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Recent advances in mammalian synthetic biology—design of synthetic transgene control networks

Marcel Tigges and Martin Fussenegger

Capitalizing on an era of functional genomic research, systems biology offers a systematic quantitative analysis of existing biological systems thereby providing the molecular inventory of biological parts that are currently being used for rational synthesis and engineering of complex biological systems with novel and potentially useful functions—an emerging discipline known as synthetic biology. During the past decade synthetic biology has rapidly developed from simple control devices fine-tuning the activity of single genes and proteins to multi-gene/protein-based transcription and signaling networks providing new insight into global control and molecular reaction dynamics, thereby enabling the design of novel drug-synthesis pathways as well as genetic devices with unmatched biological functions. While pioneering synthetic devices have first been designed as test, toy, and teaser systems for use in prokaryotes and lower eukaryotes, first examples of a systematic assembly of synthetic gene networks in mammalian cells has sketched the full potential of synthetic biology: foster novel therapeutic opportunities in gene and cell-based therapies. Here we provide a concise overview on the latest advances in mammalian synthetic biology.

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Introduction

Originally designed over a decade ago as functional genomic research tools enabling precise fine-tuning of target gene expression in mammalian cells and transgenic animals, heterologous transcription control systems are currently used and considered for gene-function analysis [1], drug discovery [2••], biopharmaceutical manufacturing of difficult-to-express protein therapeutics, [3,4] and for pharmacologic control of therapeutic transgenes in gene-based and cell-based therapies [5–7]. Owing to their importance and success in basic and applied research a panoply of such heterologous transcription control modalities have been developed that are responsive to all kinds of small molecules. Since most mammalian transcription control systems have a generic circuit topology and a common genetic design they are functionally compatible and therefore seen by synthetic biologists as biological parts that could be assembled to higher order genetic control devices and regulatory networks that are approaching the complexity of electronic circuits [8,9,10••]. Such synthetic networks reveal novel control dynamics [11] or provide prosthetic circuits for future therapeutic applications. This review covers recent advances in synthetic mammalian gene network design and highlights progress in applied mammalian synthetic biology.

Transgene regulation systems—essential building blocks for the design of synthetic gene networks

Precise transcription control of specific transgenes is the functional basis for the design of synthetic gene networks and heterologous transcription control circuits represent the fundamental biological parts for their construction, no matter what complexity. Following generic master assembly plans, involving heterologous transcription factors (transactivators, transrepressors, and transsilencers) conditionally binding to specific hybrid promoters, a panoply of tunable mammalian transgene control circuits have been designed that could either be induced (ON-type systems) or repressed (OFF-type systems) in response to a variety of molecular or physical cues [12,13] (Figure 1a). There are three distinct design principles by which transcription factors and chimeric promoters can be assembled to enable adjustable transcription control of target genes: (i) trigger-inducible DNA-binding proteins are fused to a mammalian cell-compatible transactivation domain (TAD, for example, *Herpes simplex* virus protein 16 [VP16]) to generate a synthetic transcription factor, a so-called transactivator (TA), which activates a chimeric promoter (P_{TA}) harboring TA-specific operator modules 5′ of a minimal version of a eukaryotic promoter [14] (Figure 1a). Depending on whether TA binds or is released from P_{TA} in response to the trigger signal the circuits generates an inducible (ON-type) or repressible (OFF-type) transcription response. (ii) Trigger-inducible DNA-binding proteins acting as transrepressors (TR) repress upstream constitutive eukaryotic promoters (P_{const}) when bound to their cognate operator site in a road block-like manner. Trigger-mediated release of TR

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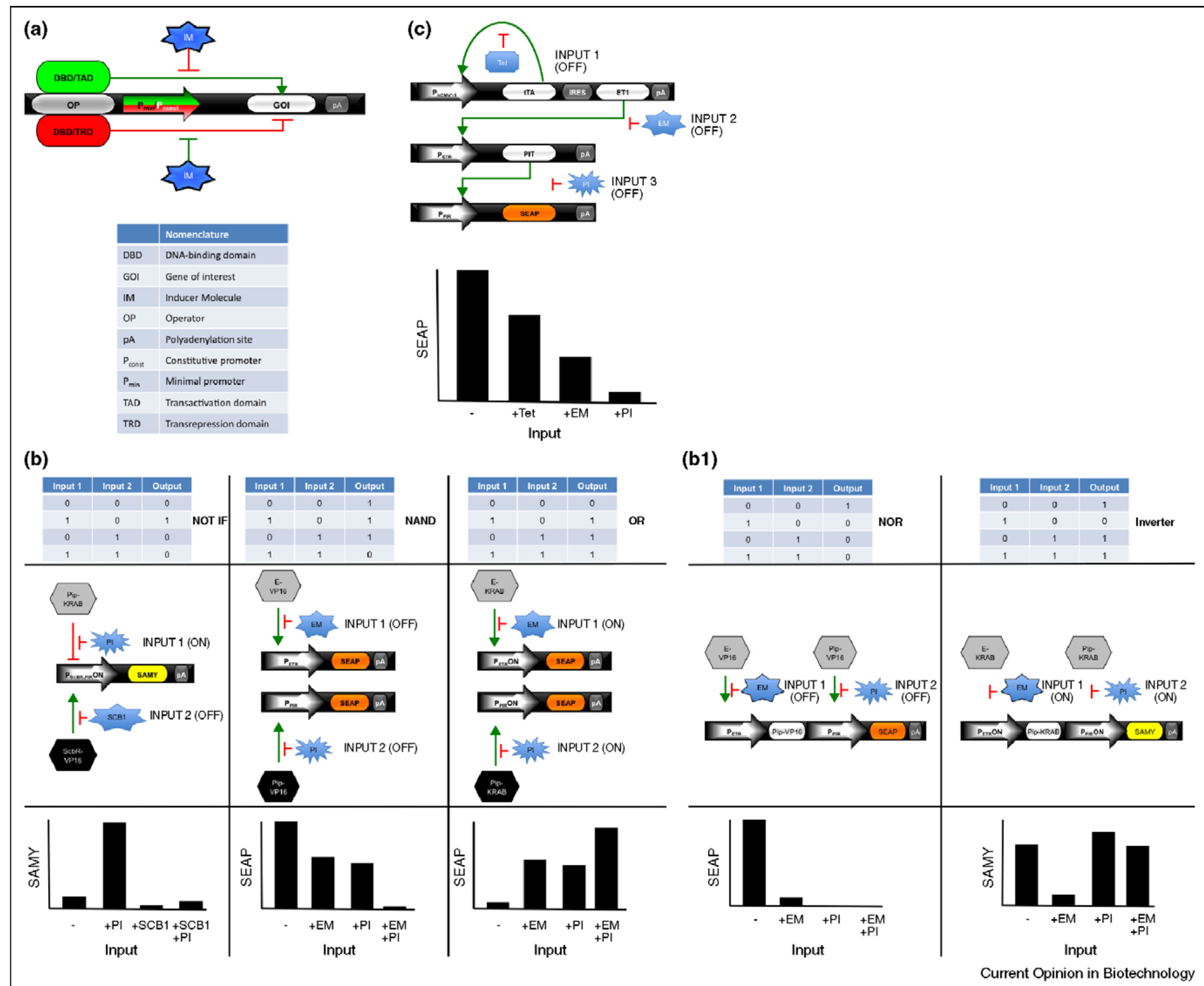
Figure 1



Minimal genetic building blocks typically used for the design of synthetic mammalian gene networks. **(a)** Schematic representation of basic transcription control components that can be assembled in alternative ways to provide different transcription control profiles (green: activation, red: repression). Such control circuits are modular devices that can be assembled to higher order control circuits. **(b)** Modular assembly of basic control circuits to BioLogic gates showing expression profiles with strict Boolean logic in response to two different input signals. Schematic representation of SAMY (*Bacillus stearothermophilus*-derived secreted α-amylase) or SEAP (SEAP; human placental secreted alkaline phosphatase) expression profiles confirm the mammalian expression logic associated with each two-input Boolean truth table. Different basic control circuits consisting of chimeric promoters targeted by small molecule dependent transactivators/transsilencers were combined to the design BioLogic gates: (i) the chimeric $P_{SCBR,PIR}$ON (scbR-PIR3-P_{MIN}) promoter containing operator sites (scbR, PIR3) for the streptogramin-dependent transsilencer (Pip-KRAB, responsive to pristinamycin (PI)) and the butyrolacetone-dependent transactivator (ScbR-VP16, responsive to 2-(1α-hydroxy-6-methylheptyl)-3-(hydroxymethyl) butanolide (SCB1)), (ii) the P_{ETR} (ETR3-P_{MIN}) promoter harboring operator sites (ETR3) for the macrolide-dependent transactivator (E-VP16, responsive to erythromycin (EM)), (iii) P_{PIR} (PIR3-P_{MIN}), a promoter with operator sites (PIR) for the streptogramin-dependent transactivator (Pip-VP16), and (iv) P_{ETR}ON and P_{PIR}ON promoter variants harboring E-KRAB and PIP-KRAB-specific operator (ETR, PIR) sites 3′ of a constitutive promoter. The NOT IF gate enables SAMY expression only in the absence of SCB1 and the presence of PI, a situation in which ScbR-VP16 binds $P_{SCBR,PIR}$ON, while Pip-KRAB remains unbound. The NAND gate enables SEAP expression when either of the two transactivators (E-VP16 and Pip-VP16) is bound to their cognate promoters (P_{ETR}, P_{PIR}). Consequently, transgene expression remains induced unless both PI and EM are present. The OR gate is exclusively repressed in the absence of both antibiotics when each of the transsilencers (E-KRAB and Pip-KRAB) is bound to their target promoters (P_{ETR}ON, P_{PIR}ON). The NOR gate, consisting of a two-level cascade, is exclusively induced in the absence of any input signal when both transactivators (E-VP16, Pip-VP16) are bound to their target promoters (P_{ETR}, P_{PIR}). The INVERTER gate is exclusively repressed in the presence of EM when E-KRAB fails to repress P_{ETR}ON so that Pip-KRAB is constitutively produced and represses P_{PIR}ON-driven SAMY expression. **(c)** Design principle and regulation performance of the pioneering mammalian transcription cascade, a three-level regulatory network consisting of three daisy chain-connected transactivator-based transcription-control circuits: tTA expression is initiated by an autoregulated $P_{hCMV^{*}-1}$ (tetracycline-responsive promoter) driven feedback loop also controlling production of co-cistronically encoded macrolide-dependent transactivator ET1. ET1 triggers P_{ETR} (macrolide-responsive promoter) driven expression of the streptogramin-dependent transactivator (PIT) that finally initiates P_{PIR} (streptogramin-responsive promoter) driven SEAP expression. The cascade can be interrupted at any level by administration of a

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from P_{const} induces transgene transcription in an ON-type manner. (iii) Alternatively, TR can be fused to a transsilencing domain (e.g. KRAB transcription-silencing domain derived from the Krueppel-associated box protein of the human *kox1* gene) to generate a synthetic transsilencer (TS) that represses target gene transcription when bound to specific operator sites placed upstream or downstream of P_{const} by steric means (downstream operator) and/or chromatin-based epigenetic silencing (upstream or downstream operator). Road block-like and epigenetic silencing are de-repressed in the presence of the trigger signal and transgene expression is induced in an ON-type fashion. A panoply of different transgene control circuits have been designed simply by using different DNA-binding protein-operator sets, preferably of heterologous (mostly prokaryotic) origin to reduce pleiotropic effects in mammalian cells. Non-limiting examples include transgene control modalities responsive to antibiotics [13,15–17], hormones, and hormone analogs [18], immunosuppressive and anti-diabetic drugs [7], vitamins [19], amino acids, and different (secondary) metabolites [20,21] including gaseous acetaldehyde [22] and the apple-derived phloretin mediating transdermal gene induction after topical application [23]. Since all of these control circuits share an identical genetic set-up they are compatible and could be functionally liked to construct higher order gene networks showing more complex control dynamics than the basic ON/OFF-type transcription switch.

Physiologic and traceless trigger signals—the future of remote transgene expression control

Although most established transcription-control systems show excellent regulation performance in mammalian cells cultivated in culture dishes [24], grown in bioreactors [19,22], or implanted into mice [25] their trigger molecules often exhibit (therapeutic) side effects (e.g. antibiotics, hormones, immunosuppressive drugs) and are therefore incompatible with biopharmaceutical manufacturing or human therapeutic use. The latest generation of adjustable transgene expression circuits are thus controlled by endogenous physiologic metabolites, food-derived compounds, vitamins, or pathologic signals to adjust production of protein therapeutics, fine-tune therapeutic transgene transcription, or to manage a therapeutic response coordinated to metabolic disorders [26••]. Highlights of transcription factor/promoter combinations responsive to physiologic trigger molecules include: (i) AlcR/P_{AIR} that is responsive to gaseous acetaldehyde (ON-type system), a signal that can be broadcast over the air and simply be 'smelled' by cell cultures grown in bioreactors or animals harboring transgenic cell implants [22]. (ii) ARG/P_{ART} that is responsive to the amino acid L-arginine (ON-type system) [20]. Unencumbered by endogenous L-arginine this circuit might be used to control L-arginine homeostasis and attenuate newborn pulmonary hypertension, hypercholesterolemia, and arteriosclerosis [27–29]. (iii) BIT/P_{BIT} that is responsive to biotin (ON-type system), also known as vitamin H, was shown to control product genes in bioreactor cultures and cell implants in mice without any detectable interference with host-cell metabolism and endogenous vitamin H levels [19]. (iv) TtgR/P_{TtgR} that is responsive to the plant defense metabolite phloretin contained in apples and therefore regularly consumed by humans. This OFF-type system has been shown to control transgenes in cell implants following transdermal and topical delivery of phloretin-containing skin lotions, which would increase convenience, patient compliance, and elimination of hepatic first-pass effect in future therapies [23]. (v) mUTS/P_{UREX} (ON-type system) that is responsive to pathologic uric acid levels and could be engineered into a therapeutic network providing self-sufficient automatic control of uric acid homeostasis by preventing crucial urate accumulation (leading to gouty arthritis) while preserving basal uric acid levels essential for oxidative stress protection (preventing development of cancer, Parkinson's disease, and multiple sclerosis) (Kemmer *et al.*, unpublished).

While all aforementioned transgene control modalities are responsive to side effect-free physiologic trigger signals they all require small molecules for transcription control. Although some of these molecules can seamlessly be administered via a skin lotion or simply be inhaled, the future will be in molecule-free or traceless remote control of transgene expression. In principle, temperature-induced expression control [30,31] is traceless; however, natural exposure to environmental temperature changes will prevent these systems from being applied in any therapeutic setting.

A major advancement in traceless therapeutic transgene control has recently been achieved by the design of a miniaturized electro-genetic device that enabled electronic control of transcription by linking electrochemical oxidation of ethanol to acetaldehyde to acetaldehyde-inducible expression fine-tuning [10••]. This electro-genetic device provided electric power-adjustable transcription control, which is able to integrate the intensity of a direct current over time, to translate the amplitude or frequency of an alternating current into an adjustable genetic readout or to modulate the beating frequency of primary heart cells following electro-induced

(Figure 1 Legend Continued) specific antibiotic. The resulting SEAP expression (schematic representation) depends on the impact of overall leakiness that varies depending on the intervention point: (i) no addition of antibiotics, 100% SEAP expression, (ii) inactivation of tTA (intervention at the first level of cascade) by addition of tetracycline results in leakiness amplification by later stages thereby limiting the extent of expression reduction and resulting in 75% SEAP expression, (iii) inactivation of ET1 (intervention at the second level of cascade) by addition of erythromycin results in 40% SEAP expression and (iv) inactivation of PIT (intervention at the third level) by addition of pristinamycin I reliably represses the transgene (0%).

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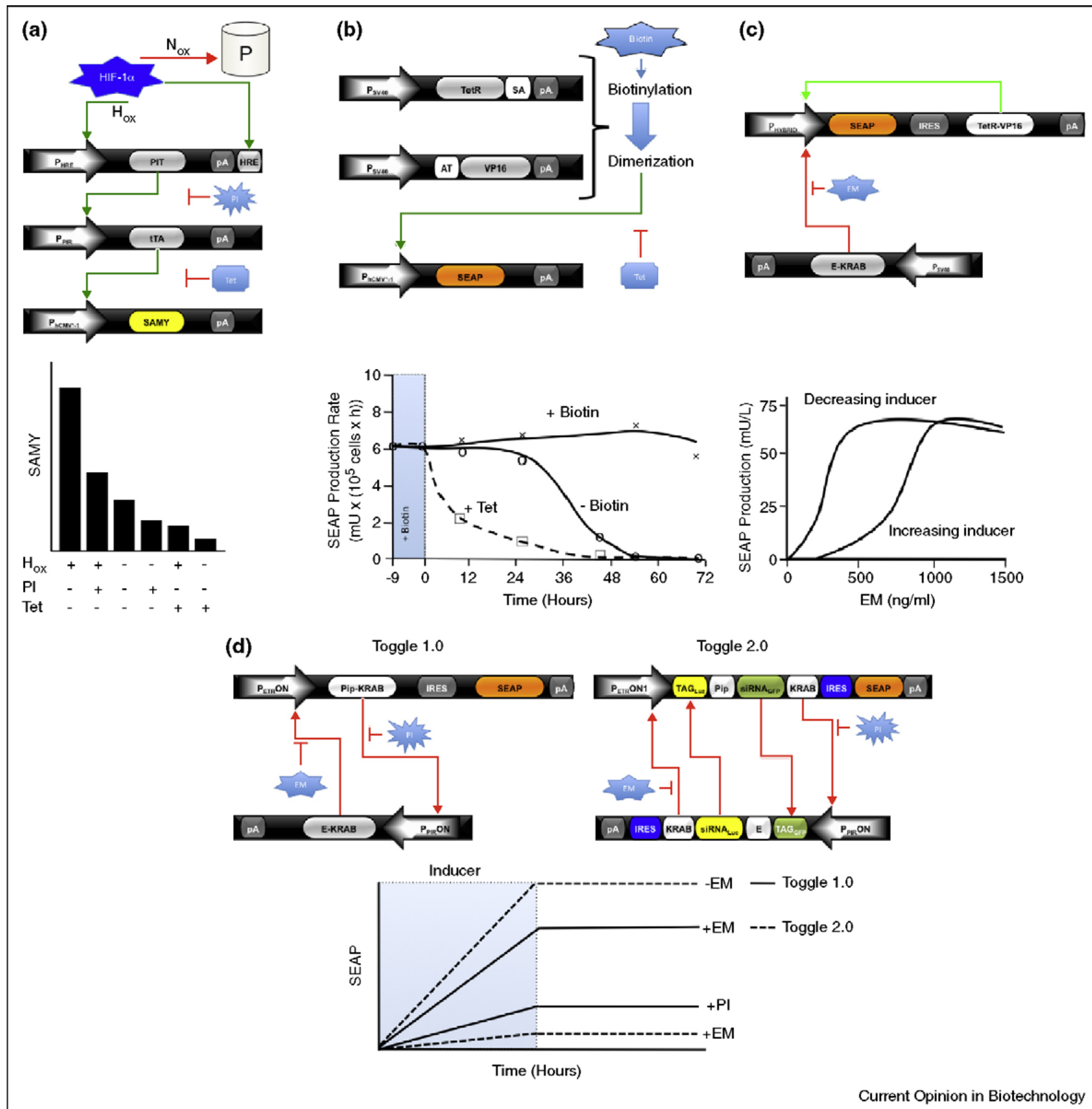
Figure 2



Higher order synthetic networks with complex regulation characteristics. **(a)** Genetic design and schematic reporter protein expression profile of a semi-synthetic regulatory network that is capable of sensing oxygen levels via endogenous HIF-1α mediated transactivation and of processing heterologous input via pristinamycin I (PI) and tetracycline (Tet). Under hypoxic conditions (H_{OX}) HIF-1α localizes to the nucleus, where it binds and activates a synthetic promoter (P_{HRE}) containing hypoxia-response elements (HRE). In case of normoxic physiological conditions (N_{OX}) HIF-1α is rapidly degraded via proteasomal targeting (P). HIF-1α-mediated P_{HRE} activity drives the expression of the streptogramin-dependent transactivator PIT (Pip-VP16) that subsequently triggers P_{PIR} (streptogramin-responsive promoter) driven tTA (TetR-VP16) expression. tTA, in turn, promotes $P_{hCMV^{*}-1}$ (tetracycline-responsive promoter) driven expression of SAMY (*Bacillus stearothermophilus*-derived secreted α-amylase). Similar to the basic transcriptional cascade shown in Figure 1c, the semi-synthetic cascade produces up to six discrete SAMY expression levels in response to different combinations of hypoxia, PI, and Tet. **(b)** Design and dynamics of a synthetic time-delay circuit consisting of a synthetic multi-domain transcription factor heterodimerized in a biotin-responsive manner. When the DNA-binding protein TetR is fused to streptavidin (SA) and the transactivation domain VP16 is linked to the AVITAG biotinylation signal (AT), biotin, constitutively ligated to AT by ectopic expression of *E. coli* BirA, heterodimerizes TetR-SA and AT-VP16 in a dose-dependent manner, thereby reconstituting a chimeric transcription factor (TetR-SA-biotin-AT-VP16) that triggers transcription from $P_{hCMV^{*}-1}$. Since biotin is incorporated into this transcription factor it will remain active even after removal of biotin that results in time-delayed target gene expression that is entirely dependent on the half-life of the transcription factor. **(c)** Design and expression dynamics of the hysteretic expression switch. The core hysteretic expression unit consists of a hybrid promoter (P_{HYBRID}) that is responsive to the transactivator TetR-VP16 and the transsilencer E-KRAB. P_{HYBRID} drives a discistronic expression unit encoding SEAP and TetR-VP16 in an auto-regulated manner. E-KRAB, which is constitutively expressed, represses P_{HYBRID} in an erythromycin-responsive manner. SEAP expression controlled by this gene network depends on historical EM concentrations. Lower EM-concentrations allow for ON to OFF switches, whereas switching from OFF to ON requires high EM-concentrations that demonstrates that the system is capable of memorizing historic inducer levels. **(d)** Design and expression dynamics of two different epigenetic toggle switches: toggle 1.0 (left panel) and toggle 2.0 (right panel). The toggle 1.0 consists of two streptogramin-dependent and

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expression of the bone morphogenetic protein 2. Although electric signal processing has evolved to manage rapid information transfer in neuronal networks or muscular contraction such control mechanisms are based on ion gradients rather than on transcription control. Functional interconnection of electro-genetic transcription control with microelectronic devices, which are among the most sophisticated man-built equipment, holds great promise for traceless gene-based therapeutic interventions in the not-so-distant future.

BioLogic gates

BioLogic gates were among the first synthetic mammalian gene networks. They consist of several transcription control circuits interconnected in different ways to integrate multiple input signals and produce different expression outputs following a strict logic that is reminiscent of electronic circuitry and can be described using Boolean operator truth tables [8]. Parallel or serial combination of different compatible ON-type or OFF-type control devices produces synthetic networks that can process two different input signals (input 1, input 2): (i) NOTIF gates are exclusively induced IF input 1 is present but NOT input 2. (ii) NAND gates are always ON unless input 1 and input 2 are present. (iii) OR gates are induced when either input 1 OR input 2 are present. (iv) NOR gates are repressed when either input 1 or input 2 are present. (v) INVERTER gates are only promote expression when input 1 is present and input 2 is absent (Figure 1b). Following the example of the above networks it is conceivable that by varying the interconnectivity of simple transcriptional control devices responding to highly specific input signals would result in other novel and potentially useful logic expression patterns.

Transcriptional cascades

Serial interconnection of multiple transcriptional units responsive to different trigger molecules has been applied by several groups to improve regulation performance by reducing inherent leakiness of basic control circuits [32] or accurately and reliably titrate target genes to multiple levels [33]. For example, serial linking of the tetracycline-dependent, streptogramin-dependent, and macrolide-dependent transactivators with respective promoters resulted in a three-level cascade in which each transactivator induces the next system until the terminal reporter gene is transcribed. Since each system operates at a different level and can be repressed by a specific antibiotic the impact of overall leakiness on target gene expression varies depending on the intervention point and enables up to four discrete expression levels: (i) no intervention, 100% expression; (ii) intervention at the first level of cascade (addition of tetracycline) results in leakiness amplification by later stages thereby limiting the extent of expression reduction and resulting in 75% expression; (iii) intervention at the second level (addition of erythromycin), 40% expression; (iv) intervention at the third and terminal level (addition of pristinamcin I) reliably represses the transgene (0%) [33] (Figure 1c).

The above-mentioned transcriptional cascade processes entirely external signals to create the desired function, successful design of prosthetic networks that seamlessly integrate into host metabolic networks will require connection of synthetic networks to endogenous physiologic signals. A pioneering host-interfaced ‘semi-synthetic’ regulatory cascade that plugs into the mammalian oxygen response system has recently been reported. Oxygen control is mediated by the hypoxia-induced factor 1α (HIF-1α) that through a series of interactions activates promoters harboring hypoxia-response elements (HRE). Under normoxic condition HIF-1α is rapidly degraded, which prevents any transcription response [34]. Coupling of the oxygen system to a synthetic mammalian transcription cascade results in multi-level control of the terminal target gene influenced by endogenous signals (e.g. oxygen) as well as heterologous external signals (e.g. antibiotics). When combining three different input signals up to six discrete expression levels could be achieved [26••] (Figure 2a)

Time-delay circuits

Time-delay circuits modulating the precise timing of gene expression are essential natural network components for rhythmic gene expression required for NF-κB activation and operation of the circadian clock [35–37]. The synthetic time-delay circuit consists of modified tetracycline-dependent transactivator (tTA) whose individual domains TetR and VP16 have been fused to streptavidin (TetR-SA) and avitag (AT-VP16), respectively. Biotin, constitutively ligated to AT in the presence of the *Escherichia coli* biotin ligase BirA and able to non-covalently bind to SA, will heterodimerize TetR-SA and AT-VP16, thereby reconstituting a functional tetracycline-responsive transcription

(Figure 2 Legend Continued) macrolide-dependent transrepressor systems (Pip-KRAB/P_{PIR}ON, streptogramin-dependent transsilencer/streptogramin-inducible promoter; E-KRAB/P_{ETR}ON, macrolide-dependent transsilencer/macrolide-inducible promoter) mutually repressing each other's expression: E-KRAB represses a P_{ETR}ON-driven dicistronic expression unit encoding Pip-KRAB and SEAP. Pip-KRAB, in turn, represses P_{PIR}-driven E-KRAB expression. By transient administration of either pristinamycin I (PI) or erythromycin (EM) SEAP expression can be locked in either an ON (+/− EM) or the OFF (+/− PI) state. Transient administration of EM produces high SEAP and Pip-KRAB levels that are able to continuously repress E-KRAB expression even upon withdrawal of EM. Likewise, after transient administration of PI, Pip-KRAB is unable to repress E-KRAB expression that, in turn, represses SEAP and Pip-KRAB and so locks SEAP expression in a repressed state even after withdrawal of PI. The toggle 2.0 is a toggle 1.0 variant containing transsilencers harboring intronically encoded siRNAs (either GFP ($siRNA_{GFP}$) specific or Luciferase ($siRNA_{LUC}$) specific) directed against TAGs placed in the untranslated transcript immediately upstream of the encoded opposing transsilencer. siRNA-mediated RNA interference complements transsilencer activity that reduces the leakiness of the system and increases the dynamic SEAP expression range compared to toggle 1.0.

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factor (TetR-SA-biotin-AT-VP16) [9,38] (Figure 2b). Owing to the irreversibility of the biotinylation reaction, heterodimerized transcription factors retain their functionality after biotin removal and continue to sustain target gene expression for some 30 h until the transcription factor is completely degraded. This time-delayed shutdown of transgene expression is reminiscent of a capacitor whose discharge results in an immediate decrease in voltage [9].

Bistable expression networks

All aforementioned networks process current environmental information to directly produce specific outcomes. The next step of complexity is that synthetic mammalian gene networks not only react to present cues but also are capable of remembering past environmental changes and modulate target gene expression accordingly. A gene network enabling such an epigenetic expression memory requires feedback loop expression circuits (e.g. a transactivator driven by its own target promoter) that enable self-sustained bistable target gene expression [39].

Hysteresis

Hysteresis is the prototype of bistable expression memory, which manages many important natural processes such as the cell cycle [40] and cell fate control [41], exhibits the following two control characteristics: (i) Threshold levels for 'ON' and 'OFF' states depend on the starting state (expression memory). (ii) Different inducer concentrations are required for OFF-to-ON and ON-to-OFF switches that insulates target gene expression from small inducer concentration changes (expression buffer). A first generation of hysteretic mammalian gene networks consisted of a transactivator (TetR-VP16) and a transsilencer (E-KRAB) competing for the same hybrid promoter thereby modulating expression of a dicistronic expression unit encoding TetR-VP16 and a reporter gene. Whereas TetR-VP16 is expressed in a positive feedback loop, E-KRAB is constitutively produced from another cistron [42]. The prevalence of E-KRAB-mediated inhibition versus TetR-VP16-mediated positive feedback depends upon historical erythromycin (EM) concentration: High historical EM levels resulted in high TetR-VP16 levels that require greater E-KRAB activity and therefore lower EM concentrations for an ON-to-OFF switch. Conversely, low historical EM concentrations resulted in minimal TetR-VP16 levels that require maximum inactivation E-KRAB, hence relatively much higher EM concentrations, for an OFF-to-ON switch (Figure 2c). This first generation synthetic mammalian hysteresis network was significantly simplified by the design of a tetracycline-responsive autoregulated dicistronic expression unit encoding the reverse tetracycline-dependent transactivator (rtTA) and the enhanced green-fluorescent protein (EGFP). This network exhibited hysteretic EGFP expression in response to different levels of the tetracycline derivative doxycycline [43•].

Toggle switch

While hysteretic networks remember stimulus concentrations and mediate expression switches between binary states at different signal thresholds, toggle switches memorize and lock ON or OFF states after transient exposure to a specific inducer. The first mammalian toggle switch was designed by assembling two antibiotic-responsive transsilencers, the erythromycin-dependent transsilencer E-KRAB [44,45] and the pristinamycin-dependent transsilencer PIP-KRAB [15] such that each transsilencer is transcribed from the other transsilencer's cognate promoter thereby establishing a mutually inhibitory network in which each transsilencer inhibits the promoter of the opposing transsilencer. A reporter gene, co-cistronically encoded with PIP-KRAB, was monitoring the expression status of the toggle switch that was either ON, following transient administration of erythromycin inactivating E-KRAB and locking target gene expression in an induced state (PIP-KRAB prevails and represses E-KRAB transcription), or OFF, following a short pulse of pristinamycin that inactivates PIP-KRAB and locks target gene expression in a repressed state (E-KRAB action prevails and represses PIP-KRAB and reporter gene expression). The network behaves as a bistable 'toggle' switch that maintains ON or OFF expression without the on-going presence of trigger molecules even across cell generations indicating that the network memory can be inherited to progeny [46] (Figure 2d, left panel). The dynamic expression range of this first-generation mammalian toggle switch could be significantly increased by supporting the transcription-inhibiting activity of the respective transsilencer with RNA interference using intronically encoded siRNA eliminating the leaky transcripts that escaped transsilencer-mediated repression [47] (Figure 2d, right panel).

Coupled transcription–translation networks

The combination of heterologous transcription control with siRNA-based RNA interference mechanisms reduces the leakiness of control circuits and improves their overall regulation performance. For example, when extending classic OFF-type tetracycline-responsive target gene expression with ON-type erythromycin-inducible expression of an siRNA specific for the target gene-encoding mRNA the regulation window can be significantly improved since leaky transcripts under repressed conditions can be eliminated by RNA interference [48]. A similar network topology combining Lac switch-controlled target gene expression with tetracycline-responsive siRNA production reduced leaky transcription below 1% [49•]. Instead of using siRNA specific for the coding sequence of the target gene, generic sets of siRNA/target sequences can be used whereby the siRNA target sequence is engineered into the 5′ or 3′ non-translated

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region of the target mRNA. When siRNAs are engineered as synthetic introns into transcription factors, coupled transcription–translation control can be encoded in a very compact format as recently shown by a multi-gene engineering strategy enabling simultaneous expression and knockdown of six transgenes off a single platform [50].

Dynamic instability—the mammalian oscillator

In contrast to the aforementioned control networks that were designed for maximum stability and could be fine-tuned by external stimuli, dynamic instability such as occurring in an oscillatory network produces periodic transcription pulses in a self-sufficient manner without any trigger input. In humans, several processes including endocrine production and release, body temperature modulation, and immune responses show circadian oscillatory behavior controlled by a master circadian clock consisting of a set of feedback-controlled autoregulated circuits producing a rhythmic signal output with 24-h periodicity [51,52].

Several synthetic oscillators have been designed in bacteria during the past decade: (i) the repressilator, in which three repressors are interconnected to form a cyclic negative feedback loop [53•], (ii) the modified toggle switch, which consists of an activator auto-inducing its own expression along with expression of an antagonizing repressor [54,55], (iii) the metabolator that captures signals of the *E. coli* acetate pathway to drive a synthetic network able to monitor and visualize oscillating metabolic activity [56], (iv) the tunable oscillator exhibiting self-sustained trigger-inducible frequency modulation as well as synchronized and hereditary oscillations based on the differential activity of two feedback loops [57••]. Basic oscillating transgene expression was recently established in NIH/3T3 transgenic for a TetR-mediated auto-inhibitory feedback circuit consisting of a native mouse β-actin promoter (P_{actin}) with 3′TetR-specific operator sites that generates a multi-functional transcript containing a non-coding intron of variable length, a TetR variant N-terminally fused to a destablized YFP (YFP-PEST) and C-terminally to a nuclear localization signal (NLS) and terminated by an AU-rich element (ARE) in the 3′untranslated region [58]. This circuit generated pulsed YFP-mediated fluorescence with periods depending on intron length: longer introns increased the time-delay of the auto-inhibitory feedback loop of the TetR fusion protein and so decreased the frequency (Figure 3a).

Although many components of the mammalian circadian clock are well established [52,59–63] it was impossible to reproduce a functional mammalian oscillator by assembling CRY, PER, BMAL1, and CLOCK following the natural circuit design [64]. This suggested that details on how cellular clocks are assembled, maintained, operated, and regulated still remain largely elusive and that a synthetic biology approach using heterologous transcription control systems would be more suited to design a mammalian oscillatory network emulating circadian clock-like behavior. Since the precise molecular mechanism and expression dynamics of the most basic mammalian circadian clock is not fully understood we have designed a synthetic oscillating network that manages autonomous, self-sustained, and tunable cyclic transgene expression in mammalian cells. The mammalian oscillator consists of a central sense/antisense expression unit encoding the tetracycline-dependent transactivator (tTA). Whereas sense expression of tTA is autoregulated by a positive feedback loop involving the tTA-specific tetracycline-responsive promoter ($P_{hCMV*-1}$), antisense transcription is controlled by a two-step time-delaying negative-feedback transcription cascade in which $P_{hCMV*-1}$-driven expression of the pristinamycin-dependent transactivator (PIT) triggers tTA antisense transcripts from a streptogramin-responsive promoter (P_{PIR}). When set to specific parameters and using $P_{hCMV*-1}$-driven green fluorescent protein expression synchronized with tTA sense expression, oscillating tTA levels could be monitored in real time and in individual mammalian cells [65] (Figure 3b). Intriguingly, even when the ratios of the oscillator components were held constant, absolute plasmid concentrations could be used to modify the period and amplitude of oscillation (Figure 3c).

Engineering of synthetic intercellular communication

All aforementioned synthetic gene networks were designed to function within single cells. However, in order for synthetic biology to reveal operation dynamics essential for development or hormone systems of multicellular organisms as well as for host–pathogen interactions and entire ecosystems, higher order control systems will be required that manage information transfer between different cell populations and across different species.

The first synthetic mammalian communication device entirely assembled from genetic parts was recently shown to manage information transfer from a sender to a receiver cell by airborne transmission of gaseous acetaldehyde [22] Such 'wireless' airborne broadcasting included either a sender cell, engineered for constitutive expression of alcohol dehydrogenase to convert traces ($^{0}/_{00}$ range) of spiked ethanol into acetaldehyde, wild-type *E. coli*, *Saccharomyces cerevisiae*, or the plant *Lepidium sativum* (the garden cress) all of which produce volatile acetaldehyde as part of their normal metabolism, which diffuses via the gas or liquid phase to the receiver cells engineered for acetaldehyde-inducible transgene expression [66••] (Figure 3d). When microencapsulated sender and receiver cells were implanted at different sites into mice, the sender cells were able to instruct the remote receiver cell population to produce a specific reporter protein. Such synthetic information transfer across a living animal is reminiscent of hormone systems in which a sender cell

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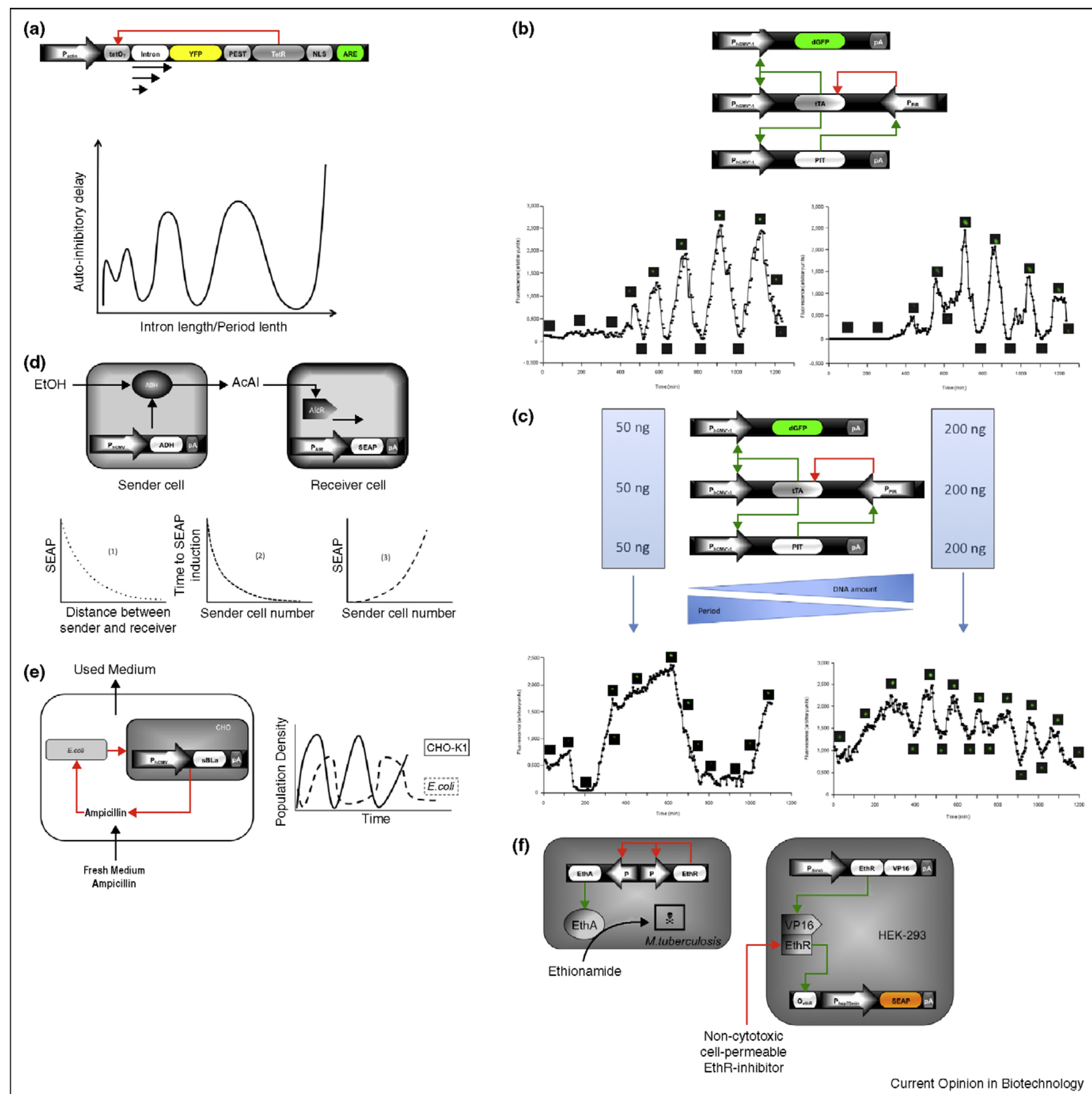
Figure 3



Synthetic networks displaying dynamic instability. **(a)** Schematic representation of a basic synthetic mammalian oscillator showing intron length dependent variations in the period of fluorescent protein expression. The core oscillator consists of a multi-domain fusion protein harboring a destabilized yellow fluorescent protein (YFP-PEST), an *E. coli*-derived tetracycline-dependent repressor (TetR) and a nuclear localization signal (NLS) that is expressed from the P_{actin} promoter engineered to contain a TetR-specific $tetO_7$ operator immediately 3′. Furthermore, the transcription unit contains an intron of different length in the 5′ untranslated region and AU-rich element (ARE) in the 3′ untranslated region to reduce the half-life of the mRNA. The TetR-mediated negative feedback loop generates rhythmic activation and repression of P_{actin} that can be monitored by YFP-mediated fluorescence intensity. The oscillation period correlates with the length of the intron (schematic representation). (**(b)** and **(c)**) Design and single cell trajectories of a tunable mammalian oscillator. (b) The clock-like mammalian oscillator consists of three independent transcription units: (i) a tetracycline-dependent transactivator (tTA), whose sense expression is driven by the tetracycline-responsive promoter ($P_{hCMV^{*}-1}$) in an autoregulated manner and the antisense transcription of which is coordinated by the streptogramin-responsive promoter (P_{PIR}), (ii) a $P_{hCMV^{*}-1}$-driven PIT (streptogramin-dependent transactivator) and (iii) a $P_{hCMV^{*}-1}$-driven expression unit encoding a destabilized green fluorescent protein (dGFP). tTA sense expression triggers a time-delaying two-level transcription cascade producing PIT that then mediates tTA antisense transcription. Therefore, tTA levels oscillate, akin to a pendulum of a mechanic clock, which is visualized by dGFP expression plugged into the circuit via $P_{hCMV^{*}-1}$

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population programs a target tissue for a particular activity.

A recent example of a synthetic ecosystem showing oscillating predator–prey population size dynamics was designed by co-cultivating the predator (wild-type *E. coli*) and the prey (CHO-K1 engineered for constitutive production of a secreted β-lactamase) while maintaining a semicontinuous supply of fresh medium supplemented with ampicillin. Upon start of the ecosystem ampicillin limits growth of *E. coli* that enables proliferation of CHO-K1. As the CHO-K1 population increases in size it produces secreted β-lactamase that lowers the ampicillin level. As the ampicillin concentration decreases, the *E. coli* population recovers and resumes growth, which compromises viability of the mammalian cells owing to bacteria-mediated nutrient depletion. Since the declining CHO-K1 population produces lower amounts of secreted β-lactamase, ampicillin level increase again and the *E. coli* population is decimated, which leads to the recovery of the mammalian cell population. As this sequence of events repeats the ecosystem exhibits phase-shifted oscillations in the size of the prokaryotic and mammalian populations [66••] (Figure 3e).

Applications of mammalian synthetic biology

Although the emerging field of synthetic biology is a success story in providing novel dynamic information on complex systems by assembling components originating from the '-omics' era [67], synthetic biologists will have to deliver useful applications in order to satisfy the expectations and hope put into this discipline and enable a sustained development of this new field. The discovery of novel anti-tuberculosis compounds is a paradigm of how the engineering principles of synthetic biology could be applied to provide solutions to long-standing therapeutic challenges. Using a pathogen-derived mammalian gene network, small molecules were discovered that switched off the resistance of mycobacteria to antibiotics [2••] (Figure 3f). *Mycobacterium tuberculosis* is inherently resistant to the antibiotic ethionamide, which must first be activated by *M. tuberculosis*' Baeyer-Villiger monooxygenase EthA. However, activation tends to be inefficient, since EthA production is repressed at the transcriptional level by the TetR/CamR-type repressor EthR [68]. Therefore, inactivation of EthR was expected to be correlated with increased EthA expression and higher sensitivity of this pathogen to ethionamide [69]. In order to screen for compounds that inactivate EthR but that are not toxic to mammalian cells and can penetrate the mammalian cell membrane to reach the intracellular pathogens, an EthR-based synthetic genetic circuit was designed and engineered into human embryonic kidney cells [2••]. A chimeric transcription factor was constructed comprising EthR fused to the VP16 transactivator that binds the EthR-specific operator O_{ethR} and triggers activation of the downstream minimal promoter, resulting in expression of the reporter gene SEAP. This configuration was used to screen a chemical library, revealing several compounds, which inhibit EthR, most of which were excluded from the list of targeted compounds, since viability of the screening cell line was non-specifically compromised. The small-molecule ester 2-phenylethylbutyrate, which is a licensed strawberry flavor met all the above criteria and was also shown to activate EthA expression in *M. tuberculosis* and to render this pathogen

(Figure 3 Legend Continued) and transactivated by tTA. (c) Tunability of the clock-like mammalian oscillator. Interestingly, the amount of clock vectors transfected into mammalian cells influenced the period of the oscillator: lower amounts of clock components resulted in lower frequencies and higher amplitudes. **(d)** Cell-to-cell communication networks. Intercellular communication devices consist of 'sender cells' engineered for constitutive expression of the alcohol dehydrogenase (ADH) that enables them to convert spiked ethanol into gaseous acetaldehyde. Acetaldehyde then diffuses through the gas or liquid phase to 'receiver cells' that are transgenic for acetaldehyde-responsive target gene expression: they constitutively express the *Aspergillus nidulans*-derived acetaldehyde-dependent transactivator AlcR and an AlcR-specific promoter (P_{AIR}) harboring AlcR operator modules 5′ of a minimal eukaryotic promoter. Binding of AlcR to P_{AIR} occurs in the presence of acetaldehyde, is dose dependent and triggers expression of SEAP (top panel). This cell-to-cell communication network provides the following SEAP expression dynamics: (1) SEAP expression is dependent on the distance between sender and receiver cells, (2) the ratio of sender and receiver cell numbers determines the timing of SEAP expression: a high number of receiver cells mediates a faster onset of SEAP expression while fewer sender cells delay SEAP expression, (3) SEAP expression is dependent on the size of the sender cell population. A given receiver cell population can, therefore, sense the population size of the sender cells. **(e)** Schematic representation of a synthetic oscillating predator–prey ecosystem. When co-cultivating wild-type *E. coli* and Chinese hamster ovary (CHO-K1) cells transgenic for production of a secreted β-lactamase in a mammalian cell culture medium with semi-continuous inflow of ampicillin-containing medium both species show phase-shifted oscillating population size dynamics: While the *E. coli* population is initially decimated by ampicillin thereby facilitating the expansion of the CHO-K1 population, the growing number of mammalian cells secrete increasing levels of β-lactamase and therefore reduce the concentration of ampicillin that promotes *E. coli* proliferation and results in nutrient depletion and a consequent decrease in the CHO-K1 population size. As the CHO-K1 population declines ampicillin concentration increases thereby initiating another predator–prey cycle starting by ampicillin-mediated elimination of *E. coli*. **(f)** Screening for compounds that switch off intrinsic etionamide resistance of multidrug-resistant *Mycobacterium tuberculosis*. In *M. tuberculosis* EthA converts ethionamide into a cytotoxic InhA-inhibitor. EthR binds and by default represses the bi-directional promoter driving EthA as well as EthR expression (left panel). Compounds, which are able to release EthR from its target promoter would trigger de-repression of EthA and so increase the sensitivity of the intracellular pathogen to etionamide. To enable efficient screening for EthR-releasing molecules a designer cell line (HEK-293, human embryonic kidney cell) was constructed that contains a synthetic transcription factor (EthR fused to the VP16 transactivation domain) binding and activating a chimeric promoter (O_{ethR} fused to a minimal version of the *Drosophila* heat shock protein 70 promoter) that drives expression of the reporter gene SEAP. When screening a library of compounds using such a designer cell line, only non-cytotoxic, cell-permeable, and EthR-specific inhibitors can specifically inhibit SEAP production. These compounds may inhibit the EthR-O_{ethR} interaction in *M. tuberculosis* and may therefore switch off the inherent EthA-mediated etionamide resistance of this pathogen.

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more sensitive to the antibiotic ethionamide, thereby providing novel therapeutic options for the treatment of tuberculosis.

Outlook

The serial or parallel combination of minimal transcription-control building blocks resulted in logic gates or transcriptional cascades and time-delay circuits, capable of following strict logic in signal integration, very similar to electronic circuits. The next level of complexity was reached with transcriptional networks enabling bistable behavior or dynamic instability, characteristics of toggle switches, exhibiting memory, or hysteretic switches, which buffer the stochastic influence and regularly timed expression of oscillatory circuits. The current number of available single modular building blocks for synthetic biology is already impressive. On the basis of continuous refinement and re-invention of existing networks, new generations of gene regulation systems, improvement of the predictions of theoretical models and approaches aimed at obtaining more detailed biochemical information about core elements, the next level of synthetic biology as a tool for gene therapy, or biopharmaceutical manufacturing might be reached in the not-too-distant future. Although there are still few *in vivo* studies that prove this applicability, increasing knowledge, and experience of synthetic biology, especially mammalian synthetic biology, will probably accelerate the development of synthetic networks towards solutions for unmet needs in medicine [70], environmental protection [71,72], biotechnology [73], and agriculture [74].

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