathways that have been long sought out but difficult to create thus far.

Synthetic biology may also allow for the creation of models that were once considered too risky and may eventually allow for the creation of organisms that live orthogonally to our world and therefore are not subject to our diseases nor are capable of harming us(125).

Synthetic biology has already developed a number of powerful tools for treating chronic diseases as well as uncovering intricate biological relationships. It has allowed for the creation of tightly controlled, orthogonally inducible systems and highly specific systems that can integrate as many as 6 factors together. In this review, the current and prospective impact of synthetic biology on basic research has been discussed. Synthetic biology, together with systems biology, allows for the modeling and the exhaustive characterization of complex systems(28). Using a number of synthetic tools that have been developed, complex systems could be integrated in vivo(54) or in a mouse model(102). Inducible systems using inert chemical ligands such as food additives(62) and common cosmetics components(63) or physical controls such as light(117) and radio waves(84) allow for the precise spatiotemporal control of a system of interest. Due to the modular nature of synthetic biology, it is possible to combine a detector and an output to create a self-regulated smart system or to even integrate a number of different inputs prior to making a decision or producing an output(114). Synthetic biology integrates the modularity of electrical engineering with tools designed over billions of years by nature to create powerful therapeutic and investigatory systems. With the

current push to make printing genomes an affordable endeavour, the possibilities in synthetic biology are expanding “at the speed of light”(126).

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Figure Legends

Figure 1. Synthetic biology has allowed for the development of test circuits that allow for the precise quantification of biological phenomena. **A.** Huang et al developed a synthetic system where two synthetic transcription factor binding sites were positioned at incrementally increasing distances. They measured the production of an output reporter induced by the action of these transcription factors. They noticed an oscillatory response, that showed a maximal synergistic activation when the transcription factors bound on opposite sides of the DNA strand. This effect also showed a distance dependency, as activation was weaker when the distance was too big. **B.** Culler at al examined how protein binding inside an intron affects splicing. They developed a synthetic test system, with three exons, the second of which gets included only under certain circumstances, and contains a stop codon. They inserted an MS2 site into 12 different positions in the introns flanking the stop-codon containing exon, and observed the effect on the selectively-spliced exon. If the exon was included in the mRNA, no reporter was produced. Its exclusion allowed for the generation of a detectable output. **C.** Hathaway et al designed a system that allowed for the inducible recruitment of a heterochromatin-inducer (labelled 'Inducer 1'). They inserted a modified promoter upstream of the Oct4 allele, and a fluorescent readout in one of the exons. Using this system they were able to track the kinetics of heterochromatin spread and its persistence. Further, upon adding 'Inducer 2', they were able to trigger activation of an output gene and track the dissipation of heterochromatin upon gene induction.

Figure 2. This figure illustrates some of the more complicated induction circuits that have been developed. **Panel A** illustrates a time delay circuit that has been developed. The tetracycline dependent transactivator (TetR-VP16) controls the expression of erythromycin dependent transrepressor (E-KRAB) that subsequently controls output expression. Upon the addition of tetracycline, TetR-VP16 is unable to bind to its operator and the production of E-KRAB is switched off. But SEAP expression remains quiescent until the E-KRAB reservoir is degraded. This generates a time-delay profile. Upon the further addition of erythromycin the residual E-KRAB is unable to bind to its operator and SEAP expression is turned on rapidly. **Panel B** illustrates the function of a toggle switch. The pristinamycin dependent transrepressor (PIP-KRAB) in the first cistron and SEAP in the second cistron are both regulated by erythromycin responsive promoter (PETR). The erythromycin dependent transrepressor (E-KRAB) is regulated by the pristinamycin responsive promoter (PPIR). By coupling these transcriptional units, the expression of SEAP can be toggled by the addition of erythromycin (EM) or pristinamycin (PI). In both cases, a transient administration of the ligand is sufficient to switch between the two states. **Panel C** illustrates an oscillator system that has been developed. The mammalian oscillator consists of TetR-VP16 driven by tetracycline responsive promoter (P_{TET}) in the sense direction and pristinamycin responsive promoter (P_{PIR}) in the antisense

direction. The expression of the output dGFP (destabilized GFP protein) and Pip-VP16 is controlled by P_{TET}. Auto regulated TetR-VP16 amplifies itself and triggers the production of Pip-VP16 and dGFP. Pip-VP16 triggers the production of antisense RNA that subsequently knocks down TetR-VP16 and the output dGFP expression is shut off along with Pip-VP16. As the antisense RNA is no longer produced, TetR-VP16 can amplify itself and the oscillations of dGFP continue.

Figure 3. Using synthetic biology, one can program cell-based screening systems. In **panel A**, the histamine sensor is illustrated. A patients' blood would be incubated with an allergen of interest, and if the patient is allergic, their basophils should produce histamine. This histamine can be detected by the histamine receptor, and using a re-wired signalling cascade, can be quantified by a synthetic cell-based system which produces a readily-detectable fluorescent marker. **Panel B,** illustrates a novel approach to detect drugs that will increase Mycobacterium tuberculosis's sensitivity to a last-line therapy. It was necessary to identify a drug that could inhibit the activity of an inhibitory transcription factor, EthR. Using a synthetic circuit, where EthR was modified to be a transcriptional activator, drugs could be screened that showed low cytotoxicity, good cell-permeability, and good inhibitory activity.

Figure 4. Synthetic biology has developed a number of systems for detecting a specific inducer and reacting to it. **A.** A strength of synthetic biology is that its constructs are highly modular, and the designed components can be combined to generate a number of different detection/response systems. Panel A illustrates three different potential classes of inducer-detection schemes that could be used: (i) an endogenous or synthetic GPCR binding to its ligand and the subsequent recruitment arrestin; (ii) two peptides dimerizing only in the pretense of some ligand; (iii) light-induced dimerization. In all three cases, the induction induced the two peptides being brought to proximity. This brings together two components that together act to produce the desired effect. One can recruit: (vi) a transcription activation to a DNA-binding domain; (vii) two protein domains together such that they complement each other; (viii) a protein and a degradation signal; (ix) a protein and a specific localisation signal; or induce protein dimerization, thereby inducing its activity. One can ensure the affect is (v) transient, lasting only for the duration of the induction; or (iv) more persistent, limited by protein half-life. Other, more persistent, methods of maintaining induction have also been developed, but are not diagramed here. **B.** Another induction scheme involves the use of GPCRs and a cell's endogenous signalling machinery to detect signalling. An endogenous, exogenous or synthetic GPCR is used, and its endogenous signalling pathway activation is detected via synthetic promoters. **C.** Selective stabilization systems have also been developed, where a degradation-inducing protein domain is added to a protein of interest. Upon the addition of a select inducer, such as TMP or Shld1, the domain's degradation-induction ability is quiesced.

Figure 5. By combining a detection system with an inducible output system, one can create circuits that automatically detect and react to certain conditions. **A.** Rössger et al, combined together a dopamine sensor and a therapeutic output, they were able to couple the reward machinery of the brain with high-blood pressure control. They exogenously expressed a dopamine receptor in a cell line, and took advantage of the cell line's endogenous signalling pathways to a synthetic output system that was engineered to react to the signalling pathway. **B.** In another work, Rössger et al developed a fatty acid system that could react to blood-stream fatty acids, and automatically produce and release a therapeutic (Pramlinitide), which suppresses appetite. This system does not depend on the presence of the endogenous signalling machinery. **C.** Kemmer et al, developed a similar autonomously-responsive system that allowed for the detection and reaction to high urate levels in the blood. In the absence of urate, a transcription inhibitor is bound to the output promoter. Upon urate detection, the inhibition is released, and Uricase is expressed, which acts to reduce urate level. This system was most effective when a human Urate transporter was also expressed in the cells, but other modifications were not required.

Figure 6. Synthetic biology has allowed for the creation of complicated logical systems to create better models and detect more specific conditions. **A.** An example of an 'AND' gate. Two component, protein A and protein B, are both inducible by different conditions. These could be endogenous promoters, which take advantage of a cell's endogenous regulatory machinery, or this regulation could be based on synthetic gene regulations systems. Activation of an output requires the presence of both, protein A and protein B, which function together. In the example mentioned in the text, protein B is be a DNA-binding domain (e.g. GAL4), while protein A is be a transcription activation domain (e.g. VP16). **B.** A simple boolean 'NOR' gate can be constructed using RNAi, where the binding of any RNAi to its recognition site on an mRNA will prompt mRNA degradation, and output inhibition. **C.** Using the two basic building blocks presented in panels A and B, one can make very complicated systems that allow for very fine discrimination between cell states. In this panel, using a combination of two inducible/endogenous promoters, and 5 inducible RNAis, one can create a gate that requires the induction of F or G, and if G is not induced, also requires the absence of the induction of A, B, C, D, and E. **D.** Inducible systems, both synthetic and natural, are often 'leaky'. One way to reduce the un-induced expression of a gene of interest was proposed by Deans et al as illustrated in this panel. In the absence of an inducer, promoter B is active, which inhibits the activity of promoters A and D. Furthermore, promoter C is also active, which also inhibits the production of D via a different mechanism. When inducer is added, it induces the repression of promoter B, which allows for production of A and D. The production of A, inhibits the production of C, simultaneously removing two sources of inhibition on D, allowing its production.

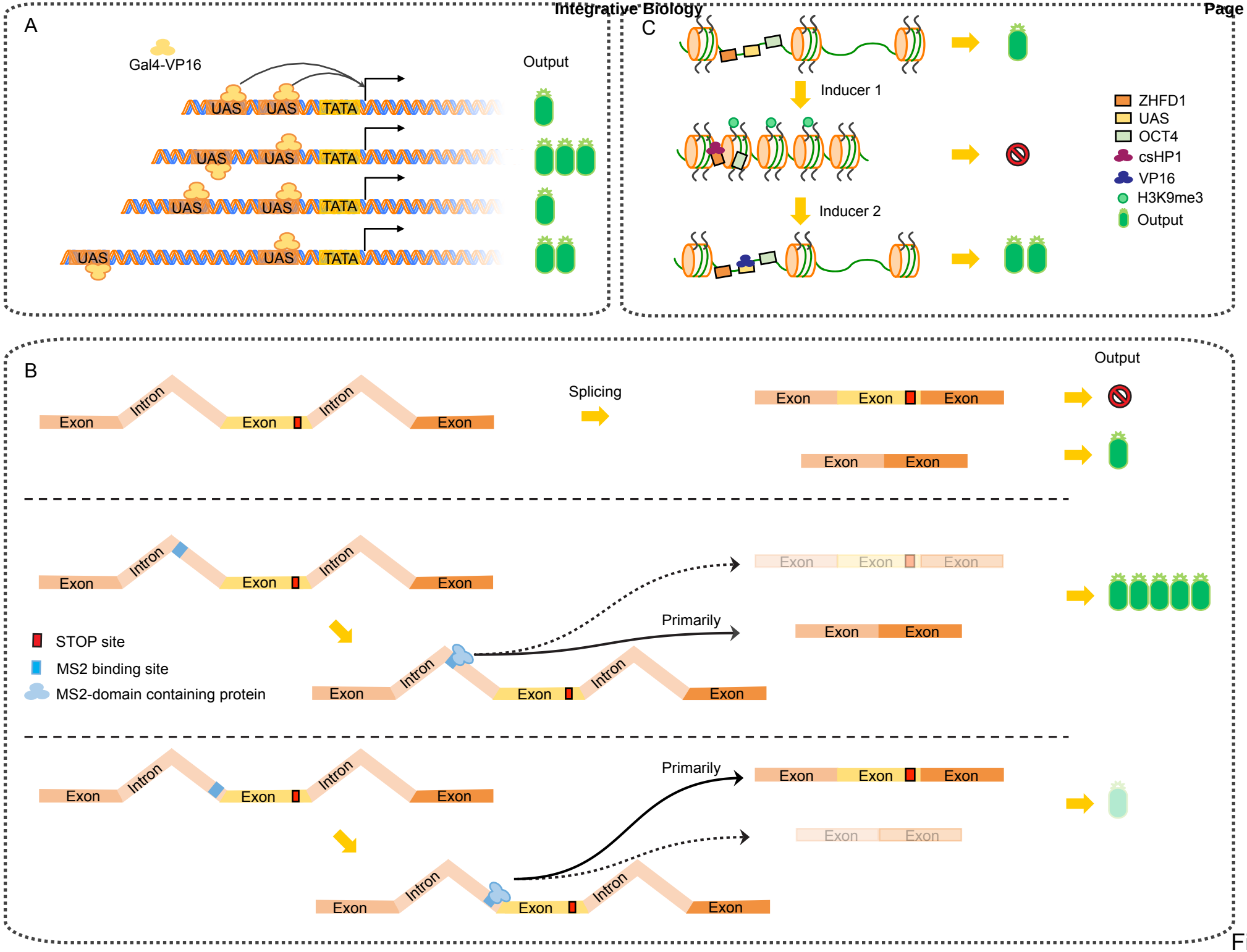
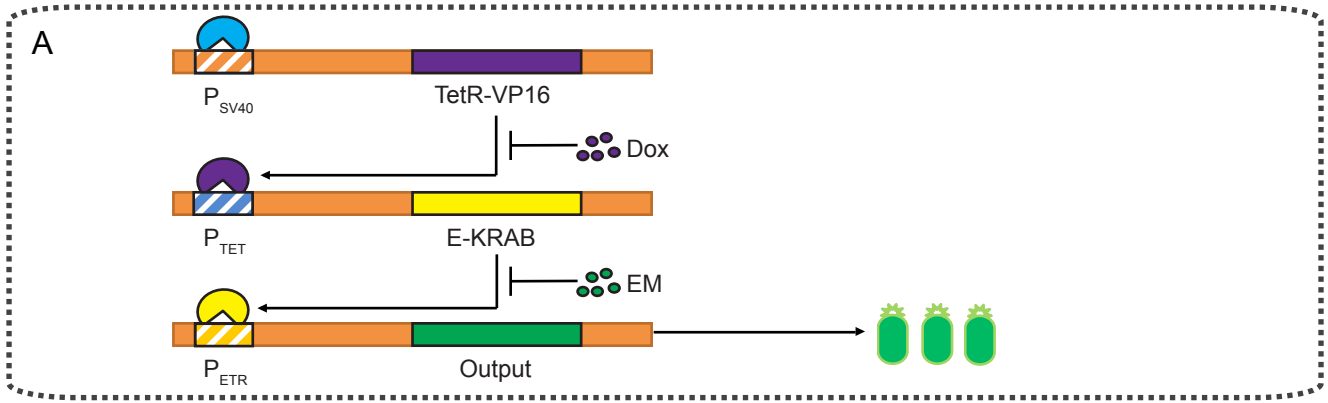
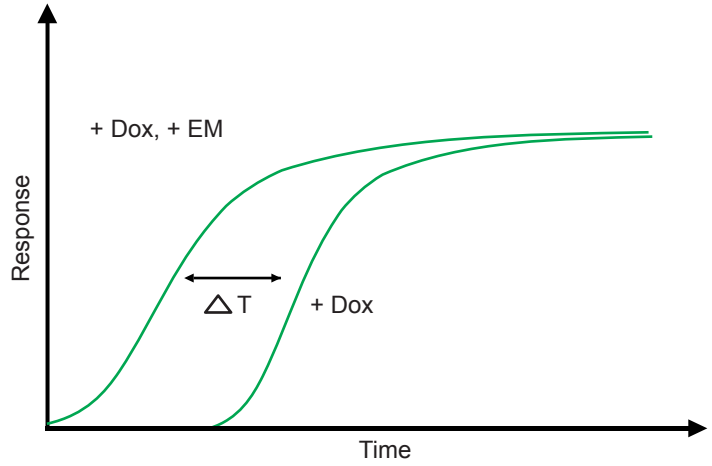
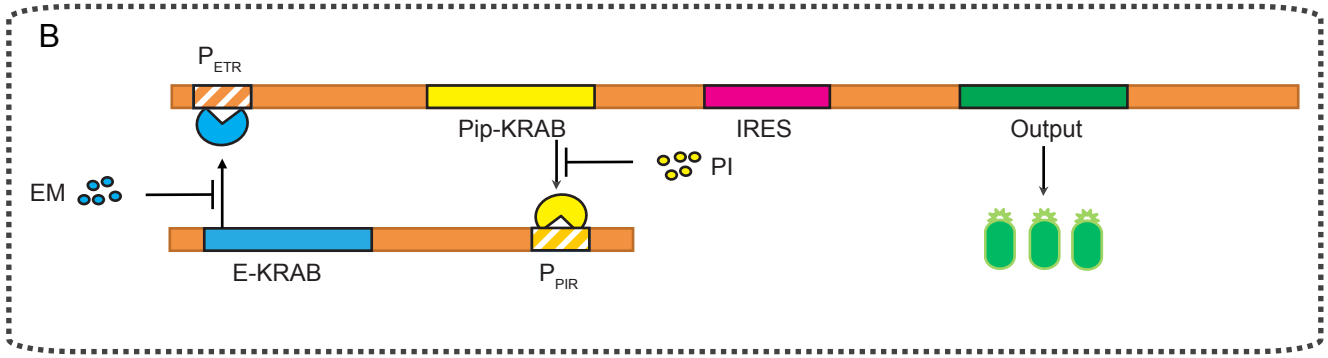
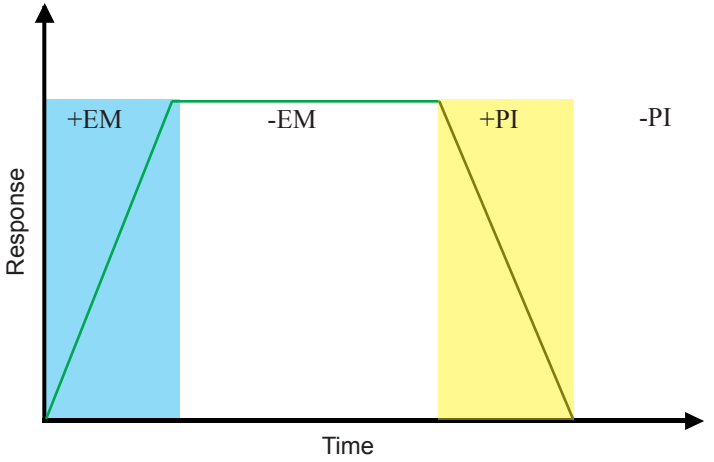

A
Gal4-VP16
UAS
TATA
Output
C
Inducer 1
Inducer 2
ZHFD1
UAS
OCT4
csHP1
VP16
H3K9me3
Output
B
Exon
Intron
Splicing
Output
STOP site
MS2 binding site
MS2-domain containing protein
Primarily

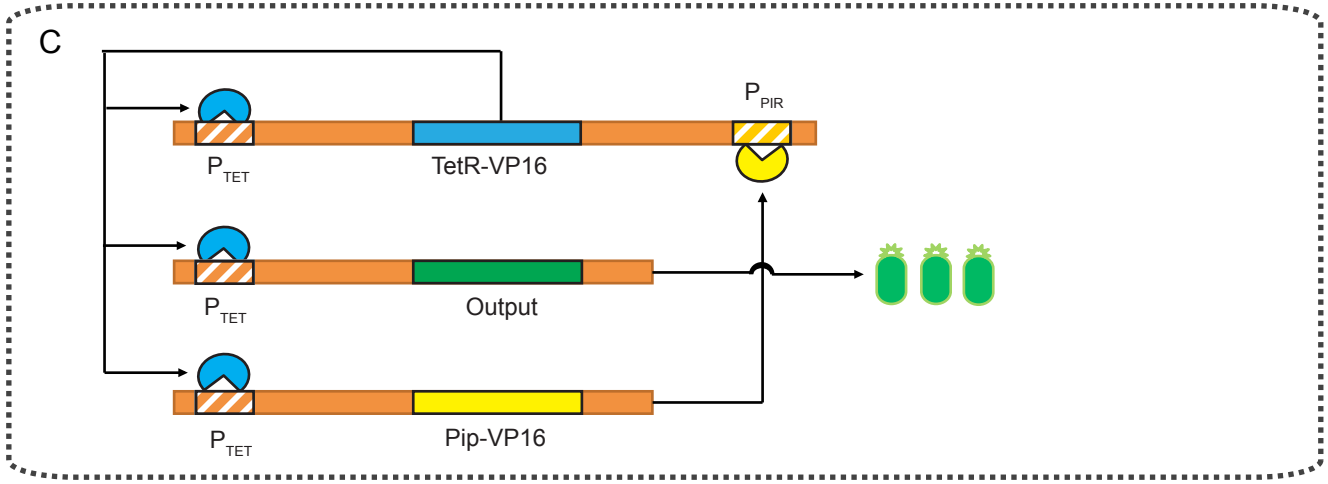
Figure 1


A
P SV40
TetR-VP16
Dox
P TET
E-KRAB
EM
P ETR
Output


+ Dox, + EM
Response
T
+ Dox
Time


B
P ETR
Pip-KRAB
IRES
Output
PI
EM
E-KRAB
P PIR


+EM
-EM
+PI
-PI
Response
Time


C
P PIR
P TET
TetR-VP16
P TET
Output
P TET
Pip-VP16

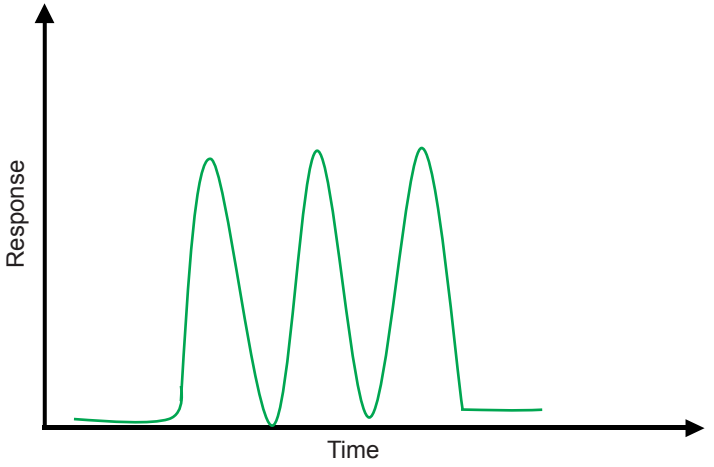

Response
Time

Figure 2

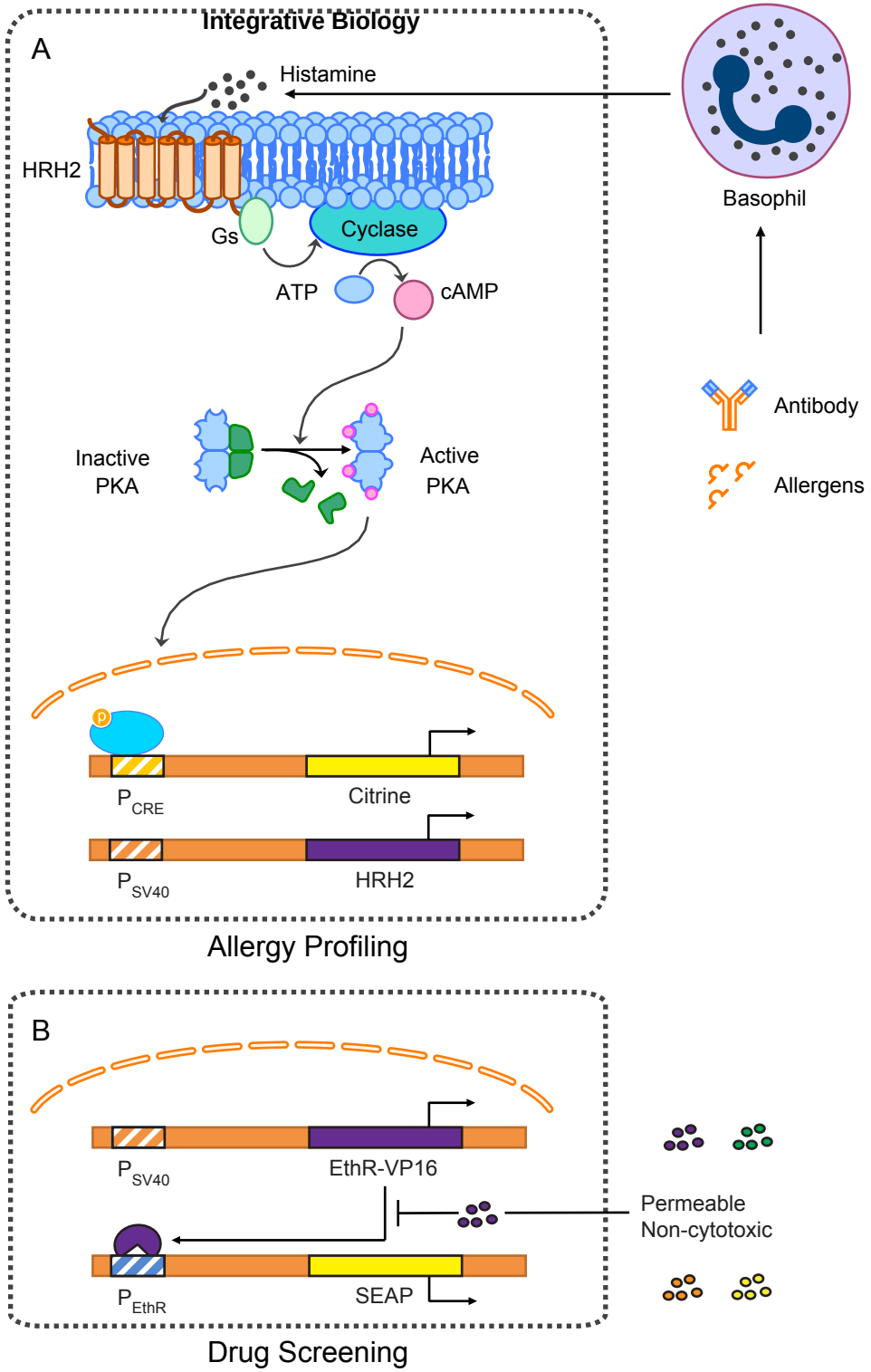

Integrative Biology
A
Histamine
HRH2
Gs
Cyclase
ATP
cAMP
Basophil
Antibody
Allergens
Inactive PKA
Active PKA
P
P CRE
Citrine
P SV40
HRH2
Allergy Profiling
B
P SV40
EthR-VP16
Permeable
Non-cytotoxic
P EthR
SEAP
Drug Screening

Figure 3

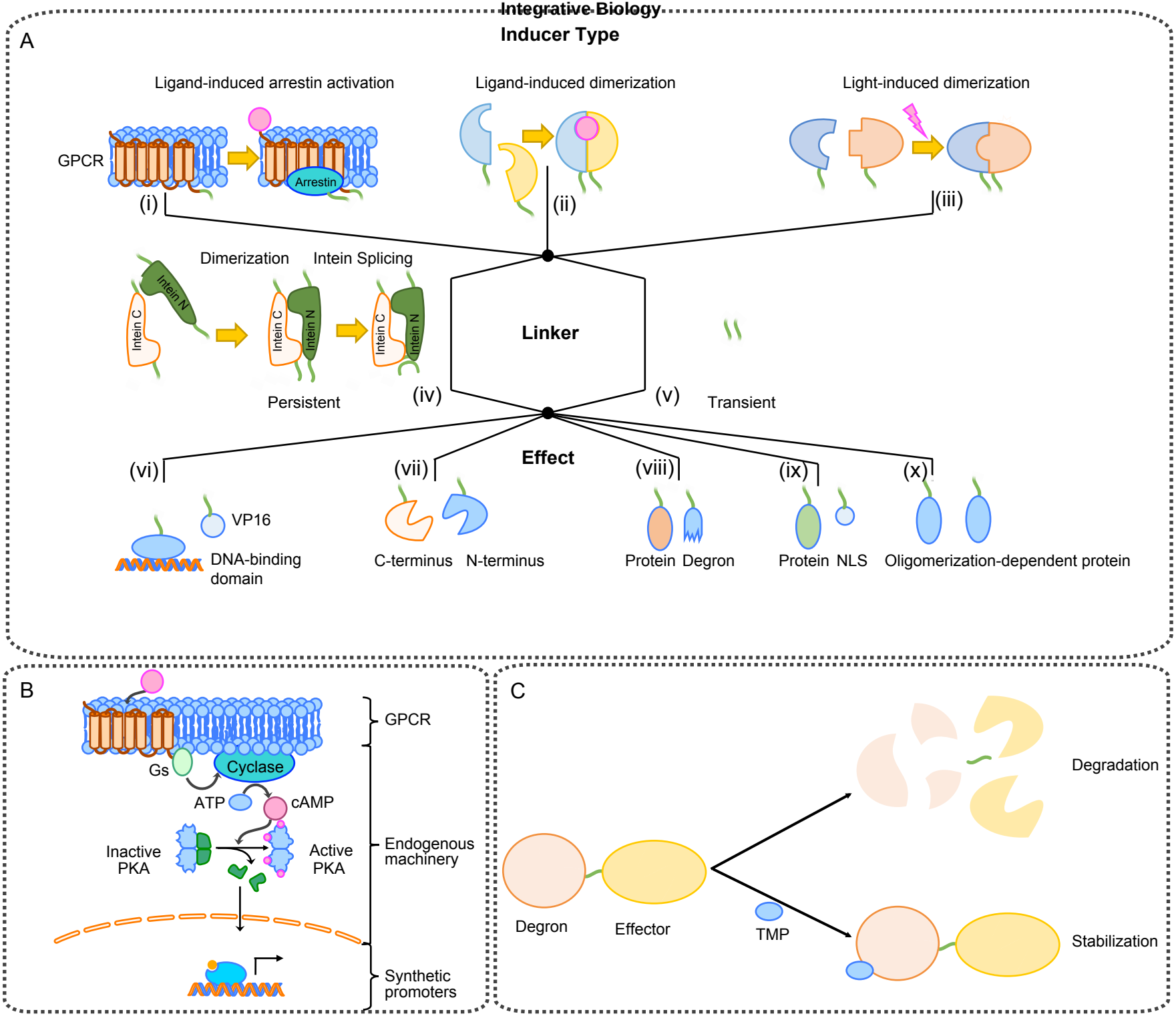

A
Inducer Type
Ligand-induced arrestin activation
Ligand-induced dimerization
Light-induced dimerization
GPCR
Arrestin
(i)
(ii)
(iii)
Dimerization
Intein Splicing
Intein N
Intein C
Persistent
(iv)
Linker
(v)
Transient
Effect
(vi)
(vii)
(viii)
(ix)
(x)
VP16
DNA-binding domain
C-terminus
N-terminus
Protein
Degron
Protein
NLS
Oligomerization-dependent protein
B
Gs
Cyclase
ATP
cAMP
Inactive PKA
Active PKA
GPCR
Endogenous machinery
Synthetic promoters
C
Degradation
Degron
Effector
TMP
Stabilization

Figure 4

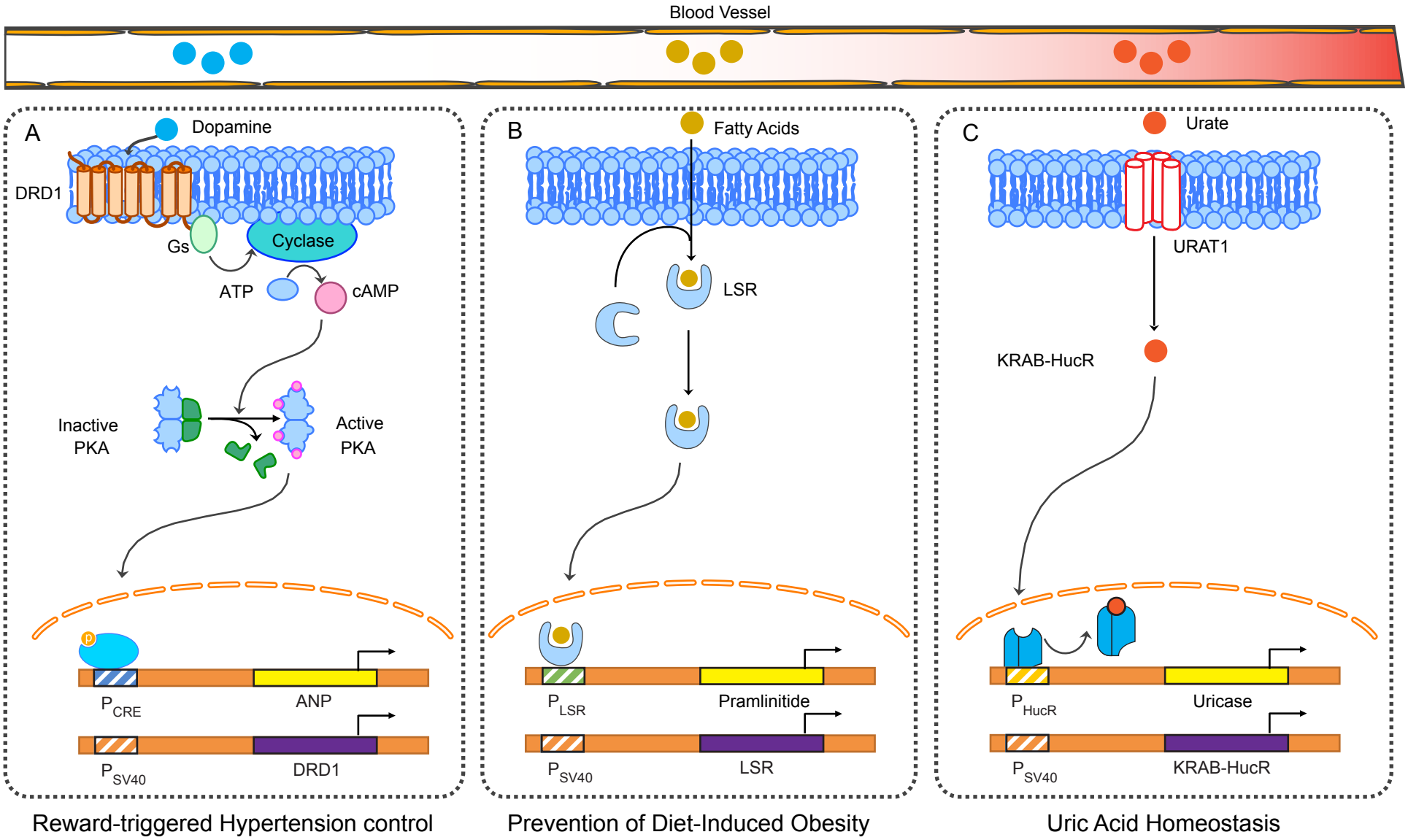

Blood Vessel
A
Dopamine
DRD1
Gs
Cyclase
ATP
cAMP
Inactive PKA
Active PKA
P
P CRE
ANP
P SV40
DRD1
Reward-triggered Hypertension control
B
Fatty Acids
LSR
P LSR
Pramlinitide
P SV40
LSR
Prevention of Diet-Induced Obesity
C
Urate
URAT1
KRAB-HucR
P HucR
Uricase
P SV40
KRAB-HucR
Uric Acid Homeostasis

Figure 5

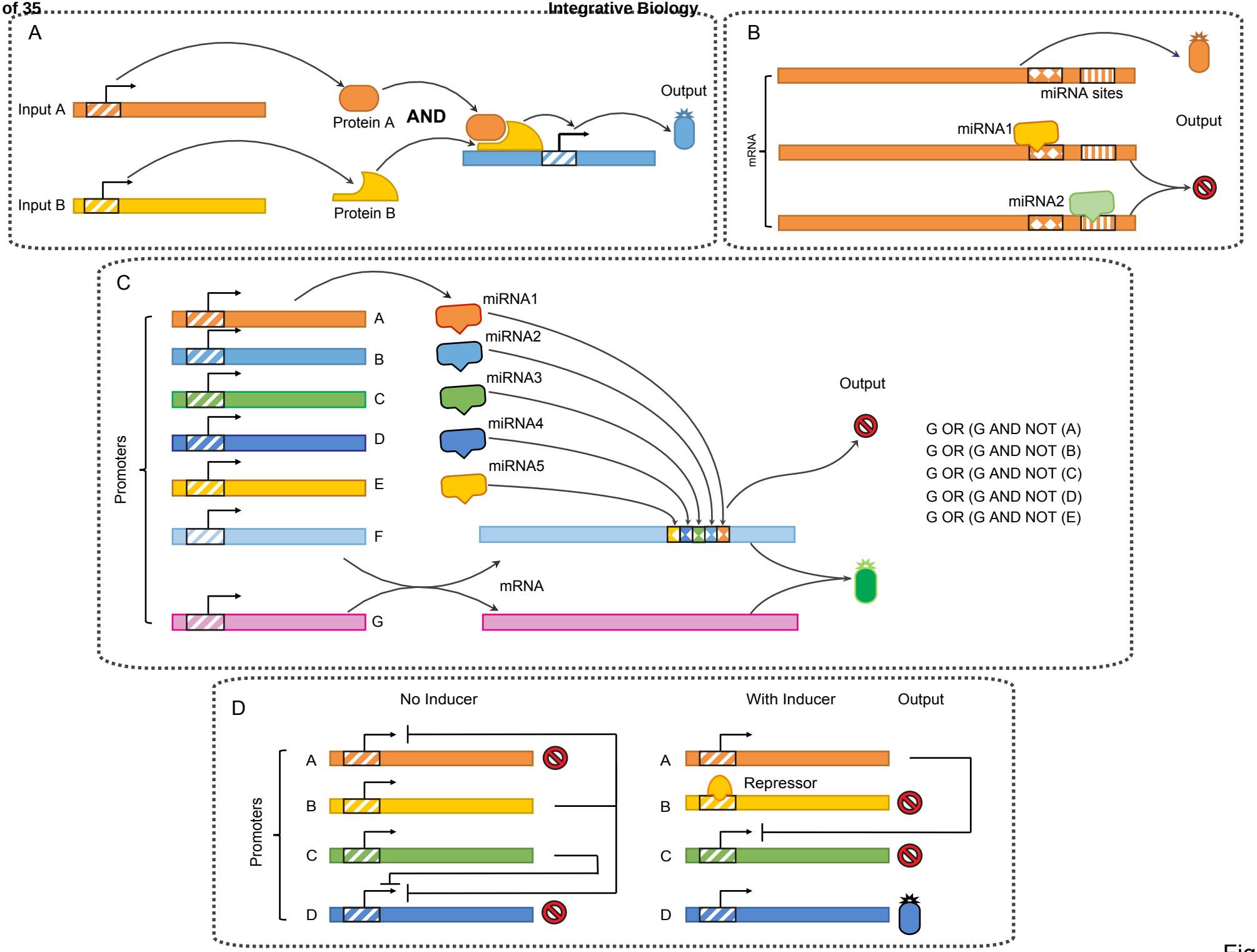

A
Input A
Input B
Protein A
Protein B
AND
Output
B
miRNA sites
miRNA
miRNA1
miRNA2
Output
C
Promoters
A
B
C
D
E
F
G
miRNA1
miRNA2
miRNA3
miRNA4
miRNA5
mRNA
Output
G OR (G AND NOT (A)
G OR (G AND NOT (B)
G OR (G AND NOT (C)
G OR (G AND NOT (D)
G OR (G AND NOT (E)
D
No Inducer
With Inducer
Output
Promoters
A
B
C
D
Repressor

Figure 6