# Context-aware synthetic biology by controller design: engineering the mammalian cell

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**Abstract**

The rise of the field of systems biology has ushered a new paradigm: the view of the cell as a system that processes environmental inputs to drive phenotypic outputs. Synthetic biology provides a complementary approach, allowing us to program cell behavior through the addition of synthetic genetic devices into the cellular processor. These devices, and the complex genetic circuits they compose, are engineered using a design-prototype-test cycle, allowing for predictable device performance to be achieved in a context-dependent manner. Within mammalian cells, context effects impact synthetic genetic device performance at multiple scales, including the genetic, cellular and extracellular levels. In order for synthetic genetic devices to achieve predictable behaviors, approaches to overcome context-dependence are necessary. Here, we describe control systems approaches for achieving context-aware devices that are robust to context effects. We then consider the application of cell fate programming as a case study to explore the potential impact of context-aware devices for regenerative medicine applications.

# The cell as a processor

Cells are dynamic units of life that rely on microenvironmental cues to drive their decision-making. A cell's behavior – to divide, die, move, or otherwise – is driven by social interactions with neighboring cells, binding to the extracellular matrix (ECM), and by messages in the form of soluble signals. Whether a member of the multicellular societies that compose our tissues or solo explorers in the unicellular world, each cell is a processor that must map these dynamical chemical and mechanical inputs to phenotypic outputs (**Figure 1A**). Rooted in the field of systems biology (Kauffman 1969; Milo et al. 2002; Barkai and Leibler 1997; Bhalla and Iyengar 1999; Hartwell et al. 1999) the view of the cell as a processor offers a basis on which synthetic biology can build, manipulating cellular behavior by engineering the processor.

The cell relies on an internal network that consists of molecular players (DNA, RNA, and proteins) that act in concert with microenvironmental inputs to define "cell state". At any point in time, cell state can be captured by the cellular transcriptome, proteome, epigenome and metabolome – the concentrations and chemical status of the cell's molecular players. As in other dynamical systems, the current cell state is shaped by three critical elements: (1) the inner regulatory network (the cell's processor), (2) inputs from the cell's microenvironment, and (3) the initial state of the regulatory network itself. The rules that govern cell state are encoded in the cell's genome, which gives rise to the RNA and proteins that take part in the regulatory network. Specifically, this network is composed of dynamical processes (transcription, translation, and modifications to the molecular players in the network) that engage in regulatory interactions with one another — a so-called "hairball". These interactions modulate the dynamical processes in the cell. For example, transcription rates can be regulated through the binding of transcription factors (TFs) to promoters; post-transcriptional regulation can involve RNA degradation by microRNA (miRNA); translation rates can be influenced by modifications to messenger RNA (mRNA) untranslated regions (UTRs); post-translational control can be achieved through modifications to protein stability; and epigenetic changes to the DNA itself can result in compaction or methylation of regions of DNA (Del Vecchio and Murray 2014; Alberts et al. 2014; Alon 2019; Allis et al. 2007). While 'omics strategies have been used to probe the transcriptome, proteome, epigenome, and metabolome, they only offer a static image of the dynamic nature of the cell's regulatory network.

As a result of the starting cell state and environmental inputs that interact with receptors on the cell surface, many cellular decisions, such as the fate of the cell, are made. Cellular decisions then shape phenotypic changes in features, such as proliferation, death, morphology, polarity, metabolism, secreted factors, size, motility, and cell type specification (Balázsi et al. 2011). Thus, one must consider the initial cell state as well as the cell's regulatory network when predicting the impact of microenvironmental cues on a cell's phenotype. Cytokine pleiotropy – in which the same soluble ligand inputs result in different phenotypic outcomes for cells, depending on the state of the cellular processor (Nicola 1994; Sánchez-Cuenca et al. 1999) – provides an example for the impact of the initial cell state on cellular decision-making. Consider, for example, the fibroblast growth factor (FGF) superfamily of cytokines, which is known to exhibit strong action on a number of different cells, due to the diversity of interactions between FGF ligands and their receptors (Kosaka et al. 2009). In the mouse, FGF-4 is first expressed in the inner cell mass of the preimplantation mouse blastocyst. For these pluripotent mouse cells, microenvironmental FGF-4 drives cell proliferation. However, the impact of FGF-4 on phenotypic outcomes changes as the cells in the developing mouse embryo undergo specialization. Later in mouse development FGF-4 instead plays a role in directing mesenchymal cell differentiation during tooth development. The ability of FGF-4 to drive different phenotypic outcomes is due to changes in cell state as pluripotent cells undergo differentiation.

Cellular decision-making thus depends on the concentration and modification status of key molecular players, such as DNA, RNA, and proteins, together determining cell state. It has been shown that the binding pattern of TFs differs between cell types, suggesting that changes to the cellular epigenome can change regulatory processes in the cell, allowing these processes to evolve over time as a function of accessibility of DNA binding domains and regulator concentrations (Tsankov et al. 2015). TF and coactivator binding throughout the genome is a function of accessible binding sites, where the relative binding affinity and concentration of competing binding partners determines the dominant regulatory interactions (Hosokawa and Rothenberg 2020). Indeed, systems biology has demonstrated the utility of modeling to better understand the impact of cell state on cellular decisions (Emmert-Streib et al. 2014; Davidson and Peter 2015; Liu et al. 2018). These efforts have aimed to predict the phenotypic behaviors of cells, including mammalian stem cells (Dunn et al. 2014; Kinoshita et al. 2018), by computationally modeling the cell's processor and its initial state. Through the addition of microenvironmental inputs, cellular outcomes have been predicted using models. These models can be augmented to yield probabilistic predictions of cellular outcomes by including different sources of cellular noise (Quarton et al. 2020). In these stochastic models, fluctuations in biochemical reactions involved in the dynamical processes and regulatory interactions within the cellular processor (Raser and O'Shea 2005) serve as an additional stochastic input that influences cellular decision-making (Wilkinson 2009; Balázsi et al. 2011; Zechner et al. 2020). To this end, combined experimental and computational techniques have helped to improve our understanding of the molecular players in the cell's regulatory network.

In this review, we summarize the progress made by the field of mammalian synthetic biology, which adopts the systems biology view of the cell as a dynamical system, to program novel functions into the cellular processor (Khalil and Collins 2010). Synthetic biology applies genetic engineering, mathematical modeling and computational approaches to design and construct genetic circuits that produce predictable cellular outcomes. Many early genetic circuits were developed in bacteria, including the toggle switch and oscillator (Gardner et al. 2000; Elowitz and Leibler 2000). Given that cell state and the inner regulatory network are key drivers of cellular decision-making, the behaviors of synthetic genetic circuits that are transplanted into cells are inevitably shaped by these drivers. Here, we specifically focus on challenges that the mammalian cell context imposes, providing an overview of context effects that have important implications for synthetic genetic device design. We then explore strategies involving control systems approaches towards context-aware device design, with a particular emphasis on applications in cell fate programming.

## Cell fate programming: the promise of stem cells

Owing to two cardinal properties – the ability to self-renew and to give rise to all of the cell types of the body – pluripotent stem cells (PSCs) have generated excitement as a powerful substrate for regenerative medicine. Stem cell potency has been conceptually visualized through the classic Waddington landscape (Waddington 1957). As cells roll down the hills on the landscape, they lose their potential and commit to specialized cell types, which are epigenetically stabilized in valleys that represent their endpoint fate (**Figure 1B**). The ability to reliably control the differentiation, growth and death dynamics of stem cells and their progeny has been a key focus of stem cell bioengineers (Tewary et al. 2018). Cell fate programming of PSCs to clinically relevant cell types has opened the door to new classes of off-the-shelf cell therapies, in which viable cells are implanted into a patient in order to effectuate a medicinal effect. Cellular therapies are a booming biotechnology industry, valued at over $6 billion USD in 2020 and projected to reach a global market share of $9 billion by 2027 (Grand View Research report GVR-2-68038-701-8). They offer an exciting paradigm shift towards the treatment of chronic and acute diseases through the transplantation of living cells and are a compelling example of the clinical implications for mammalian synthetic biology.

Given that cells are dynamical systems whose outputs depend on microenvironmental cues, cell therapies open the door to co-opting the native function of cells to deliver therapeutic function in a context- and site-specific manner while allowing for regeneration of damaged tissues. A prime example of this is the advent of chimeric antigen receptor (CAR) T cells, which demonstrate the ability to devise designer cells on-demand by engineering their function (June et al. 2018). Specifically, CAR T cells are created by genetically engineering autologous (or patient-derived) T cells to express a CAR specific to a target cell, such as B lymphocytes, allowing the engineered T cell to bind to and kill aberrant cells like B cell lymphoblastic leukemia and lymphoma cells. The addition of the CAR to the T cell membrane represents a relatively simple genetic maneuver that has profound impacts on the phenotypic function of the cell by allowing a new environmental input to interface with the T cell's regulatory network. CAR T cells represent the tip of the iceberg for how engineering of the cell's processor can unlock designer cells. Looking forward, synthetic biology will allow for cell therapies to be genetically equipped with new functions – such as the ability to sense and kill cancer cells (Rafiq et al. 2020) – while also offering a strategy for manufacturing allogeneic cell therapies through the efficient directed differentiation of PSCs (Lee et al. 2020; Tewary et al. 2018; Prochazka et al. 2017).

The ability to reliably predict and program cellular decisions is a central goal in mammalian synthetic biology (Kitaada et al. 2018; Prochazka et al. 2017; Ho and Chen 2017; Black et al. 2017; Xie and Fussenegger 2015; Lienert et al. 2014). This capability is critical for both understanding how changes to the cell state and cellular inputs drive cell fate changes, as well as for engineering cell-based therapies. Specifically, reliable programming of cellular functions would have profound implications for our basic understanding of how genetic rules at the single cell level shape the dynamics of multicellular systems, like our tissues and organs. It also opens the door to a new class of engineered cells for therapeutic use, where synthetic genetic devices can be used to encode desired behaviors in cells in a predictable and robust manner, both *in vitro* and post-transplantation (Kis et al. 2015; Kitaada et al. 2018; Tewary et al. 2018).

Despite their promise, PSC-derived cell therapies are not yet in prominent clinical use. A major barrier to the translation of stem cell bioengineering efforts has been our inability to predictably and reproducibly control cell fate changes. This includes challenges in guiding the trajectory of cells as they change from one cell type to another, such as in the conversion of PSCs into specialized cell types, as well as challenges in controlling the cell-cell interactions that shape the outcomes of multicellular populations. Synthetic biology offers a unique opportunity to redirect trajectories of seemingly committed cell fates by opening up new channels and routes on the Waddington landscape (**Figure 1C**). Cell fate control applications exemplify the potential impact of synthetic biology for programming mammalian cells and is featured as a case study in this review.

## Cell fate programming: views from inside and outside of the cell

Recognizing that both environmental inputs and the cellular processor influence cell fate trajectories, cellular engineering has involved both niche and genetic engineering (Tewary et al. 2018). Genetic engineering approaches to cell fate programming represent an "inside-out" approach, where portions of the cellular processor are manipulated: either the receptors and signaling pathways (pathway engineering) or the regulatory networks themselves (regulatory gene network engineering). Niche engineering, on the other hand, represents an "outside-in" approach, where the cellular microenvironment is programmed through the addition of native or synthetic extracellular signals such as cytokines, small molecules, and engineered cellular matrices (**Figure 2**). These environmental cues provide chemical and mechanical inputs into the cellular processor, thus driving phenotype. Indeed, niche engineering strategies that guide the differentiation trajectory of stem cells has been inspired by our expanding knowledge of the spatiotemporal

microenvironmental cues that shape embryonic development, which can be mimicked *in vitro* to give rise to specialized cell types on demand (Keller 2005; Williams et al. 2012; Zhu and Huangfu 2013). Stem cell bioengineering has focused on guiding the trajectory and outcome of these cells as they transition between fates. For example, human pluripotent stem cells (PSCs) can be successfully differentiated to a beta cell state through a 7-stage protocol, where each stage introduces cells to media containing a careful concoction of soluble factors (Rezania et al. 2014). The staged addition of extracellular signals, which act as cellular inputs, can help guide cells on a trajectory of changing cell fate.

Outside-in and inside-out engineering represent complementary approaches, as exemplified by recent advances in cell fate programming through genetic engineering. Indeed, the foundational work of Yamanaka and Takahashi challenged the field's perception of the programmability of the cellular processor by demonstrating the ability of inside-out engineering to break the boundaries of cell fate plasticity (Takahashi and Yamanaka 2006). Through the overexpression of four key endogenous TFs (Oct4, Sox2, Klf4, c-Myc), fibroblasts were reprogrammed to pluripotency, moving cells up the Waddington landscape and allowing them to stabilize in an induced PSC (iPSC) state through the presence of key cytokines in the microenvironment. This technically simple genetic manipulation, which perturbs the expression rates of core pluripotency genes and morphs the Waddington landscape in a way that has not been achieved through niche engineering efforts alone, showcases the power to engineer cell fate by targeting the cellular processor (Del Vecchio et al. 2017; Zhou and Huang 2011; Huang et al. 2007). Reprogramming cell fate through the forced overexpression of key genes unlocked the gateway for inside-out cell fate programming and lays the groundwork for synthetic biology approaches to enter the stem cell bioengineering arena.

The degree to which synthetic biology can be used to program the cellular processor can vary (**Figure 2**). CAR T cells represents an example of pathway engineering, where an engineered receptor interfaces with existing downstream cellular machinery. On the other hand, cell fate programming involves the manipulation of the core regulatory network, representing genetic engineering, allowing us to reprogram the cell's identity – a property that was historically thought to be rigid. Through the development of synthetic biology tools, we have the potential to allow the cell to traverse novel fate trajectories that may otherwise not be achievable through outside-in approaches alone, and to do so in a predictable manner. Future prospects for synthetic biology in mammalian cell programming also include the addition of synthetic regulatory networks (circuits) that allow for novel processing capabilities in cells. A preliminary example of novel cellular states is the derivation of so-called "fuzzy" iPSCs, which were derived through forced overexpression of key TFs. Fuzzy iPSCs have the ability to give rise to cells in all three germ layers while exhibiting the resilience to survive in the absence of cellular neighbors, making them an attractive potential substrate for suspension-based cell manufacturing pipelines (Tonge et al. 2014). While the derivation of fuzzy iPSCs did not involve the use of genetic circuits, the ability to derive a novel PSC state (that has not been observed naturally) through genetic manipulation provides further motivation for the implications of synthetic biology in cell fate programming. Through synthetic biology, inside-out engineering provides an avenue to direct cellular decisions, programming new functions into cells and efficiently acquiring existing and novel target cell states for downstream applications.

# The genetic device as the core unit of synthetic biology

Synthetic genetic devices are the basic dynamical unit that can be used to engineer the cellular processor (**Figure 3A**). Through the application of engineering principles, such as from dynamical systems and control theory (Åström

and Murray 2008; Del Vecchio and Murray 2014), it has been possible to achieve circuits with desired temporal dynamics in gene expression and dose response (Gardner et al. 2000; Elowitz and Leibler 2000). Indeed, a key aspect of synthetic biology is the aim to design and construct genetic circuits by wiring genetic devices together in a manner to achieve desired input/output (I/O) temporal responses (Yosef and Regev 2011; Ang et al. 2013).

Given that the genetic device is the core unit of genetic circuits, careful attention should be paid to its design and characterization. A basic genetic device includes a single transcriptional unit whereby a promoter drives the expression of a coding sequence that is flanked by UTRs. The genetic device is composed of four key dynamical processes: transcription, post-transcriptional regulation, translation, and post-translational regulation (**Figure 3B**). Transcription is the process that generates mRNA from DNA; post-transcriptional changes to mRNA include processes such as mRNA degradation; translation is the process that produces protein from mRNA; and post-translational changes to proteins include processes, such as protein degradation or post-translational modification (*i.e.*, phosphorylation). The rates of each of these processes are shaped by the values of physical parameters that can be used for design (**Figure 3C**). For example, the transcription rate can be tuned by the choice of promoter (Ede et al. 2016; Ponjavic et al. 2006; Haberle and Stark 2018) and terminator (Proudfoot 2016; Cheng et al. 2019), while the translation rate can be tuned by the sequence in the 5' and 3' UTRs (De Nijs et al. 2020) (such as with the addition of binding sites for endogenous miRNAs (Gam et al. 2018; Michaels et al. 2019)). Similarly, protein degradation can be tuned by adding protein degradation domains (Trauth et al. 2019). These choices, being hard-coded in the DNA, represent static design parameters that cannot be manipulated once the genetic device is constructed.

Each of the processes can be further regulated by suitable inputs, which can change with time (**Figure 3D**). For example, the rate of transcription can be dynamically modulated by recombinases (Weinberg et al. 2017) and TFs (Gaber et al. 2014; Kiani et al. 2014; Nissim et al. 2014; Stanton et al. 2014; Li et al. 2015; Donahue et al. 2020; Israni et al. 2021); the rates of mRNA translation or degradation can be modulated by small molecules/aptamers (Yokobayashi 2019), ribosome binding proteins (RBPs) (Wroblewska et al. 2015; Wagner et al. 2018; DiAndreth et al. 2019), and miRNAs (Cottrell et al. 2017; Michaels et al. 2019); and protein degradation and activity levels can be modulated by proteases (Cella et al. 2018; Gao et al. 2018), engineered protein-protein interactions (Langan et al. 2019; Chen et al. 2020), and post-translational modifications (Prabakaran et al. 2012).

Finally, each genetic device has the molecular species it produces as outputs: RNA and protein. These can, in turn, function as input regulators for other genetic devices, allowing circuit designers to wire genetic devices together through output-to-input connections. The elements enumerated above thus serve as basic parts for building genetic devices and regulating their functions, and can be composed together to make sophisticated genetic devices, such as the control systems that we describe later. The degree to which such elements can be composed together depends on the degree of context-dependence in their functions, which we discuss in more detail in the next section.

During the earliest days of the mammalian synthetic biology field, genetic devices were connected in simple ways to derive desired functionality, including oscillators, memory, and digital logic gates (Khalil and Collins 2010; Kitaada et al. 2018). Since then, the field has developed complex circuits composed of increasing numbers of devices that are inter-connected to give rise to more sophisticated functions such as multi-input classification (Xie et al. 2011; Prochazka et al. 2014), cell-cell communication (Johnson et al. 2017; Kojima et al. 2020), and directed development (Guye et al. 2016; Prochazka et al. 2017), among other possibilities (Black et al. 2017; Kitaada et al. 2018).

Given that the genetic device, and the circuits that it constitutes, are embedded in a cell and the cell, in turn, is influenced by its extra-cellular context (**Figure 4**), the properties of a synthetic genetic circuit will often vary with

respect to those initially prescribed. In order to facilitate robust and predictable behaviors of synthetic genetic circuits, design-prototype-test (Khalil and Collins 2010) cycles can be achieved in mammalian cells by applying optimized transfection pipelines that allow for quick and easy multifactorial quantification of device properties (Gam et al. 2019). Specifically, modular cloning is a key tool that enables rapid prototyping of genetic device designs (Lienert et al. 2014). Nevertheless, the design-prototype-test approach can involve lengthy iterative processes due to poorly known context effects, often with poor outcomes wherein a circuit's function is conditioned to specific intra- and extra-cellular contexts. These contexts, however, are difficult to control in most realistic applications of mammalian synthetic genetic circuits. In the next section, we describe known sources of uncertainty coming from the cellular context and introduce solutions proposed to make genetic devices insulated from specific context effects.

# Challenges of context-dependent gene expression in mammalian cells

Applications of synthetic biology to cell therapies, regenerative medicine, and beyond, all critically require key challenges from the mammalian context to be addressed before we can achieve robust and predictable control of cell behavior. Ideally, we could engineer cell behavior like a computer program, stitching together increasingly complex functions and modules until we achieve the desired phenotype. However, this form of bottom-up design, which is a bedrock of other engineering disciplines, is challenged by the unique environments inside and outside of cells and by the properties of the programming substrate itself: nucleic acids. In any engineered system, whether it be mechanical, electrical, or biological, there is always a discrepancy between the desired and actual system behaviors. Most of the reasons for this discrepancy can be classified into three basic types: uncertainty in the values of physical parameters, unmodeled dynamics, and externally acting perturbations that cannot be directly controlled or anticipated. Specific to the biological substrate, uncertainty in the values of physical parameters can be orders of magnitude larger than found in mechanical or electrical systems, the extent of dynamics that remain unmodeled in the design process is substantial, and, most of all, the number and strength of unforeseen external perturbations acting on the engineered system is unprecedented (Del Vecchio and Murray 2014). These external perturbations arise from the *context* (genetic, cellular, and extracellular) in which the genetic device is placed (**Figure 5A**). Each perturbation affects certain rates of the processes within genetic devices (**Table 1**), and thus influences observed emergent behaviors, ranging from the operation of one cell to the phenotype of an entire tissue. In the following sections, we describe in greater detail these perturbations that act on the genetic device by adopting a control systems view of the problem, wherein context perturbations are depicted as disturbance inputs to the synthetic genetic device.

## Genetic context: perturbations from the local DNA environment

As its name implies, the "genetic context" encapsulates the immediate genetic environment of the device (**Figure 5B,C**). Within mammalian cells, there are four main factors to consider with respect to genetic context. The first factor is the genetic substrate. In most cases, genetic devices are encoded in DNA, though it is also possible to encode programs in RNA (Beal et al. 2014; Wroblewska et al. 2015; Wagner et al. 2018). The second factor is the localization of the substrate within the cell. Specifically, DNA-encoded devices are generally integrated into the genome or kept outside of the genome within the nucleus, as in episomes (Ehrhardt et al. 2008). The position within the genome can have substantial effects on gene expression, especially across cell types (Mitchell et al. 2004). RNA-encoded devices may move among the nucleus, cytoplasm, and/or specialized compartments in the cellular membrane, depending on

| Rate | Context effect |
|---|---|
| **Transcription ($\alpha$)** | • Off-target TF activity<br>• Gene copy number<br>• Genomic integration site<br>• Transcriptional resource availability<br>• DNA torsion<br>• DNA epigenetic state<br>• Nearby enhancers/silencers |
| **mRNA decay ($\delta$)** | • Off-target miRNA, ribonuclease, & deadenylase activity<br>• RNA sequence and chemical modifications<br>• mRNA localization<br>• mRNA degradation resource availability<br>• Change in dilution due to cell growth rate |
| **Translation ($\beta$)** | • Off-target miRNa & RBP activity<br>• RNA sequence and chemical modifications<br>• mRNA localization<br>• Translational resource availability<br>• Codon usage<br>• UTR sequences |
| **Protein decay ($\gamma$)** | • Off-target protease activity<br>• Covalent modifications<br>• Protein localization<br>• Protein degradation resource availability<br>• Cell growth rate |
| **PTMs ($\varphi$)** | • Off-target kinase, phosphatase, & ubiquitin ligase activity<br>• Covalent modifications<br>• Protein localization<br>• Protein co-factors |

**Table 1:** Effect of context on gene expression and function

the type of RNA (Beal et al. 2014; Ryder and Lerit 2018). The third factor is how the encoding DNA or RNA is replicated (or not) within the cell and propagated across progeny during cell divisions. Genomically-integrated DNA is naturally replicated with the cell's genome and thus inherited by cellular progeny, but episomal DNA and RNA require special sequences and proteins to be replicated (Beal et al. 2014; Ehrhardt et al. 2008). Finally, the fourth factor is how genetic sequences nearby (or even further away within the genome) interact with the encoded device and its function (Grigliatti and Mottus 2013; Laboulaye et al. 2018; Liu et al. 2015). Here, large-scale genomic architecture, epigenetics, and proximal effects of being near other genes can all affect device behavior.

**Expression stability of synthetic genetic devices.** One of the most common techniques used for prototyping genetic devices is transient transfection, the process of introducing non-integrating DNA or RNA into the cell (Kim and Eberwine 2010). Being *transient*, the DNA or RNA is not replicated by the cell, and can be degraded by nucleases and diluted out during cell division. This lack of temporal stability in the expression of the genetic device can impose challenges for quantifying its steady-state behavior. Transfected DNA or RNA can be made stable through the inclusion of sequences and protein factors in the cell that enable replication. For example, plasmid DNA becomes able to self-replicate through the expression of the large T antigen from the SV40 virus and inclusion of the SV40 origin of replication on the plasmid (Mahon 2011). RNA can self-replicate through expression of non-structural proteins from alphaviruses and inclusion of genetic sequences from those viruses in the RNA (Wahlfors et al. 2000). An important consideration for both of these technologies is that they can have profound effects on the cell's growth and development (Ahuja et al. 2005; Beal et al. 2014), which can feed back onto the device itself and affect its performance through cellular context (explored in detail in the next section). Given that they are not integrated into the genome, episomal DNA and RNA experience limited interactions with other genetic sequences in the cell, which is advantageous for design. Nevertheless, multiple genes or protein coding sequences delivered in one strand of RNA or DNA can still affect each other's expression (Yeung et al. 2017; Wagner et al. 2018; Liu et al. 2017). While typically ignored, plasmid DNA can interact with histones and thus form chromatin (Tong et al. 2006). Thus, more rapid epigenetic effects (Bintu et al. 2016) should not be ignored in transient transfections. Overall, episomal devices can be relatively isolated from the cell's genetic context, but tend to degrade or dilute out after several cell divisions.

To ensure that the DNA on which a genetic device is encoded is stably maintained within a cell over time while not depending on exogenous proteins, genomic integration is widely used. Genomic integration can be achieved through directed DNA repair, recombination, transposition, or viral integration (Smith 2007; Nayerossadat et al. 2012; Yamaguchi et al. 2011; Rutherford and Van Duyne 2014; Duportet et al. 2014), all of which affect the efficiency of integration and ultimately the genetic context of the device. Genomic integration of synthetic devices can introduce significant positional effects, where transcriptional activity in the local DNA environment, such as read-through from upstream transcription (Loughran et al. 2014; Li and Zhang 2019), DNA torsional effects (Yeung et al. 2017; Sevier and Levine 2018), DNA looping (Hao et al. 2019), and the activity of enhancers and silencers (Liu et al. 2015) interfere with the prescribed function of the genetic device (**Figure 5C**). Transposons and viral integration strategies generally lead to semi-random integration of exogenous genetic materials (Yant et al. 2005; Staunstrup et al. 2009; Vranckx et al. 2016), with little to no control over the genetic position of the device in the mammalian cell genome. In an effort to overcome positional effects, recent developments in genetic engineering technologies have enabled more predictable control over integration sites of exogenous genetic material, including the landing pad DNA integration platform (Duportet et al. 2014; Gaidukov et al. 2018) and targeted CRISPR-based approaches for insertion into "safe harbor" loci, which experience reduced epigenetic silencing and ensure the device does not interfere with core cell functions such as cell cycle regulation (Papapetrou and Schambach 2016). Thus, while delivery of genetic devices via such random integration methods is often more efficient than targeted integration, the expression and regulation

of the devices will have a larger dependence on location of integration, as seen from bacterial studies (Segall-Shapiro et al. 2018). Device performance will vary from cell to cell and among multiple integrated copies of a device per cell (Jordan et al. 2001).

**Epigenetic modifications in the synthetic device locale.** Another critical facet of eukaryotic genetic context is the local epigenetic state, including DNA methylation, histone tail modification, chromatin spreading and compaction, and DNA coiling, all of which can interfere with gene expression and regulation, even to the point of gene silencing (Allis et al. 2007; Allis and Jenuwein 2016; Yeung et al. 2017). Protection mechanisms against the local genetic environment have been proposed in the form of insulator elements, which aim to decouple the activity of genes from inappropriate transcriptional signals (West et al. 2002; Liu et al. 2015) or block epigenetic silencing (Müller-Kuller et al. 2015). Despite these advancements, genetically integrated devices cannot be completely decoupled from their local DNA context. To enable this decoupling, the creation of an artificial chromosome has been posited as a potential carrier for embedded synthetic genetic devices (Lienert et al. 2014; Prochazka et al. 2017), though such a system has not yet been developed. These advancements in genetic engineering technologies provide strategies to help overcome perturbations from the genetic context by physically shielding the device from these anticipated effects.

**Copy number variability.** For both episomal and integrated genetic devices, a significant source of cell-to-cell variability comes from differences in the copy number of genetic devices delivered per cell. For example, transient transfection leads to large differences in plasmid delivery, with some cells receiving just one plasmid that then quickly dilutes out, and other cells receiving upwards of a thousand plasmids (Bleris et al. 2011; Jones et al. 2020). This variation comes from the method of preparing nucleic acids for delivery as well as from how cells receive them. Though it can impose challenges, the variance in genetic device delivery by transfection also allows for the exploration of device behavior at different copy number regimes and part stoichiometries using quantitative single cell analysis pipelines (Gam et al. 2019). Thus, variance can be a source of both frustration as well as information about genetic device design that should be minimized where needed and exploited where possible. For viral integrations, the copy number of device integrations is determined by the multiplicity of infection (MOI), which represents the number of infectious viral particles per cell during transduction. The distribution of copy numbers across cells can be well-modeled with a Poisson distribution (Ailles et al. 2002; Prasad et al. 2011), where the coefficient of variation (standard deviation divided by mean) decreases as a function of the MOI. Thus, high MOI infections can be used to ensure high integration efficiency, reduce noise from copy number variation, and also reduce the relative impact on expression of any one integration position. However, the use of high MOI transductions has the trade-off that high copy numbers of the genetic device increases demand for limited cellular resources and can be expected to increase toxicity.

**The evolving DNA substrate.** The evolving nature of DNA also contributes to genetic context effects, where random mutations to DNA-based devices can cause them to evolve over time and lose functionality (Liao et al. 2019) or cause unexpected, environmental-dependent discrepancies in their behavior (González et al. 2015). Overall, activity and change in the local genetic context imposes perturbations to the performance of the engineered genetic device. These perturbations can then impact the temporal stability and predictability of the I/O response of a genetic device while also creating cell-to-cell heterogeneity in device function. While such mutations may be useful for directed evolution experiments, they can be detrimental to forward design and necessitate sophisticated strategies to maintain circuit function (Liao et al. 2019).

**Cell fate programming and the dynamic genetic context.** Large-scale cell state changes that accompany movements between cell types often involve the overexpression of TFs (Jopling et al. 2011), which drive heightened transcriptional activity. This may increase the perturbations caused by genetic activity nearby the synthetic device, such

as read-through or physical torsional effects. As the cell undergoes fate transformation, the epigenetic state of the cell will also change. Changes to the epigenetic landscape impact DNA accessibility, influencing the ability to target loci for stable integration of synthetic devices into the genome. The epigenetic changes that accompany cell fate programming may also result in silencing of genetic devices inserted into the genome in the starting cell state. Indeed, this has been used as a hallmark of successful cell fate transition in the context of somatic cell reprogramming (Takahashi and Yamanaka 2006), indicating that the final cell state is stabilized in a manner that is independent of exogenous TF expression.

## Cellular context: hidden interactions with the regulatory hairball

Synthetic genetic devices are situated within the cellular processor, whose state is shaped by molecular players (DNA, RNA, and protein) that engage in dynamical regulatory interactions with one another in the cell, often described as a hairball due to its highly interconnected nature (Yan et al. 2016) (**Figure 5A**). The complex nature of this regulatory hairball makes it fertile ground for unexpected or "hidden" interactions among synthetic genetic devices and the cellular processor. These hidden interactions are not specified by the device's design but rather originate from uncontrolled and unmodeled mechanisms. Although there are myriad hidden interactions, here we focus on those that can be grouped into one of the following two categories: *direct* interactions with the cell state and other devices and *indirect* interactions with the cell state and other devices, where "other devices" can be both endogenous and synthetic. The first group includes effects such as unknown cross-reactivity with molecular species within other devices or in the cellular processor, direct ways in which the cell state affects the genetic device, and direct ways in which the genetic device affects cell state. The second group includes issues of resource sharing, growth inhibition, and retroactivity. In either case, they can be represented within a block diagram formalism as input disturbances to the genetic device, which cause deviations from expected device behavior (**Figure 5B**).

**Direct interactions with the cell state and other devices.** In the human genome, there are tens of thousands of genes encoding proteins and functional RNAs (Pertea et al. 2018). For many of these genes, there are numerous transcript variants that can be generated via different transcription start sites, alternative splicing, and RNA modifications (Pertea et al. 2018; Grosjean 2005; Fisher and Beal 2018). In each different cell type, a unique combination of these RNA variants are expressed, ultimately producing the protein and RNA regulators that define the given state of the cell. These regulators affect the level of cellular resources that the genetic device uses as well as the state of the DNA or RNA on which it is encoded. For example, changes in cell state often drive large-scale changes to chromatin, which among other things affects the ability of TFs to accomplish their intended functions by limiting their access to DNA (Cheedipudi et al. 2014). Thus, cell state changes can severely affect biochemical parameters describing transcription and translation rates within the synthetic genetic devices. These devices can, in turn, also affect cell state by design, such as through reprogramming cellular identity (Takahashi and Yamanaka 2016). In these cases, hidden interactions can emerge between the device and the cell processor.

Genetic devices can further interfere with cell state through unwanted activation of the cell's innate immune responses (Kitaada et al. 2018). Many of the genetic devices used in mammalian cells are composed of DNA, RNA, and proteins derived from non-human sources, such as bacteria and yeast. The use of bacterial proteins in mammalian cells has been reported to elicit an immune response (Prochazka et al. 2017). To get around this problem, recent efforts in synthetic biology have aimed to develop humanized genetic devices, composed of parts that are designed based on bacterial counterparts without containing their components. For example, a recent report developed a CRISPR/Cas-

inspired RNA targeting system that allows for RNA editing, degradation, or translation (Rauch et al. 2020). Further developments in this space can help to overcome interference from the innate cell response following synthetic device operation.

A key property of synthetic genetic devices is that the intended input and output regulators are designed to be orthogonal to the cellular processor and to other genetic devices. Orthogonality requires that (1) regulatory outputs of a genetic device do not directly regulate endogenous genes unless specified by design, (2) genetic devices are not regulated by endogenous genes unless specified in the design, and (3) regulatory outputs of one synthetic genetic device are inputs only to a specified set of devices and not to others. Collectively, we refer to unexpected regulatory interactions among synthetic and cellular genetic devices as "off-target" interactions, which challenge orthogonality (**Figure 5B**). Off-target interactions can significantly affect genetic device function and the expression of natural genes (Rowland et al. 2012; Singh et al. 2016; Meyer et al. 2019). Orthogonality is difficult to achieve within cells because most molecular species have the chance to come in contact with each other throughout their lifetimes. Thus, much work has gone into finding and engineering regulators that work orthogonally to one another and to the cell's processor (Gaber et al. 2014; Stanton et al. 2014; Li et al. 2015; Weinberg et al. 2017; Cella et al. 2018; Gao et al. 2018; Gam et al. 2019; Langan et al. 2019; DiAndreth et al. 2019; Chen et al. 2020; Donahue et al. 2020).

The eukaryotic cell context presents a further contextual challenge due to the non-homogeneous distribution of cellular parts and resources in cellular compartments, often requiring shuttling of components for synthetic genetic parts between the nucleus and cytoplasm. As a result, design of exogenously expressed parts needs to consider additional requirements to ensure their correct spatial localization following expression (Alberts et al. 2014; Barajas and Vecchio 2020). For example, nuclear localization signals and nuclear export signals are used to direct proteins into and out of the nucleus, respectively. However, hidden interactions may interfere with mechanisms that regulate spatial arrangement of parts, leading to their incorrect placement in the cell. A lack of spatial compartmentalization within the nucleus and cytosol may also increase off-target interactions, allowing for gene regulators to interact with DNA, RNA, and proteins indiscriminately, with the probability of interaction dependent on the strength of binding.

**Indirect interactions with the cell state and other devices.** Another way in which genetic devices affect cellular state is by indirectly impacting the survival and growth dynamics of the cell, which in turn affects the dynamics of the devices, creating another feedback loop that is challenging to analyze (**Figure 5B**). For example, overexpressing genes that demand large amounts of the cell's resources can be detrimental to cell viability (Berger et al. 1992; Gilbert et al. 1993; Baron et al. 1997; Lin et al. 2007). For cells that survive, selection against resource-demanding genetic devices can cause changes to the cell processor or to the genetic device itself (González et al. 2015; Gouda et al. 2019). Due to resource depletion, where the cell lacks sufficient resources for its housekeeping processes, resource loading can reduce cellular growth rates, which has been observed in both bacterial (Ceroni et al. 2015) and mammalian cells (Jones et al. 2020). Cell cycle has been shown to drive transcriptional noise and stochasticity (Zopf et al. 2013) and more recently cell cycle length has been suggested as a mechanism to control the transcription of genes based on their sequence length (Chakra et al. 2020). As a consequence, any perturbations to cell proliferation dynamics will also indirectly affect transcriptional regulation. At a more global level, molecule dilution due to cell division impacts the decay rate of all molecules, which dictate key temporal dynamics of genetic devices (Del Vecchio and Murray 2014). Hence, genetic device-induced growth rate changes globally influence the function of both cellular and synthetic genetic devices, causing a feedback loop that is very difficult to untangle (**Figure 5B**).

Indirect interactions also arise due to problems such as competition for shared factors in the cell. One such way that this manifests is through competition for broadly-shared gene expression resources (which has been noted

in both mammalian and bacterial cells), such as transcriptional machinery (Courey 2008; Jones et al. 2020), post-transcriptional processing factors (Grimm et al. 2006; Castanotto et al. 2007; Boudreau et al. 2009; Munding et al. 2013), translational machinery (Gyorgy et al. 2015; Frei et al. 2020a), and factors affecting protein stability (Lobanova et al. 2013). Individual genes and proteins can also compete for shared regulatory inputs, such as gRNAs competing for shared pools of dCas9 to regulate target promoters (Fontana et al. 2018; Zhang and Voigt 2018). Even with a well-designed genetic device that operates orthogonally to the cell processor and uses individual components that are themselves orthogonal to one another, resource competition can induce unintended changes in gene expression and disrupt the function of genetic devices and circuits (Del Vecchio 2015; Qian et al. 2017; Sabi and Tuller 2019; Jones et al. 2020; Frei et al. 2020a).

In most cases, resource loading by one gene reduces the expression level of all other genes through the sequestration of factors needed for transcription and translation. In bacteria, the most limiting resource is the ribosome, with RNA polymerase also being important (Gyorgy et al. 2015). In mammalian cells, transcriptional resources appear to be most limiting (Jones et al. 2020), though RNA-level effects have been observed in several cases (Munding et al. 2013; Frei et al. 2020a). However, production resources are not the only factors that genes can compete for. As with changes in cell growth rate, competition for RNA or protein degradation machinery can influence gene expression dynamics, thereby altering genetic device behavior (McBride and Vecchio 2017; Cookson et al. 2011). A special case of resource competition comes from competition among genes (potentially both synthetic and natural) for a shared gene regulator. Specifically, when a TF binds to target DNA, the temporal dynamics of the free TF concentration changes and leads to significant alterations in the behavior of genetic devices regulated by it. This phenomenon is referred to as retroactivity (Del Vecchio et al. 2008), and is typified by the addition of downstream genetic devices disrupting the behavior of upstream modules. Retroactivity is caused by the sequestration (loading) of an output molecule by multiple downstream targets. This can cause effects ranging from changing the bias of bistable switches to going so far as to destroy sensitive temporal behaviors such as oscillations (Del Vecchio et al. 2008; Jayanthi et al. 2013; Lyons et al. 2014; Mishra et al. 2014; Pantoja-Hernández and Martínez-García 2015; Menon and Krishnan 2016). Inevitably, genetic devices that share the same regulatory inputs become indirectly coupled together through resource loading while genetic devices with multiple outputs experience retroactivity.

All of the above described interactions lead to disturbance inputs to the genetic device (**Figure 5B**). Since unaccounted for and largely unknown, these disturbances dramatically reduce device predictability in the mammalian context. Many off-target interactions are expected to result from our incomplete understanding of the physical properties of the molecular players that compose the cellular processor, as well as of the reactivity between them (Zechner et al. 2020). To this end, emerging synthetic biology tools can help to elucidate the regulatory hairball (explored in a later section).

**Cell fate programming and the dynamic cell state.** The dynamic nature of the transcriptional and epigenetic environment during cell fate conversion imposes the additional complexity of time-varying context effects. As such, the degree and nature of perturbations to the synthetic device's performance can be expected to be dynamic in nature. In fact, epigenetic changes that allow cells to exit their identity and stabilize as a new cell type during cell fate programming may result in the silencing of genes that were relevant and accessible in the starting cell state but not in the new attained state (Plath and Lowry 2011; Teshigawara et al. 2017). Further, genetic devices that affect cell state will directly alter cellular context itself. Competition for resources, undesirable interactions among genes, burdens imposed on cell growth, and alterations in cell-cell signaling may all vary depending on the state or type of the cell. Just as the changing cell state impact device function, so too do synthetic devices have unintentional impacts on cell

state, particularly in cell fate applications. Indeed, it is understood that the derivation of PSCs through reprogramming, as well as their culture *in vitro* can result in mutations and genetic/epigenetic abnormalities (Hussein et al. 2011; Lund et al. 2012; D'Antonio et al. 2018; Merkle et al. 2017).

## Extracellular context: niche interactions and population effects

Mammalian cells naturally live in multicellular societies, where they are regularly engaging in interactions with neighboring cells and receiving microenvironmental cues. Since the state of the cellular processor is shaped by microenvironmental signals, the behavior of a synthetic genetic device that is transplanted into the endogenous context is inevitably also influenced by signals from the cellular niche. The impact of microenvironmental cues on the changing cell state thus indirectly affects synthetic devices (**Figure 5B**).

The microenvironmental context also offers challenges for properly testing and characterizing synthetic genetic devices. In particular, there is a growing awareness that multicellular populations are not a sum of their parts and thus careful quantification of underlying single cell dynamics is required when measuring the function of synthetic devices. Just as the microenvironmental context can impact the behavior of synthetic circuits, so too can synthetic devices drive changes to the growth and death dynamics of multicellular populations. This is increasingly relevant for mammalian cell system, where cell-cell killing has recently been uncovered , driven by the differential expression of key "fitness" genes, such as Myc (Clavería et al. 2013; Sancho et al. 2013; Dejosez et al. 2013; Díaz-Díaz et al. 2017). Thus, if synthetic devices perturb the genetic fitness landscape of the cell – perhaps through direct or indirect regulation of fitness genes – they may drive the selection of a subset of cells. By impacting cell fitness, synthetic devices can also drive changes to a cell's proliferation rate and metabolic profile (Lawlor et al. 2019), thus driving changes to cell state. In turn, the synthetic devices can then drive the clonal dynamics of the multicellular population and lead to unanticipated consequences.

The impact of genetic manipulations on mammalian cell survival dynamics have not yet been thoroughly explored. In fact, the insertion site of the device itself in the genome has been shown to influence the growth dynamics of the engineered cells, possibly interfering with the function of oncogenes and leading to preferential growth of a subset of clones (Maldarelli et al. 2014; Wagner et al. 2014). There is growing evidence of this in the context of CAR-T cells, where lentiviral insertion site of the exogenous CAR has been shown to impact the preferential survival and dominance of a subset of clones (Shah et al. 2019; Nobles et al. 2019) and specific knock-in to the *TRAC* locus can improve anti-tumor therapy (Eyquem et al. 2017). The relationship between integration site and clonal dominance has also been reported in the context of retrovirus-mediated gene therapy (Hacein-Bey-Abina et al. 2003). Lentiviral integration has also been reported to induce changes in alternative splicing patterns and lead to aberrant transcripts (Moiani et al. 2012), while also causing insertional mutagenesis (Bokhoven et al. 2009). Thus, the synthetic device and endogenous regulatory network of the cell influence each other's states and introduce perturbation, both intentional and accidental. By influencing clonal dynamics, synthetic devices can thus shape the multicellular context and bias experimental measures of their own performance.

**Cell fate programming and the evolving multicellular society.** An additional layer of complexity for cell fate programming applications arises from the dynamic nature of multicellular populations, particularly PSCs and their derivatives. Initiatives in regenerative medicine to direct differentiation and reconstruct functional tissues yield systems with complex cell-cell and cell-niche interactions that can be difficult to model and can affect genetic device

function. Cell division is a common occurrence in cell fate programming applications, including reprogramming and differentiation to specialized cell types. Individual cells undergoing fate programming can expect to give rise to progeny through the process, which introduces extracellular context effects. Indeed, the mammalian cell biology and stem cell fields have recently recognized that seemingly homogeneous populations of cells engage in active competition with one another, leading "fitter" cells to eliminate their seemingly weaker neighbors (Shakiba and Zandstra 2017; Baker 2020). This observation has gained particular attention in PSC populations, where mouse PSC have been shown to engage in active elimination of one another both *in vitro* (Sancho et al. 2013; Díaz-Díaz et al. 2017) as well as during embryonic development (Hashimoto and Sasaki 2019; Clavería et al. 2013; Dejosez et al. 2013). Through interactions with neighbors, cell competition drives changes to the individual cell's state, triggering proliferation or death, as well as stem cell differentiation or extrusion. In fact, competitive interactions have been shown to drive the dynamics of cell populations during cell fate programming, where an elite subpopulation of cells that is primed to undertake the fate transition is selected for and dominates the cell population (Shakiba et al. 2019).

In addition to elite cell subpopulations that overtake multicellular populations undergoing fate transitions, neutral selection also plays a role in driving the growth dynamics of stem cell populations, where some clones overtake the cell population simply by chance. Indeed, neutral drift has been shown to play a role in tissue homeostasis *in vivo* and can be expected to impact *in vitro* systems as well (Krieger and Simons 2015). The local microenvironmental context of cells undergoing cell fate programming is therefore dynamic and the nature of perturbations to the synthetic device's performance will be dependent on the cues received from neighboring cells. Similarly, cell therapies that are genetically engineered to carry synthetic devices, such as CAR-T cells, will also face changes to their microenvironment as they are transitioned to *in vivo* environments in patients, bringing on substantial unknowns in terms of extracellular context. Clearly, cell fate programming applications that aim to guide the movement of cells on the Waddington landscape cannot ignore the effect of multicellularity and the extracellular context in influencing the cell's trajectory, and thus the ability to predictably control this (**Figure 1A**).

## Synthetic biology tools for resolving context dependency

Through the development and application of technologies for tracking the intra- and extra-cellular context, synthetic biology tools have facilitated efforts to reverse engineer the structure and function of the cell's processor as well as its interfacing inputs (Lienert et al. 2014; Mathur et al. 2017). For example, the development of libraries to tune endogenous gene expression (Jost et al. 2020) can provide new insights into the dose-dependent role of genes in driving cell fate decisions, as well as the regulatory interactions between genes.

Analogously, DNA-based devices for lineage tracking are powerful tools for understanding how our synthetic genetic circuits influence cell state, as well as how growth and death dynamics influence our interpretations of circuit function during testing (Shakiba et al. 2019; Gerrits et al. 2010). Clonal tracking using DNA barcoding (Kebschull and Zador 2018) has revealed that the growth and death dynamics of the clones that compose multicellular mammalian population may differ greatly, thus leading a subset of the clones to drive the dynamics of the overall population (Shakiba et al. 2019). This is an important consideration when interpreting experimental data during testing of synthetic device performance in cells, where population-averaged measurements may not provide an accurate picture of the device performance in individual cells. This is particularly the case if neutral or elite clonal dynamics in the culture give rise to a subset of dominant clones overtaking the population. Lineage tracking strategies are also powerful tools for understand whether the presence of a synthetic device influences cell fitness, which leads to the elimination or

selective propagation of a subset of cells in culture. By comparing the clonal dynamics of engineered cells that carry our synthetic circuits with wild-type cells that do not, we can assess the impact of the synthetic device on cell survival, proliferation, and cell-cell interactions.

When coupled with temporal single cell gene expression analysis, lineage information can be used to better understand the trajectory of cell state changes of individual cells (Weinreb et al. 2020). Recent advancements in genetic engineering technologies, with the discovery of CRISPR/Cas gene editing tools (Mali et al. 2013; Cong et al. 2013), has also enabled the development of DNA-based event recorder technologies, such as: mSCRIBE, in which two self-targeting gRNAs are used to record activity of the NF-kB inflammation pathway in mouse cells (Perli et al. 2016); CAMERA, which can record temporal Wnt activity of human cells in a cell-embedded DNA tape (Tang and Liu 2018); DOMINO, which also records order information of environmental stimuli (Farzadfard et al. 2019); TRACE, which allows simultaneous recording of multiple temporal stimuli in bacteria (Sheth et al. 2017); and SENECA, which enables transcriptome-scale molecular recording in bacteria (Schmidt et al. 2018). DNA-based recording devices like these open the door to unraveling the cellular hairball by allowing for temporal tracking of molecular species (such as mRNAs) within the cell's regulatory networks.

These expanding synthetic biology toolsets have catalyzed efforts to reverse engineer the cellular processor and its inputs. Undoubtedly, a more complete understanding of the cellular system will help to better anticipate perturbations to genetic device performance, allowing for synthetic genetic circuit designs to be well-informed of cellular and extracellular context effects. Nevertheless, the lingering unknowns of the mammalian cell context, as well as the stochastic features of the cellular processor, continue to challenge robust and predictable synthetic circuit design.

# Context-aware device design

Context-dependent effects across the genetic, cellular, and multicellular scales serve as perturbations to synthetic devices that seek to regulate gene expression. This necessitates the development of genetic devices that can correctly parse inputs to outputs and drive cellular behavior regardless of context. We refer to these classes of devices as "context-aware", as their design accounts for the physical sources of context effects. Though there are many sources of context-dependence, we can abstract their effects on a given genetic device by considering the effect of the contextual factors on basic gene expression processes: transcription, post-transcription (*i.e.*, mRNA decay or modification), translation, and post-translation (*i.e.*, protein decay or modification). This framework simplifies the view of context in a way that also conveniently aligns with the conception of modular genetic devices as elements in which we can modulate one or more of these rates (**Figure 3**). Thus, to build devices that are robust to specific context factors, it is necessary to desensitize the devices to either the factors themselves or to the parameters that are affected by such factors. A primary method to approach this problem is through the design of genetic controllers that, through their topology and operating regimes, desensitize genetic device output to certain parameters that are affected by disturbance inputs. Indeed, robust systems have been built through model-driven design that considers the effect of context factors on system function. Here, we discuss genetic controllers and how they can be applied to insulate genetic device function from context.

## Basic principles of genetic controllers

A controller is a module that is connected to and regulates the behavior of a system, typically to enforce a desired output despite perturbations (disturbances). Controllers can generally work via *feedforward* or *feedback* actuation (**Figure 6A**). In natural regulatory networks, both feedforward and feedback control motifs have been observed to impart high robustness and adaptation to disturbances (Barkai and Leibler 1997; Becskei and Serrano 2000; Yi et al. 2000; Ma et al. 2009; Sturm et al. 2010; Ferrell 2016; Araujo and Liotta 2018; Nunns and Goentoro 2018). A feedback controller measures the system's output, compares it to a desired output, and actuates an input to the system to decrease the measured discrepancy between the desired and actual output. By contrast, a feedforward controller directly senses disturbances and actuates an input to the system to compensate for the expected effect of these disturbances on the output. To implement effective feedforward control, it is thus necessary to have a sufficiently descriptive model of how the disturbance affects the output. Feedback controllers do not have such a requirement; however, they must be carefully designed to ensure that the closed loop system is stable (Åström and Murray 2008).

To enforce a desired output regardless of disturbances, controllers work by minimizing the effect of such disturbances on the output (Del Vecchio and Murray 2014; Del Vecchio et al. 2016). In the simplest and most common use-case, biological controllers can be used to enforce a constant level of a protein of interest. Specifically, we will say that the controller achieves robustness (adaptation) to a given disturbance if, upon presentation of such a disturbance, the level of the protein is able to reach, after some time, a value close to the pre-disturbance value. If the residual error is exactly zero, the controller is said to achieve perfect adaptation to the disturbance (**Figure 6B**) (Del Vecchio and Murray 2014). Perfect adaptation is possible with both feedforward and feedback controllers (Ma et al. 2009; Araujo and Liotta 2018). In what follows, we review basic feedback and feedforward control design principles to achieve high robustness and provide examples of how these have been implemented in either prokaryotic or eukaryotic cells.

**Feedback controllers for robustness.** At a high level, feedback controllers that regulate the level of a protein of interest work by measuring the difference between the actual and target concentration (*i.e.* the error) of the protein, then increasing or decreasing the production or decay rates of the protein as needed. A common and relatively simple way to achieve robustness to some disturbance is the use of proportional, high-gain, negative feedback (Del Vecchio and Murray 2014; Åström and Murray 2008), in which the degree to which protein production rate is adjusted is proportional to the error. For the sake of illustration, the concept of high-gain feedback can be explained by the following simple differential equation describing the rate of change of the level $X$ of a protein of interest:

$$\frac{dX}{dt} = G(u - X) + d - \gamma X,$$

in which $d$ represents a disturbance in the protein's production rate and $\gamma$ is the protein decay rate constant. Here, $u$ can be regarded as a desired protein level (*i.e. set point*) and $G(u - X)$ represents the control action with gain $G$, which is proportional to the error between desired and actual protein level. The controller action increases production of the protein of interest if $X < u$ and decreases it if $X > u$, thereby compensating the effect of $d$. In particular, at steady state, if $G \gg \gamma$, the error ($e$) due to the disturbance is given by:

$$e = |u - X| \approx d/G,$$

which can be made arbitrarily small as $G$ is increased, hence the name as "high-gain" feedback. More sophisticated and non-linear variations of high-gain feedback are possible and have been used to design insulation devices and load

drivers in both bacterial and eukaryotic cells to insulate a TF output from loads applied by its downstream targets and non-specific DNA binding (Jayanthi and Del Vecchio 2011; Nilgiriwala et al. 2015; Mishra et al. 2014). Specifically, the eukaryotic load driver implementation (Mishra et al. 2014) uses post-translational protein modification, *i.e.*, phosphorylation and dephosphorylation, in order to create a high-gain post-translational feedback controller that achieves adaptation to the presence and amounts of the output protein DNA binding targets.

High-gain feedback allows for increased robustness as the controller gain increases, but cannot achieve exactly zero error at steady state. Zero steady state error, and hence perfect adaptation, is reachable by the use of integral feedback (Åström and Murray 2008; Del Vecchio and Murray 2014) (**Box 1**, **Figure 7**). To implement integral feedback, a controller species ($Z$) is introduced such that its concentration acts as a molecular "memory" variable, where accumulation of $Z$ implies a deviation of the protein of interest from target expression levels. Specifically, the resulting control action $z$ is proportional to the integral of the error:

$$e = (u - X), \quad z \quad = k \int_0^t e(\tau) \cdot d\tau,$$

in which $k > 0$ is a control gain, which modulates both the absolute degree of discrepancy between achieved and desired protein levels as well as the temporal persistence of this discrepancy.

The simplest mathematical model of this control strategy can be captured by the following two differential equations:

$$\frac{dX}{dt} = Z + d - \gamma X, \quad \frac{dZ}{dt} = k(u - X),$$

in which the production rate of $X$ is "actuated" by the concentration of the "memory" molecule $Z$. If $u$ and $d$ are both constant with time, the system achieves steady state (*i.e.*, the rate of change of the concentrations practically reaches zero), when $X = u$, which is completely independent of the disturbance $d$. The memory of past error, accumulated in the concentration of $Z$, critically enables an integral controller to eliminate steady-state error and thus achieve perfect adaptation. While feedback controllers require an integrator to achieve perfect adaptation, feedforward controllers can do so without explicitly creating an integrator (Mangan and Alon 2003; Del Vecchio et al. 2016), although it can be shown that a "hidden" integrator is present in their structure (Shoval et al. 2011).

Feedback controllers can, in principle, achieve robustness to those perturbations that affect the genetic device *within or upstream*, but not downstream, of the feedback loop created by the controller (**Figure 6C**). A significant benefit of feedback control over feedforward control is the ability to achieve robustness to uncertainty affecting the controlled system itself. This uncertainty can include uncertain parameters, noise, and unmodeled dynamics (Åström and Murray 2008; Del Vecchio and Murray 2014).

An important consideration in feedback controller design is the way in which the set point $u$ enters the controller. If this way is itself affected by a disturbance $d$, the controller may not be able to adapt to $d$. For example, the bacterial antithetic small RNA (sRNA) feedback controller, which senses an output TF ($X$) and then produces an sRNA molecule to sequester and degrade the TF's mRNA transcript (Huang et al. 2018), can achieve almost perfect adaptation to disturbances affecting the translation of the output protein, such as from changes in the availability of ribosomes. However, because the input $u$ is a transcription rate, this design cannot desensitize the output to changes in TX resources without also desensitizing the response to $u$. To avoid the latter problem, the design employed an amplification of the transcription rate by promoter tuning, which ensures the system output responds to $u$, but also sensitizes it to transcriptional perturbations. Similar problems can appear in other feedback controllers, so the model

should always be examined in detail to ensure that the critical parameters to which robustness are needed do not feed into $u$.

**Feedforward controllers for robustness.** Feedforward controllers are commonly constituted by a molecular species that offsets the effects of disturbances on the output of a regulated device. This is achieved by sensing such disturbances in a manner that is equivalent to how they affect the device output, then eliciting a proportional negative regulation of the device's output. At a high level, feedforward controllers can be implemented by either enhancing the decay rate or by inhibiting the production rate of the target molecular species in the genetic device, typically the mRNA or protein species (Del Vecchio and Murray 2014; Shoval et al. 2011). The simplest differential equation model describing the first mechanisms is given by

$$\frac{dX}{dt} = udK - ZX - \gamma X, \quad \frac{dZ}{dt} = dK - Z,$$

in which $d$ can be regarded as a perturbation of transcription rate, such as due to varying DNA copy number or to variable transcriptional resources, which affects both the device and controller in the same way. Indeed, if $K$ is sufficiently large, we obtain that at steady state $X \approx u$, independent of the disturbance $d$, reaching almost perfect adaptation. Implementations of this principle have appeared in mammalian cells in order to make the expression level of a protein robust to variation in DNA copy number (Bleris et al. 2011; Jones et al. 2020) or to resource loading (Jones et al. 2020; Frei et al. 2020a). In both cases, the feedforward actuation enhances the decay rate of the output protein's mRNA, via either a miRNA (Bleris et al. 2011; Frei et al. 2020a) or an endoribonuclease (Jones et al. 2020).

A simple model of a feedforward controller implemented by inhibition of production rate of the output protein is given by

$$\frac{dX}{dt} = \frac{ud}{1+Z} - \gamma X, \quad \frac{dZ}{dt} = dK - Z,$$

in which if $K$ is sufficiently large, for non-zero $d$, we obtain that at steady state $X \approx u$, independent of the disturbance $d$, thereby also reaching almost perfect adaptation. This mechanism was implemented in both bacterial (Segall-Shapiro et al. 2018) and mammalian (Bleris et al. 2011) cells to obtain adaptation of protein expression levels to variation in plasmid copy number. In this design, $Z$ is a transcriptional repressor of $X$ expressed from the same plasmid as $X$ in a manner that ensures the same disturbance $d$ affects both the device and the controller proportionally. The requirement for $Z$ to repress the production of $X$ without cooperativity (*i.e.*, with power 1 in the equation) has made it difficult to implement feedforward control with transcriptional regulators (Bleris et al. 2011; Segall-Shapiro et al. 2018), which frequently bind to DNA in a cooperative manner (Zhang et al. 2013).

A feedforward controller can impart robustness to disturbances that the controller can sense (**Figure 6D**). Compared to feedback controllers, feedforward controllers have a reduced risk for instability. However, unlike feedback controlled systems, feedforward-controlled systems are, in general, not robust to parameter variability within either the controller or controlled system.

## Limitations to controller implementations in biological systems

Well-designed controllers can, in principle, allow perfect adaptation to disturbances; however, there are challenges for implementation in living cells. One major hurdle is the decay ($\gamma$) of controller species via degradation and dilution,

which for integral feedback controllers creates a leakiness in the integrator function:

$$\frac{dZ}{dt} = k(u - X) - \gamma Z,$$

This decay can arbitrarily worsen the adaptation performance. Performance can be rescued by ensuring that the controller reactions implementing the integrator are sufficiently faster than molecule decay, effectively making the decay term negligible, leading to "quasi-integral" control (Qian and Del Vecchio 2018) (see **Box 1**).

While integral feedback control can guarantee adaptation to constant disturbances, real perturbations are often time-varying. In this case, proving disturbance rejection is substantially more challenging but it is possible under suitable assumptions for time-varying disturbances that operate on a slower timescale than the controller reactions (Qian and Del Vecchio 2019). There are also tradeoffs between the rate of convergence to steady-state and the resulting steady-state error of the output relative to the set point (Olsman et al. 2019).

A common limitation in the implementation of feedback controllers is that the sensor of the output species $X$ or $M$ is not necessarily the actual output protein or RNA, and thus is only a proxy for the true output $Y$. If $X$ and $Y$ are both proteins, such a proxy can be obtained in mammalian cells by 2A-linking (Szymczak et al. 2004) $X$ and $Y$ or using an IRES (Hellen and Sarnow 2001) to separate their coding sequences, both of which ensure that $X$ and $Y$ are translated into separate peptides from the same RNA species. In bacteria, this can be done more simply by placing both coding sequences within an operon (Qian and Del Vecchio 2018; Aoki et al. 2019). Co-transcription without co-translation may cause the levels of the two proteins to become decoupled and respond differently to disturbances, therefore hampering the controller performance. This can be ameliorated by fusing $X$ and $Y$, though the feasibility of doing so is confounded by the potential for fusion to disrupt the functions of $X$ and/or $Y$. Additionally, fusions are not useful when the species must occupy different compartments inside or outside of the cell.

Feedforward controller implementations also have important limitations. Since feedforward controllers require separation between the control and output branches, they do not provide any robustness to disturbances that disproportionately affect one branch over the other. For example, if any off-target interactions affect the output branch but not the control branch, then the control branch will not be able to compensate. Thus, feedforward controllers must be carefully designed, down to the sequence level, to capture as many relevant disturbances as possible. They are also generally quite vulnerable to changes in the relative decay rates of $X$ and $Z$, which can cause the feedforward mechanism to over- or under-compensate, depending on the parameter regimes (Jones et al. 2020). This can occur if, for example, the degradation rates of $X$ and $Z$ are different, and the cell growth rate changes.

Finally, while the development of controllers for use in bacteria has advanced rapidly in the past decade, such development is lagging behind in mammalian cells. This can partially be explained by the greater intensity and longevity of research in bacterial synthetic biology compared to mammalian synthetic biology, as well as by the lengthier design cycles inherent in engineering mammalian cells. Thus, as we aim to build control systems for applications in regenerative medicine, cell therapy, programmed organoids, and other areas of mammalian synthetic biology, there will continue to be a need to port solutions from bacteria to mammalian cells and to realize new mammalian designs that can remedy the numerous unique context-dependencies found in mammalian systems.

## Applications of controllers to insulate genetic device function from context

Over the past several years, a number of synthetic genetic controllers have been developed for bacterial, yeast, and mammalian genetic devices to achieve robustness to various context-dependencies. A summary of different controllers, the organisms they were built for, and the context-dependencies solved are presented in **Table 2**. Schematics for the enzymatic feedback, antithetic feedback, and feedforward designs are provided in **Figure 9A, B, and C**, respectively.

| **Controller architecture** | $X$ | $Z$ | **Robust output rates** | **Implementations** |
|---|---|---|---|---|
| Enzymatic feedback | Phosphatase | TF | $\alpha$**, $\delta$**, $\beta$*, $\gamma^{\dagger}$, | Bacteria (Chang et al. 2013), Mammalian (Jones et al. 2021) |
| | miRNA | RBP | $\alpha$**, $\delta$*, $\beta$*, $\gamma$, | Mammalian (Bloom et al. 2015) |
| | miRNA | TF | $\alpha$**, $\delta^{\dagger}$ | Mammalian (Lillacci et al. 2018) |
| | Degron unlocker | TF | $\alpha$*, $\delta$*, $\beta$*, $\gamma^{\dagger}$ | Yeast (Ng et al. 2019) |
| Antithetic feedback | TF | Scaffold/anti-scaffold | $\alpha$, $\delta$, $\beta$, $\gamma^{\dagger}$ | Bacteria (Hsiao et al. 2015) |
| | Sigma factor | sRNA/RNA | $\alpha^{\dagger}$, $\delta$, $\beta$, $\gamma^{\dagger}$ | Bacteria (Huang et al. 2018) |
| | TF | Sigma/anti-sigma factors | $\alpha$, $\delta$, $\beta$, $\gamma^{\dagger}$, | Bacteria (Aoki et al. 2019) |
| | TF | mRNA/antisense RNA | $\alpha$, $\delta$, $\beta$, $\gamma^{\dagger}$ | Mammalian (Frei et al. 2020b) |
| Proportional feedback | TF | (Same as $X$) | $\alpha$**, $\delta$*, $\beta$**, $\gamma^{\dagger}$ | Mammalian (Bleris et al. 2011), Bacteria (Shopera et al. 2017) |
| | RBP | (Same as $X$) | $\alpha$**, $\delta$*, $\beta$*, $\gamma^{\dagger}$ | Mammalian (Stapleton et al. 2012) |
| Feedforward | DNA | TF | $\alpha^{\dagger}$, $\delta^{\dagger}$, $\beta^{\dagger}$, $\gamma^{\dagger}$ | Mammalian (Bleris et al. 2011), Bacteria (Segall-Shapiro et al. 2018) |
| | RNA | miRNA | $\alpha$, $\delta^{\dagger}$ | Mammalian (Bleris et al. 2011), (Strovas et al. 2014; Frei et al. 2020a) |
| | Protein | Protease | $\alpha^{\dagger}$, $\delta^{\dagger}$, $\beta^{\dagger}$, $\gamma^{\dagger}$ | Mammalian (Gao et al. 2018) |
| | RNA | endoRNase | $\alpha^{\dagger}$, $\delta^{\dagger}$, $\beta^{\dagger}$, $\gamma^{\dagger}$ | Mammalian (Jones et al. 2020) |

**Table 2:** Controllers to solve context-dependence. *Not measured. **Imperfect adaptation observed. $^{\dagger}$Perfect adaptation conditional on relative parameters for controller and output species.

**Robustness to DNA copy number variation**. Feedforward controllers built with miRNAs, TFs, or endoRNases have been used to make gene expression robust to DNA copy number variation (Bleris et al. 2011; Lillacci et al. 2018; Segall-Shapiro et al. 2018; Jones et al. 2020) (Figure 9C-i, -ii, -iv), a key component of the overall transcription rate of a gene ($\alpha$). These designs all operate by expressing the controller species from the same DNA strand as that of the genetic device. While different cells may have different copy numbers of the DNA, the expression of the controller species (miRNA (Bleris et al. 2011; Lillacci et al. 2018), TF (Bleris et al. 2011; Segall-Shapiro et al. 2018), or endoRNase (Jones et al. 2020)) and output protein are both proportional to the DNA copy number. Thus, as long as the controller species concentration linearly actuates decay of or inverse-proportionally actuates production of the output protein, changes in the transcription rate of the genetic device output are offset such that the output level is maintained across large ranges of DNA copy number.

Desensitizing gene expression to DNA copy number will be useful for stem cell engineering and regenerative medicine applications by enabling targeted overexpression of key gene regulators, allowing for precise programming of cell fate decisions. Currently, cell fate programming is typically achieved by overexpressing lineage-specific TFs with lentiviral- or transposon-based gene delivery systems (Takahashi and Yamanaka 2016), which can integrate a range of DNA copy numbers per cell. Precise gene expression control can be used to accelerate efforts to model diseases connected to exact gene expression levels (*e.g.* (Zhu et al. 2019)) by limiting copy number variation among cells, such that CRISPR or other targeted integration strategies are not needed.

**Robustness to resource loading**. More recently, both feedforward and feedback controllers have made gene expression robust to changes in cellular resource availability (Shopera et al. 2017; Darlington et al. 2018; Huang et al. 2018; Frei et al. 2020a; Jones et al. 2020; Jones et al. 2021). In bacterial cells, feedback control has been used to attenuate the effects of resource loading, *e.g.* from competition for ribosomes, on the level of protein produced by genetic devices (Shopera et al. 2017; Darlington et al. 2018; Huang et al. 2018). In these designs, either transcriptional or post-transcriptional control is applied to dynamically adjust protein production in response to resource fluctuations. In particular, the mechanism of (Huang et al. 2018) is a quasi-integral antithetic feedback controller, in which the controller reactions are made very fast in order to overcome integrator leakiness and reach almost perfect adaptation to resource loading (Figure 9B-ii). While sRNA are bacteria-specific, an analogous system built in mammalian cells that has antisense RNA in place of sRNA was shown to impart rejection to various disturbances, though resouce competition was not tested (Frei et al. 2020b) (Figure 9B-iv). In mammalian cells, feedforward control has been implemented to robustly set protein expression levels in the face of transcriptional resource fluctuations. In these designs, either a miRNA (Frei et al. 2020a) or an endoRNase (Jones et al. 2020) is used to sense changes in resource availability and then offset the resulting changes in output production (Figure 9C-ii, -iv)), similarly to how feedforward control had previously been used to offset changes in DNA copy number (Bleris et al. 2011; Segall-Shapiro et al. 2018). More recently, feedback control has also shown to reduce sensitivity of gene expression to resource loading and off-target regulation at the transcriptional and post-transcriptional levels in mammalian cells (Jones et al. 2021) (Figure 9A-i)

At an abstract level, differences in the expression level of any given gene among different cell types is the result of the differential expression levels of gene regulators in each cell type. Thus, to establish control over RNA and protein expression that is robust to changes in cell state throughout processes such as reprogramming, differentiation, and integration into complex environments such as tumors, it will be necessary to ensure engineered genetic circuits can operate across diverse intracellular conditions. Early evidence for controllers enabling such behavior has been observed with the endoRNase feedforward controller, with which expression levels across several cell lines were precisely aligned (Jones et al. 2020).

**Robustness to noise via linearization of the transfer curve**. In natural biological circuits, linear amplification is observed for many signaling networks (Nunns and Goentoro 2018). For example, the ERK MAPK pathway resembles an enzymatic feedback controller: Raf phosphorylates and activate MEK, which phosphorylates and activates ERK, which then feeds back by phosphorylating and deactivating Raf (Sturm et al. 2010). This feedback linearizes the response from Raf activation to ERK activation (Sturm et al. 2010). In synthetic circuits, this principle has been applied to make inducible genetic devices respond to their inducer inputs in a graded, uniform manner (Dublanche et al. 2006; Nevozhay et al. 2009; Nevozhay et al. 2013; Jones et al. 2021). Similarly, feedback control using sequestration of scaffold proteins was shown to enable concentration tracking of an output gene to a reference input (Hsiao et al. 2015), indicating a linear input-output response (Figure 9A-ii).

Input-output linearization has been successful for two inducible systems (TetR/Dox and PhlF/DAPG) in mammalian cells (Szenk et al. 2020), both of which comprise the small molecule inhibiting negative autoregulation by the TF. Transcriptional repressors feeding back on their own production are classically modeled as proportional feedback modules. However, as the binding of a TF to a small molecule resembles a sequestration reaction, a reframing of the model suggests that the topology could operate as an antithetic quasi-integral controller when the binding between the TF and small molecule is faster than all other reactions and effectively irreversible. In this case, the extracellular small molecule sets the reference ($u$), the intracellular small molecule and TF take the roles of the sequester species $Z$ and $\hat{Z}$,

and the mRNA for the TF is the controlled species $X$. In the case of TetR binding to Dox, the reverse reaction occurs on the timescale of tens of hours (Schubert et al. 2004), meaning a TetR-Dox complex dissociates about as frequently as the complex decays in normally-dividing mammalian cells. Further work will be needed to test if such controllers are also capable of robust disturbance rejection, as expected based on the antithetic feedback topology.

For regenerative medicine applications, linearized sensing systems have applications in better controlling synthetic or rewired signaling networks (Kojima et al. 2020). As engineered genetic circuits grow larger and more sophisticated, reducing noise propagation through the circuit modules will be essential to guarantee performance across individual cells. This will be especially important in applications such as cell fate programming and cell therapies, where variation among individual cells can bias cell fate potential (Strebinger et al. 2019) and possibly contribute to the breaking of "kill switches" and target specification moieties encoded in the therapeutic cells (Esensten et al. 2017), leading to potentially deleterious therapeutic outcomes.

**Robustness to variable downstream loads**. Another application example of synthetic feedback control in eukaryotic cells has been in buffering against the effects of retroactivity (Mishra et al. 2014; Jayanthi and Del Vecchio 2011), whereby loading of a device's output by binding to downstream targets slows and disrupts the dynamics of the device itself. A frequent cause of retroactivity is the binding of a TF output to the promoters of many downstream target genes (Mishra et al. 2014; Jayanthi et al. 2013). By placing a "fast" buffering device between the device's TF output and the downstream targets, thereby implementing a form of high-gain negative feedback, the dynamics of the device's output can be decoupled from the presence of DNA target sites (Mishra et al. 2014). The high-gain feedback can be implemented with a pool of rapidly-converting covalently-modified proteins that can be turned 'on' or 'off' with much faster timescales than the dynamical processes in either the upstream or downstream devices (Mishra et al. 2014). This solution to retroactivity may be useful for regenerative medicine applications where precise control is desired for a gene regulator with many downstream targets, such as lineage-specific TFs that target hundreds of sites with varying access in enhancers throughout development. In such scenarios, the buffering device could potentially be used to ensure a constant TF input to each individual enhancer as access of the TF to its genomic binding sites fluctuates over time.

**Robust regulation of cell growth rate**. Finally, an emerging body of research has used controllers to decouple cell growth from genetic device function. This is a particularly difficult challenge, because changes in cell growth rate change the decay rates of all reasonably stable RNA and protein species (*e.g.* in mammalian cells, those with half lives greater than ~10 hours). In turn, the operation of genetic devices can change cell growth rate (Ceroni et al. 2015; Jones et al. 2020). One way to approach the problem of genetic devices affecting cell growth rates has been to use feedforward and/or feedback controllers to limit the burden of genetic devices on the bacterial (Ceroni et al. 2018; Barajas et al. 2021) or mammalian cell (Lillacci et al. 2018) (Figure 9A-ii, C-ii), thus better optimizing the resource usage of a genetic device given the constraints of the cellular environment. Note that the controller by Barajas *et al.* works through regulation of ppGpp (Figure 9C-v); there is currently a limited understanding of the role of ppGpp in animal cells (Ito et al. 2020), and thus the device may not be directly translatable to mammalian cells. However, the general principle could be applied to boost availability of other types of shared resources to offset loading of such resources.

Additionally, the cell growth rate itself as a function of environmental perturbations such as temperature changes has been controlled in bacteria via feedback control (Aoki et al. 2019). Note that the device built by Aoki *et al.* operates via antithetic feedback with sigma factors (Figure 9B-iii), which are bacteria-specific, though the topology could potentially be extended to homologous general TFs in mammalian cells or to gene-specific TFs that bind form

high-affinity and inactive heterodimers. Future work will be needed to extend this solution to the mammalian cell cycle, which has much more complex regulation (Satyanarayana and Kaldis 2009). Fortunately for mammalian synthetic biologists, mammalian cells generally grow and divide much more slowly than bacteria and yeast, limiting the severity of effects on the transcriptome and proteome caused by changes in cell growth rates. Whereas many molecular species in mammalian cells have a decay rate that is dominated by degradation and thus insensitive to growth rate changes, the decay rates of molecular species in fast-dividing organisms are significantly affected by dilution due to cell growth.

For future applications, ensuring robust device function in response to changing cell growth rate will facilitate the translation of genetic device designs from fast-dividing cells like PSCs and HEKs to slower-dividing cells, non-dividing cells, and even cells that will variably grow and divide in response to environmental conditions. Limiting the effect of genetic devices on cell growth rates may also be particularly relevant for programming cell development, wherein developmental progression is intricately tied to the decay rate of staged gene regulators (Rayon et al. 2020).

# Future development and applications of context-aware design

## Applying control across biological scales

Given the complexities and interconnected nature of perturbations that impact synthetic devices during cell fate programming, there is an opportunity to build synthetic devices that are aware of context effects at all three levels: genetic, cellular, and extracellular context. These multi-scale context-aware devices would include deliberate and direct connections between the device and the cell's changing state (**Figure 10A**).

**Optimizing design of individual genes**. At the genetic level, CRISPR and landing pads (Duportet et al. 2014; Gaidukov et al. 2018) can be used to integrate genes into "safe harbor" loci that are resistant to silencing, or that become selectively active during specific stages of cellular development. Random-integration methods like lentiviruses and transposons can be made more resistant to epigenetic changes through the use of chromatin insulators (Chung et al. 1997; Liebert and Ellis 2005; Bortle and Corces 2012; Liu et al. 2015). Devices that synthetically regulate epigentic states of chromatin and DNA are emerging for synthetic control of gene expression and protection from epigenetic effects (Thakore et al. 2016; Park et al. 2019). Additionally, the cell-to-cell variability in expression caused by copy number variation among cells engineered with lentiviral, transposon, and episomal vectors may be effectively eliminated through the use of feedforward controllers (Bleris et al. 2011; Lillacci et al. 2018; Jones et al. 2020). At the cellular and extracellular level, feedback and feedforward controllers may be used to make expression of the gene robust to changes in the myriad cellular resources that direct gene expression (Jones et al. 2020; Frei et al. 2020a; Jones et al. 2021), off-target interactions from endogenous gene regulators (Jones et al. 2021), and changes in the cellular environment (Aoki et al. 2019). Tools such as LOCKR feedback control (Ng et al. 2019) (Figure 9A-iii) will also enable robust interrogation and control of existing gene networks and signaling pathways, furthering our ability to integrate synthetic and natural systems. Controllers and context-aware design more generally offers a solution to quantify the underlying physical consequences of these context effects on gene expression, and design systems that are robust to the context-dependent parameters and variables. However, since adding genetic controllers can substantially increase the complexity and resource demand of genetic devices, bioengineers will have to maximize the utility of their systems by identifying and designing around the dominant sources of context-dependence in their systems.

**Controlling gene networks**. For each gene in the network, the above context effects and solutions for solving them can be utilized. However, the goal of cell engineering is more often to achieve a desired cellular function, with the expression state of a given gene being simply one piece of the puzzle. Ensuring that a network can properly make one or more input-output calculations is a difficult task with few demonstrated bioengineering solutions (Chen et al. 2015; Veliz-Cuba et al. 2015; Aoki et al. 2019). At a high level, feedback and feedforward control can be used in the same abstract manner to sense the inputs and/or outputs to a network and feed in control signals to correct network behavior (**Figure 10A**). In this case, the behavior to be corrected can be a molecular output of the network, or the confluence of many such outputs that collectively classify network status.

**Interfacing tissues, organs, and whole organisms**. Finally, extending the network to that of multiple cells in a tissue, tissues in an organ, or organs in an organism, we can apply the same controls framework to build robust multicellular systems. In this case, the individual cells communicate with each other through signaling molecules and other means of cell-cell communication (Youk and Lim 2014; Teague et al. 2016; Toda et al. 2019; Kojima et al. 2020). Individual cells or subsets of cells sense the various inputs coming from other cells and make decisions about what outputs to produce in response. This picture brings us to the point of engineering systems that can function like homeostatic regulators in the human body. In future therapies where we deliver synthetic cells, tissues, and/or organs as therapies, we will need to encode robust behavior at all levels, ranging from regulation of our engineered genes in the designer cells/tissues/organs all the way up to how these organs interact with their host to ensure both safety and efficacy. To achieve this goal, we can utilize synthetic genetic devices for engineering cell-cell communication. Such tools including synthetic Notch receptors (synNotch) for sensing contact with specific cell types (Morsut et al. 2016), as well as synthetic soluble factor sensing systems (Kojima et al. 2020; Maze and Benenson 2019; Scheller et al. 2020). Mirroring natural morphological networks (Li et al. 2018), individual cells programmed with feedback controlled-networks to respond to signaling molecules will be useful for robustly generating tissue patterns and structures. Besides controlling specific cell behaviors, controllers can also be used to regulate the growth and survival of populations of cells (Ma et al. 2020), which one could imagine using to self-limit therapeutic cells that can be toxic if overactive (Lundh et al. 2020).

## Applications of context-aware genetic controllers for cell therapy development

Moving forward, we can imagine synthetic biology, control systems, and context-aware design becoming more broadly applicable to the entire pipeline of cell therapy development, from discovery to manufacturing to transplantation. Here, we will lay out a vision for a PSC-derived immune cell therapy for cancer as an example, but this process could be applied to efforts in treating psoriasis (Schukur et al. 2015) and diabetes (Ye et al. 2016; Xie et al. 2016), among other conditions (Kitaada et al. 2018; Scheller and Fussenegger 2019; Kojima et al. 2020).

**Prototyping therapeutic programs**. In the discovery stage of cell therapy development, synthetic biology offers tools for building and screening various genetic devices for detection and actuation of therapeutic actions. Biosensors and the genetic/cellular responses that they generate are the basic building blocks of a cell therapy that can sense and respond to cues in the body. Controllers can be applied when developing such sensors in order to linearize their I/O responses (Sturm et al. 2010; Nevozhay et al. 2013; Nunns and Goentoro 2018; Szenk et al. 2020), decrease noise (Dublanche et al. 2006; Jones et al. 2021), and ensure that the sensor functions are robust to perturbations (Müller et al. 2019; Steel et al. 2019; Jones et al. 2021). Thus, controllers can help both in the development of devices with ideal properties, as well as in ultimately yielding systems that will be more likely to work in varying contexts, such as

when moved from cell lines (*e.g.* Jurkat T-cells) to primary cells, or when *in vitro* tests with those engineered primary cells are moved *in vivo*.

**Cell manufacturing**. For the manufacturing stage of allogeneic (or off-the-shelf) cell therapies (**Figure 10B**), PSC-derived cells must follow defined developmental trajectories to reach the desired cell type. In the case of T cell derivation by directed differentiation of PSCs, controllers can help to ensure each cell autonomously receives the correct signals at each stage of differentiation, by robustly linking optimized TF expression cascades to cell state sensors. While current methods of niche engineering can generally provide many of these signals on time, controllers can ensure that extracellular context effects, such as variable niche signals and interaction with heterogeneous cells at asynchronized developmental stages and fates, will not disrupt the development of the target cell population. In addition, controllers can be used to measure the current state of the cell and modulate the levels of stimuli to enforce desirable cell fate transitions or prevent undesirable fates.

Broadening this framework to the multicellular environment of bioreactors in which PSCs are expanded and differentiated to T cells, context-aware design can help to expand or limit the specialized cell types that develop in the cell batches, avoiding the emergence of off-target cell types. For scale-up of these manufactured cells, population-limiting feedback systems (Ma et al. 2020) can be used to prevent overgrowth such that ideal densities of cells are maintained throughout fed-batch growths. In fact, production of effectors proteins such as CARs and cytokines by engineered T cells may also be improved by optimizing the gene expression programs within the cell to avoid overburdening the cell's resources (Lillacci et al. 2018). Overall, the use of controllers and context-aware engineering in cell therapy production will help to increase reliability, simplify experimental conditions, and reduce costs.

**Controlling therapeutic cell function**. Context-aware design is particularly important for the therapeutic use of cell therapies. Expected challenges such as the inability to engraft and graft-vs-host disease (GVHD) can be approached by engineering signaling proteins/processes and major histocompatibility complexes in the therapeutic cells (Deuse et al. 2019; Ferreira et al. 2019; Han et al. 2019; Raffin et al. 2020; Zhao et al. 2020). With control systems in place, the expression of such elements can be made to adapt to the local environment, further improving the chance of successful engraftment for individual cells and perhaps even entire tissues. Control systems can similarly be used to ensure that therapeutic cells home to the right part of the body, for example by sensing nearby cells and altering motility until locating a tumor (**Figure 10B**). Within tumors, context effects become extreme: between patients and even within an individual patient's tumor, the variability in cell composition and interactions among tumor, immune, and stromal cells are extremely complex, limiting the efficacy of immune and cell-based therapies (Beatty and Gladney 2015; Bielamowicz et al. 2018). We thus need to design our therapeutic cells to identify and kill cancer cells and recruit/stimulate other immune cells to help in a robust and reliable way. For example, we can imagine sensing and outputting signals from the therapeutic cell into the tumor in order to control the overall state of signaling and immune competence within the tumor microenvironment. The therapeutic cells themselves can be engineered to be robust in function relative to the suppressive immune environment of the tumor, reducing problems like T cell exhaustion (Martinez and Moon 2019) and other undesirable cell state changes that limit efficacy. Finally, the cell therapy must be designed to maximize safety; it cannot harm the patient through problematic outcomes such as cytokine release syndrome (Lundh et al. 2020). To do so, we can engineer self-limiting systems that measure the effect that the therapeutic cell is having on the body as a whole and adjust effector functions as needed to prevent toxicity while continuing to carry out prescribed tasks. This will require the development of sophisticated genetic systems that can connect intracellular and extracellular environments and apply robust, precise regulation of signaling to the tissue and body as a whole.

## Conclusion

Through the design of genetic controllers, fundamentally rooted in control systems theory, we can develop a new class of context-aware synthetic devices that are robust to the mammalian cell context. These devices and the larger circuits they compose offer a new strategy for insulation from genetic, cellular and extracellular context effect by viewing them as perturbations. With this new class of context-aware devices comes exciting opportunities to engineer therapeutically-relevant cells that have fundamentally predictable behaviors, both in manufacturing pipelines and upon transplantation into patients.

# Figures

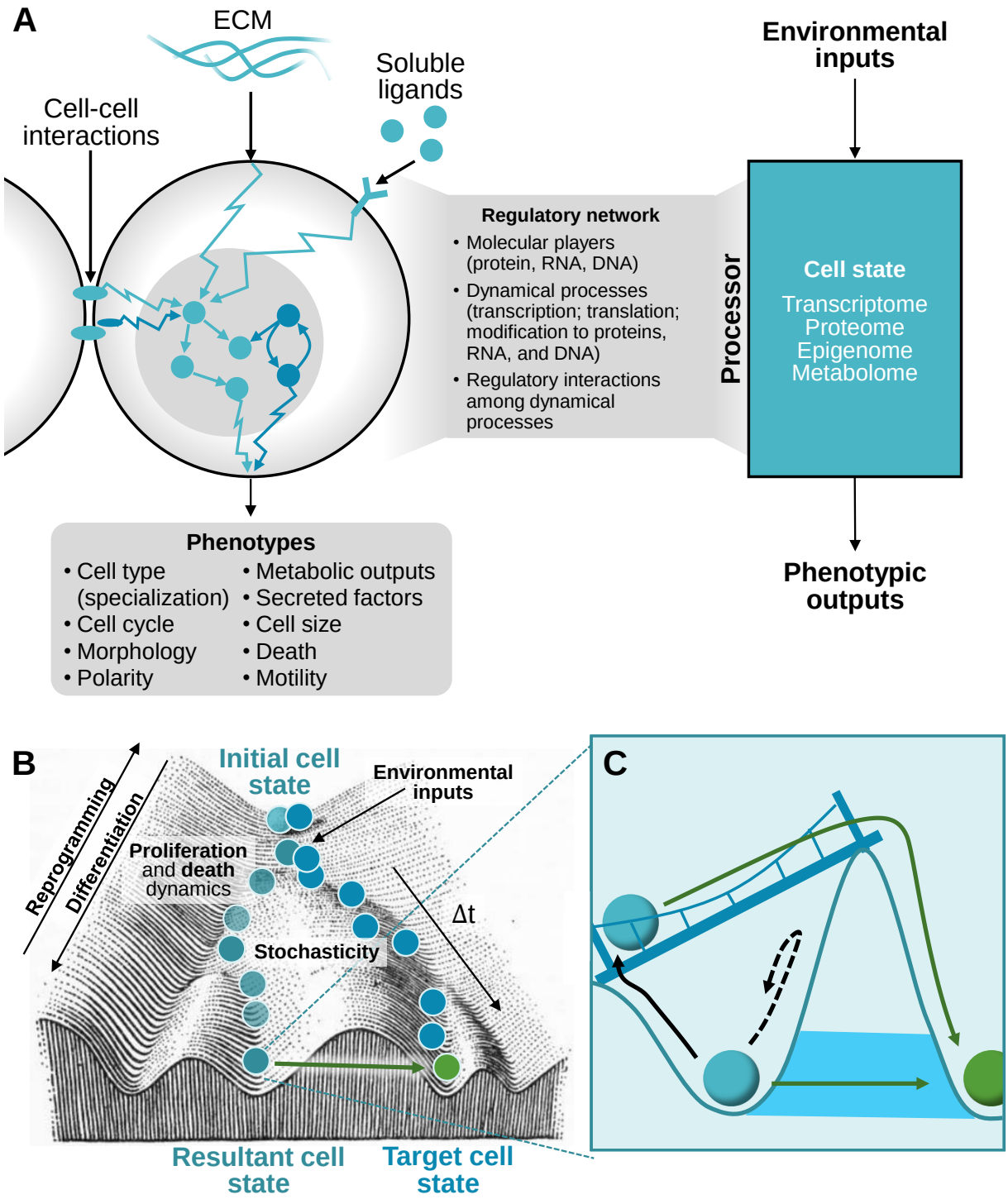
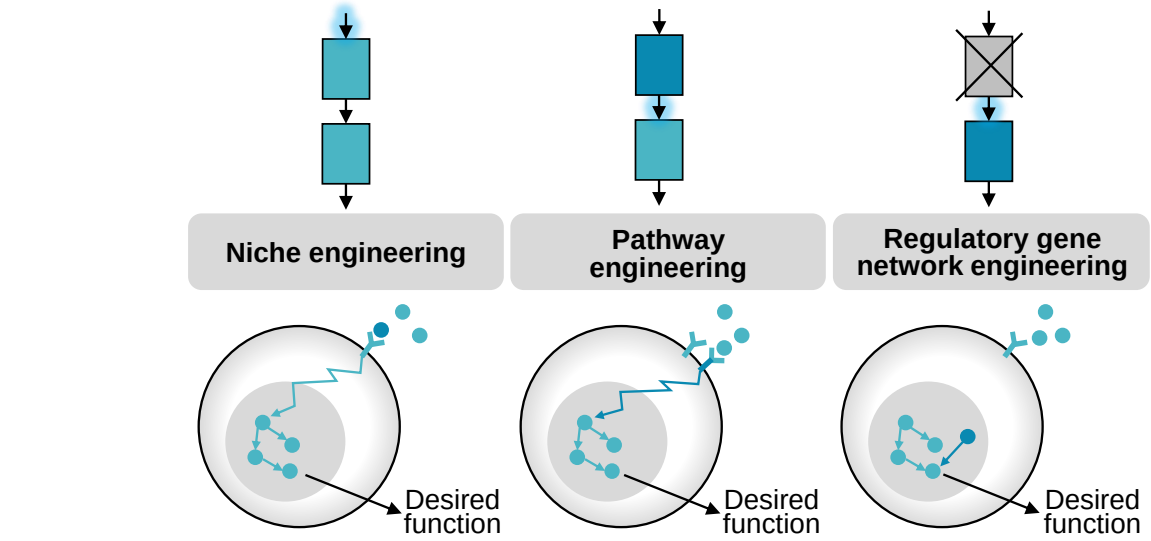



**Figure 1:** Viewing the cell as a programmable unit.




| | Niche engineering | Pathway engineering | Regulatory gene network engineering |
|---|---|---|---|
| **Manipulating endogenous processes** | • Addition of native **cytokines**, **small molecules**, **ECM**<br>• Chemical and mechanical signals | • **Rewiring** endogenous receptors and signaling pathways | • Gene over-expression and knock-down/-out<br>• Manipulating **endogenous** gene regulatory networks (**GRN**) |
| **Introducing novel processes** | • Addition of **synthetic extracellular signals** | • Design of **novel**, **orthogonal** receptors and signaling pathways | • **Genetic devices** to drive protein and RNA outputs<br>• Design of **orthogonal** synthetic GRN with novel motifs |

**Figure 2:** Outside-in and inside-out approaches to cell engineering.

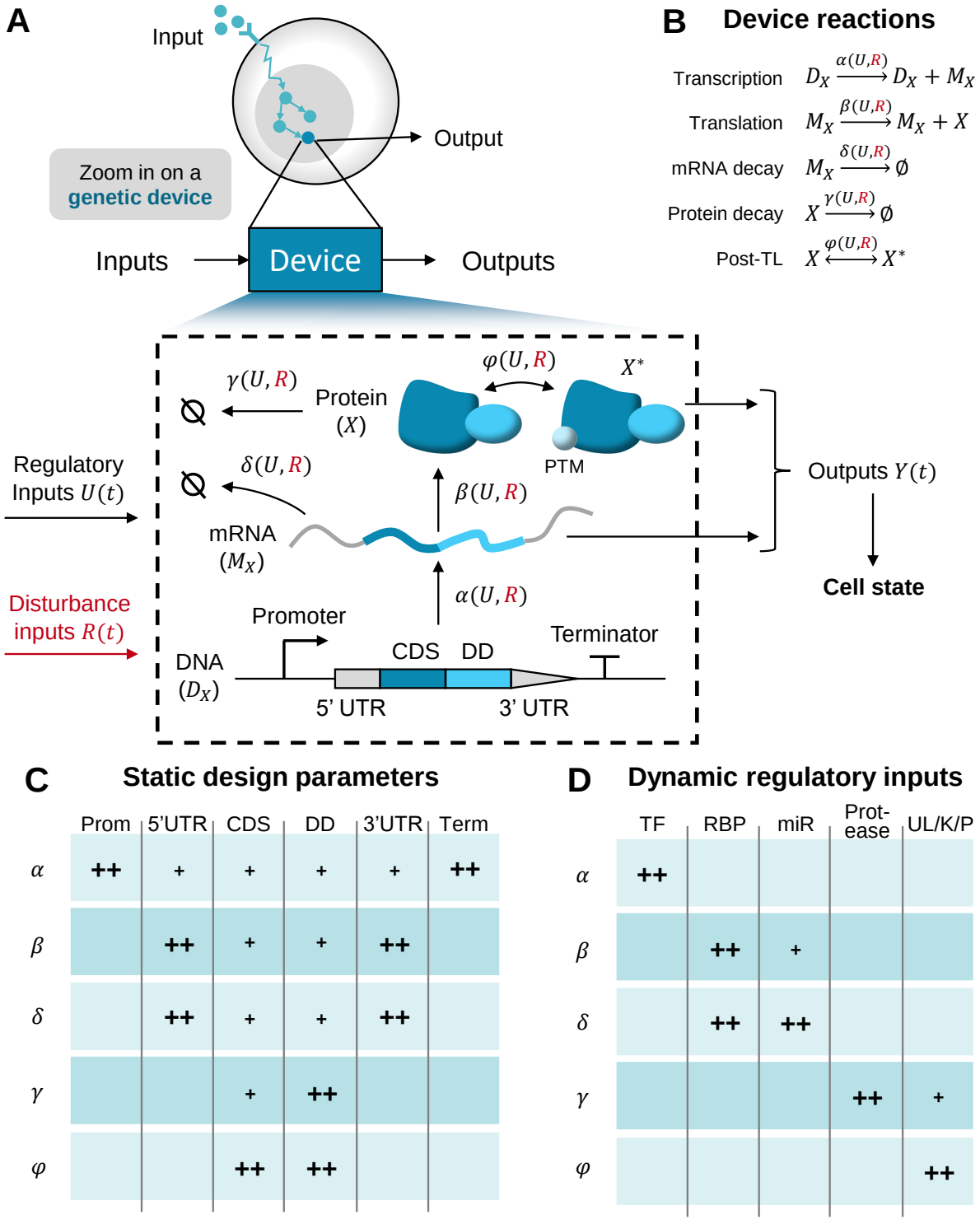



**Figure 3:** Detailed breakdown of a genetic device.

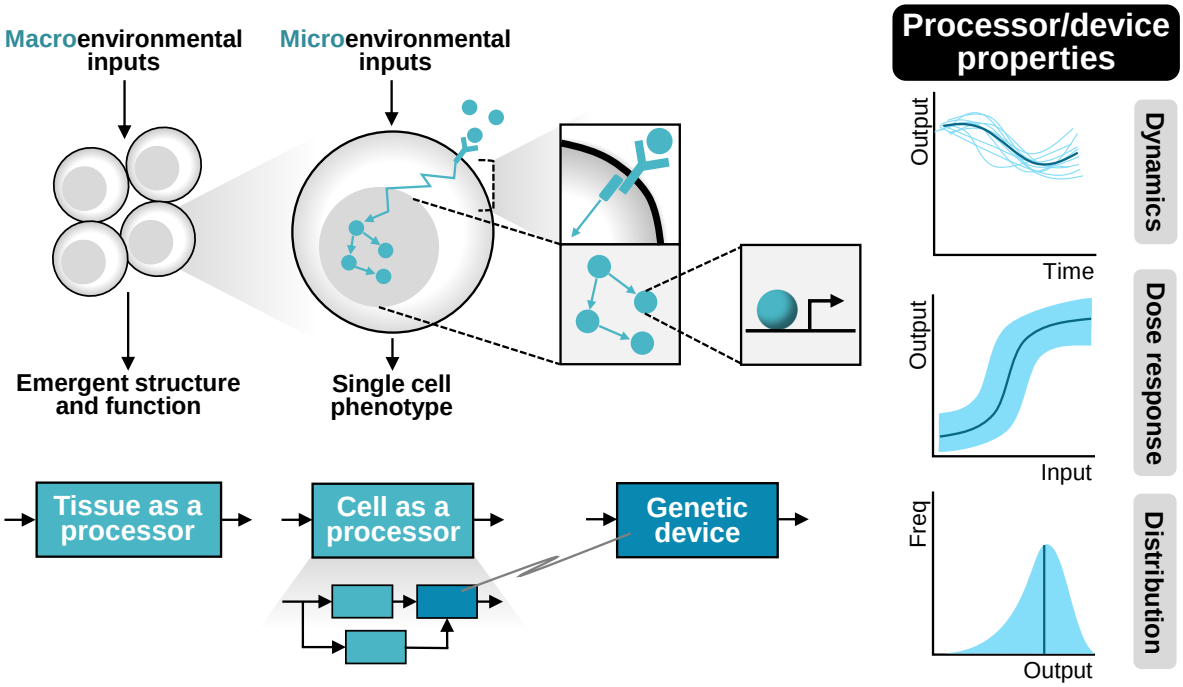



**Figure 4:** The genetic device is the core unit of synthetic biology.

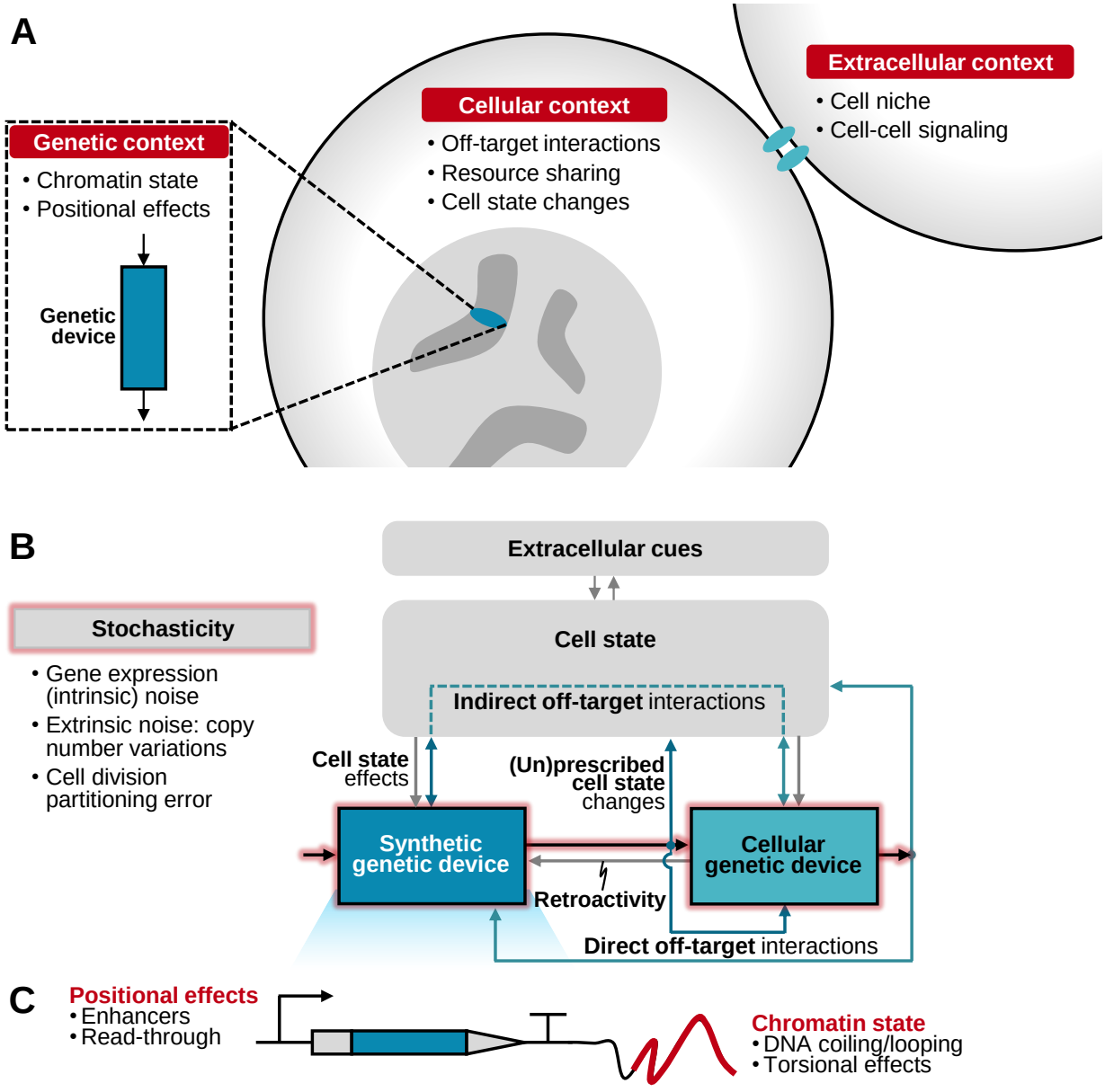



**Figure 5:** The mammalian cell context imposes context effects that challenge the function of genetic devices.

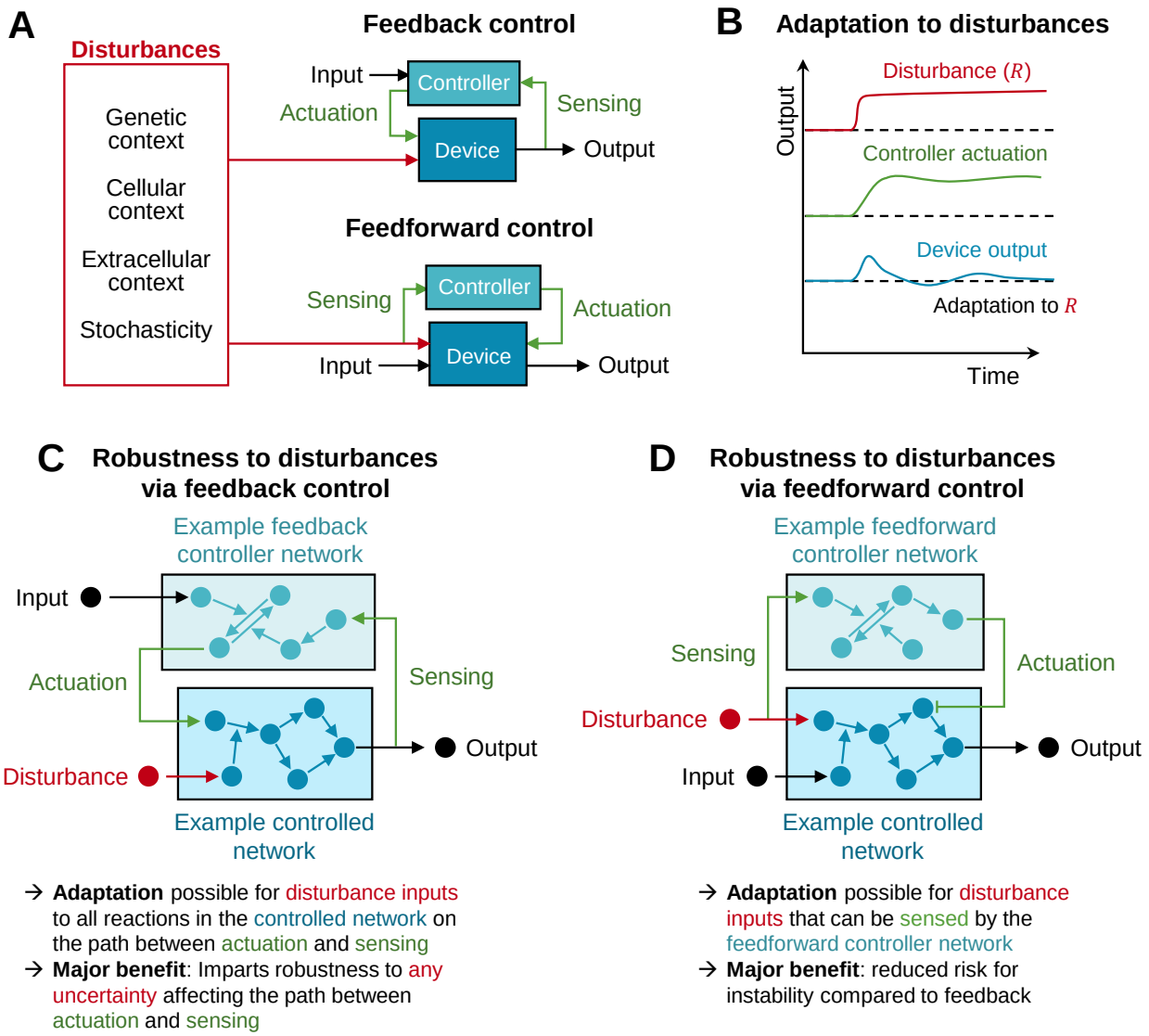



**Figure 6:** Genetic controllers adapt genetic device outputs to disturbances.

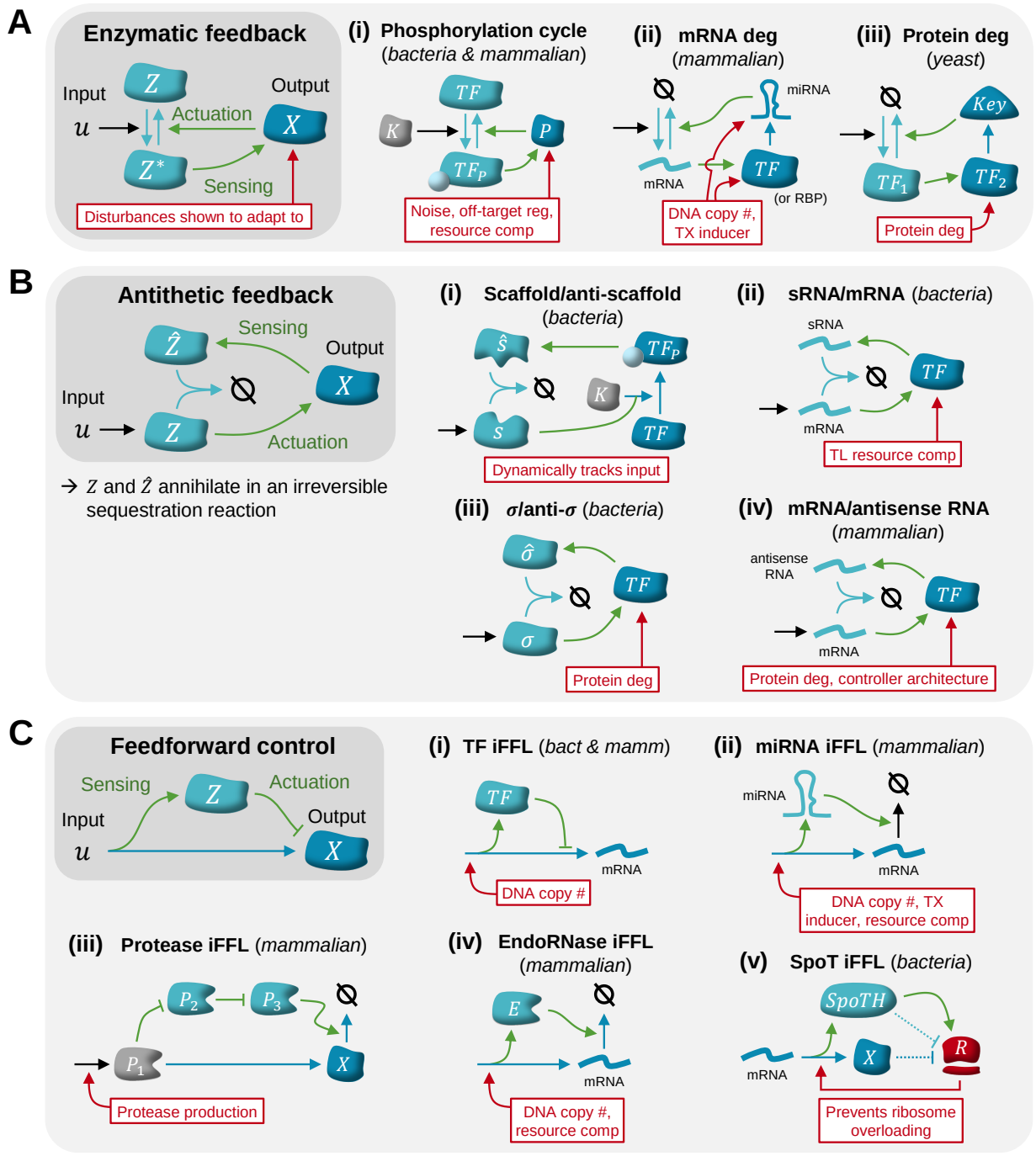



**Figure 9:** Feedback and feedforward controller implementations.

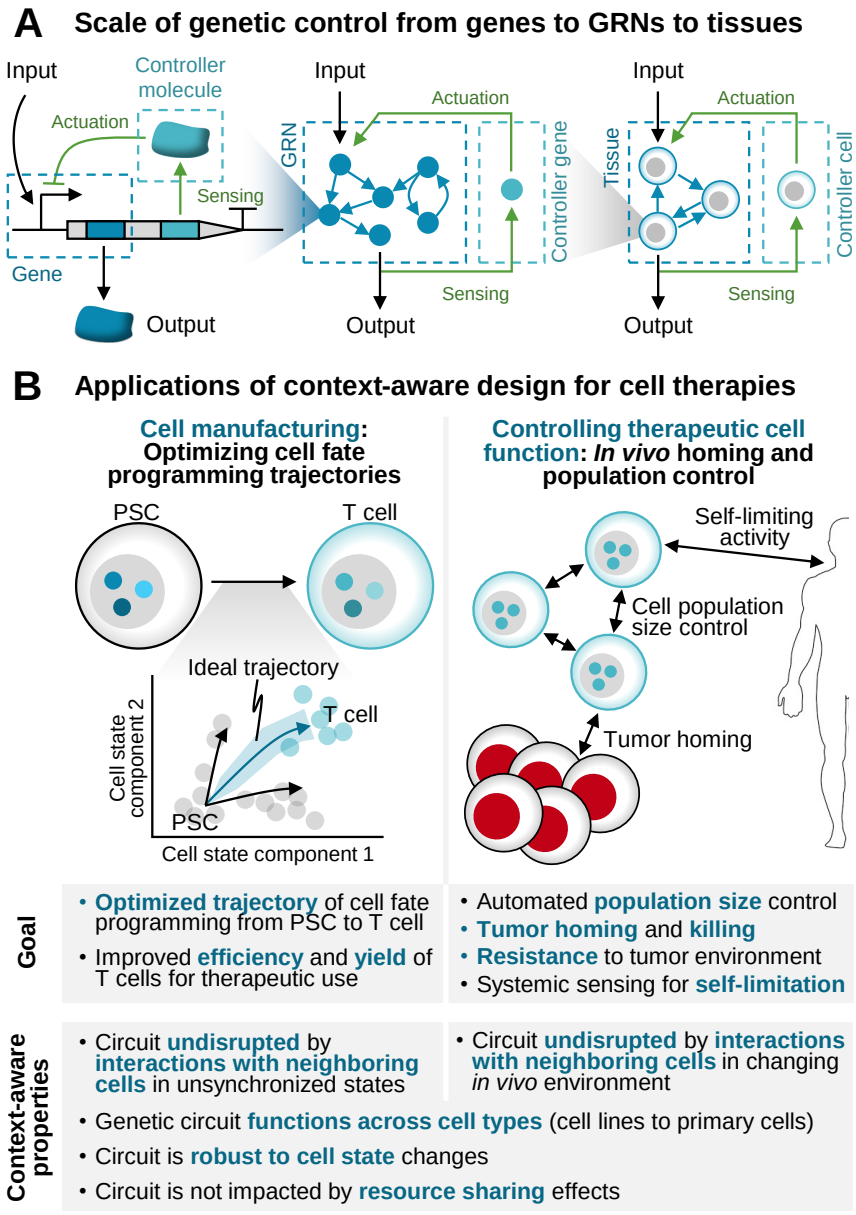



**Figure 10:** Future applications for context-aware genetic controllers in regenerative medicine.

# Figure Legends

**Figure 1: Viewing the cell as a programmable unit.** (**A**) The cell is a processor that maps chemical and mechanical inputs from the cellular microenvironment – cell-cell interactions, interactions with the extracellular matrix (ECM), and soluble factors – to phenotypic outputs. The processing activity of the cell is enabled through the regulatory network, composed of molecular players (DNA, RNA, and proteins) that interact via biochemical reactions to facilitate dynamical processes, including transcription, translation, and modifications to the players involved. Synthetic genetic circuits (green) are incorporated into the endogenous molecular network (blue) to enant new function. (**B**) Cell fate trajectory is driven by the state of the cellular processor, environmental inputs, and sources of stochasticity. Cells can be visualized on the classic Waddington landscape, moving from an initial state as they roll down the hill and stabilize in valleys representing the final cell state. Reprogramming, the conversion of somatic cells to pluripotent stem cells, is often visualized as a movement up the Waddington landscape as the cells gain potency, while differentiation is depicted by a movement downwards. (**C**) Synthetic biology offers a framework for programming the inner regulatory network of the cell, allowing for predictable cell fate changes to occur, leading cells to a desired target state.

**Figure 2: Outside-in and inside-out approaches to cell engineering.** Outside-in and inside-out engineering represent complementary approaches for cellular engineering. Niche engineering represents an "outside-in" approach, where the cellular microenvironment is programmed through the addition of native or synthetic extracellular signals such as cytokines, small molecules, and engineered cellular matrices. These environmental cues provide chemical and mechanical inputs into the cellular processor, thus driving phenotype. On the other hand, genetic engineering approaches to cell fate programming represent an "inside-out" approach, where portions of the cellular processor are manipulated: either the receptors and signaling pathways (pathway engineering) or the regulatory networks themselves (regulatory gene network engineering).

**Figure 3: Detailed breakdown of a genetic device.** (**A**) Zooming in on the genetic device constituting one node in a larger network. The basic genetic device represents one gene encoded on a strand of DNA $D_X$, which is transcribed to generate an mRNA $M_X$, which is translated to a protein $X$. After translation, $X$ can be regulated via the addition or removal of post-translational modifications (PTMs) to $X^*$. Common PTMs include phosphate group addition/removal by kinases/phosphatase and ubiquitination by ubiqutin ligases (ULs). The output ($Y$) of the genetic device can be any of the species produced by the gene that feed into a downstream process. Expression of the output(s) is affected by specified regulatory inputs ($U$) and undesirable disturbance inputs ($R$) that can act on each of the key rate processes of gene expression: transcription ($\alpha$), translation ($\beta$), mRNA decay ($\delta$), protein decay ($\gamma$), and PTM addition/removal ($\varphi$). (**B**) Summary of the reactions involved in expression of the genetic device output(s). (**C**) Each of the reaction rates are dependent to varying degree on sequences encoded in the DNA. These sequences can ideally be combined in a modular fashion to statically engineer the reaction rates of the device. (**D**) The reaction rates can also be regulated by dynamic inputs ($U$). Transcription factors (TFs) bind DNA and regulate transcription, RNA binding proteins and miRNAs (miRs) bind and regulate RNA decay and translation, proteases bind and regulate protein decay, and kinases/phosphatases/ULs bind and regulate protein PTMs (Del Vecchio and Murray 2014; Alberts et al. 2014; Alon 2019). In Panels (C) and (D), + and **++** indicate static and dynamic design parameters that influence the indicated reaction rate; **++** indicates a dominant influence on the given rate).

**Figure 4: The genetic device is the core unit of synthetic biology.** The genetic device provides a desirable input/output response, allowing for predictable control over its temporal dynamics, dose response, and the distribution of the outputs it produces. The device sits within the context of the cell's inner regulatory network, or processor,

which provides an input/output mapping within the context of the microenvironment. The cellular microenvironment provides inputs to the cell in the form of chemical and mechanical cues from neighboring cells or from the cell itself. Multicellular populations, such as tissues and organs, can also be viewed as systems that map inputs from their macroenvironment to outputs, which are seen as emergent structures and functions. These emergent properties enable the tissue to provide a function in the body through coordination between the cells composing the tissue.

**Figure 5: The mammalian cell context imposes context effects that challenge the function of genetic devices.** (**A**) The genetic, cellular, and extracellular contexts provide sources of perturbation that interfere with the genetic device's input/output performance. (**B**) Control systems view of the interactions between the synthetic genetic device and the cellular context. Direct off-target interactions between the synthetic device and the cell's regulatory network perturb both the genetic device and, indirectly, the cell state (Berger et al. 1992; Gilbert et al. 1993; Baron et al. 1997; Lin et al. 2007; González et al. 2015; Gouda et al. 2019). The sharing of critical cellular resources, such as polymerases and ribosomes, among synthetic and natural genetic devices creates indirect off-target interaction between the device and other cellular processes (Ceroni et al. 2015; Jones et al. 2020). Connections between the synthetic genetic device and other devices (whether cellular or synthetic) can lead to retroactivity (Del Vecchio et al. 2008). The synthetic genetic device is also directly influenced by changes in cell state, which alter the concentration and availability of endogenous and exogenous molecular components. The device may also drive both prescribed and unprescribed changes to the cell state. Stochasticity due to mesoscopic fluctuations in the biochemical reactions involved in genetic device activity also perturb device function. This can result from the noisy nature of gene expression(Raser and O'Shea 2005), variability in DNA copy numbers (Bleris et al. 2011), and cell division partitioning error (Del Vecchio and Murray 2014). (**C**) The genetic context imposes positional effects due to nearby transcriptional activity, such as enhancers (Liu et al. 2015) and read-through (Loughran et al. 2014; Li and Zhang 2019), as well as chromatin state effects, such as DNA coiling and torsional effects (Allis et al. 2007; Yeung et al. 2017; Allis and Jenuwein 2016; Yeung et al. 2017).

**Figure 6: Using genetic controllers to adapt genetic device outputs to disturbances.** (**A**) There are two basic architectures of controllers: feedback and feedforward. In feedback control (top), the output is compared to a set point (input) and when the output level and set point are different (for example, due to disturbance inputs), the controller actuates the device to change its output. In feedforward control (bottom), the controller directly senses disturbance inputs and then actuates the genetic device to offset the effects of the disturbances. (**B**) Adaptation to disturbances enabled by controllers. In response to a disturbance ($d$), the controller responds by actuating the device. This should restore the device output to the set point at steady-state, yielding adaptation. If there is a difference between the device output and set point at steady-state (nonzero steady-state error), then the controller provides imperfect adaptation to disturbances. (**C**) Generalized structure of a feedback controller. An arbitray controller network is connected to an arbitrary controlled network (one theoretical example of each shown) via sensing of the controlled network's output and actuation into one of its nodes. These controllers can achieve robustness to perturbations that affect genetic devices within or upstream of the feedback loop created by the controller, so long as the perturbation affects the information path between the controller actuation and sensing reactions. A benefit of feedback control is the ability to adapt to any uncertainty in the controlled system itself (again, as long as perturbations are on the path between actuation and sensing). This uncertainty can include uncertain parameters, noise, the effects of disturbance inputs, unmodeled dynamics, and other unknowns. (**D**) Generalized structure of a feedforward controller. Similarly to before, arbitrary controller and controlled system networks are connected, though in this case the controller senses the disturbance itself rather than the output of the controlled network. These controllers can impart adaptation to disturbances that the controller can sense, and have a reduced risk for instability compared to feedback controllers. However, feedforward

controllers cannot adapt to perturbations that affect the plant only but not the controller, such as parameter uncertainty.

**Figure 9: Feedback and feedforward controller implementations.** (**A**) One topology capable of quasi-integral feedback control is enzymatic feedback (see **Box 2** and **Figure 7** for mathematical treatment). In this design, two factors competitively catalyze the conversion of a gene regulator between an active and inactive state. (i) A well-studied version of this controller is implemented with a kinase and phosphatase that competitively phosphorylate and dephosphorylate a TF (Chang et al. 2013; Qian and Del Vecchio 2018; Jones et al. 2021). This design has been shown capable of adapting output expression to various inputs, including input pulses (Chang et al. 2013) and off-target regulation and resource competition (Jones et al. 2021). (ii-iii) Another conception of this basic design is through enzymatically-induced decay of the controller species at either the (ii) RNA (Lillacci et al. 2018; Bloom et al. 2015) or (iii) protein (Ng et al. 2019) level. The forward reaction in this case is encapsulated by the production of the controller species. Such designs have been shown to enable adaptation of output expression to several disturbances, including copy number variation (Lillacci et al. 2018), transcriptional perturbations (Bloom et al. 2015), and induction of protein decay (Ng et al. 2019). Note that no work thus far has demonstrated *in vivo* quasi-integral control with this design. (**B**) Another topology capable of quasi-integral feedback control is antithetic feedback (see **Box 2** and **Figure 7** for mathematical treatment). In this design, two controller species irreversibly bind and form an inert complex or annihilate (Briat et al. 2016). One species must be responsible for sensing the controlled system's output and the other sensing the reference input (Aoki et al. 2019). (i) The earliest implementation of this design used scaffold and anti-scaffold proteins to dynamically track a reference input set by one of the scaffolds, which connected a kinase to a substrate (Hsiao et al. 2015). (ii-iv) In the last few years, several successful implementations of the antithetic feedback design using (ii) bacterial small RNA (sRNA) (Huang et al. 2018), (iii) bacterial sigma factors (Aoki et al. 2019), or (iv) antisense RNA (Frei et al. 2020b) as the sequester species have been shown to achieve quasi-integral control and impart robustness to several perturbations. These perturbations range from changes in protein decay rates (Aoki et al. 2019; Frei et al. 2020b) to overloading of ribosome availability via resource competition (Huang et al. 2018). The design by Frei *et al.* was even shown to enable adaptation to network perturbations in the controller, demonstrating the *in vivo* versatility of this controller design. (**C**) Near-perfect adaptation to disturbances has been achieved using several feedforward control designs. Feedforward controllers have typically been made via the incoherent feedforward loop (iFFL) motif (Mangan and Alon 2003), in which an upstream node both positively and negatively regulates a downstream node. (i) In both mammalian cells (Bleris et al. 2011) and bacteria (Segall-Shapiro et al. 2018), feedforward control using TFs has been used to offset changes in DNA copy number. The latter was highly successful due to the use of TALE repressors, which unlike many TFs do not bind to DNA cooperatively (Segall-Shapiro et al. 2018). (ii-iv) Another class of feedforward controllers have used enzymatic degradation of an output molecule via miRNAs (Bleris et al. 2011; Strovas et al. 2014; Lillacci et al. 2018; Frei et al. 2020a), proteases (Gao et al. 2018), or endoRNases (Jones et al. 2020). These designs have also enabled adaptation to DNA copy number (Bleris et al. 2011; Lillacci et al. 2018; Jones et al. 2020), as well as resource competition (Frei et al. 2020a; Jones et al. 2020), transcriptional inducers (Strovas et al. 2014), and changes in protease production (Gao et al. 2018). (v) A recent study demonstrated feedforward control of ribosome levels to offset loading of the ribosome by the output protein by co-expressing SpoTH, the hydrolysis domain of SpoT, a positive regulator of ribosome activity in bacteria (Barajas et al. 2021). This was shown to reduce the effects of resource loading both on other genes and on cell growth rates.

**Figure 10: Future applications for context-aware genetic controllers in regenerative medicine.** (**A**) Context-aware genetic devices can provide robust control over the expression of genes of interest at the molecular level, while also embedded in synthetic regulatory networks that rely on control systems to direct cell state changes. These networks are further embedded in multicellular environments where feedback between engineered cells can be used

to control the dynamics of the multicellular system. These layers of control allow for robust and predictable cell behaviors to be achieved. (**B**) Context-aware genetic controllers can be applied to PSC-derived engineered T cells for cancer therapeutics. Two examples are proposed. The first optimizes the derivation of T cells from PSCs through the controlled overexpression of TFs, where genetic controllers provide dynamic control over the levels of these TFs that guide individual cells along this optimal differentiation trajectory through real-time sensing of the cell's state. Finally, controllers can be used to confer the ability of these engineered T cells to home to and trigger the selective killing of tumour cells in the patient's body. Further, the cells can be made to engage in self-sensing to control their population size, as well as sensing of markers of the overall systemic state, allowing the T cells to self-limit their killing activity as needed. The functions of these circuits are made robust through context-aware circuit design, which ensures that their functions are undisrupted by the changing cell state and microenvironment. These designs can also be used to mitigate resource loading effects and ensure that circuit function is preserved across cell types, where initial testing can be done in cell lines and later transitioned to PSCs.

## Box 1: Near-perfect adaptation via quasi-integral feedback control

A key approach for a system to achieve perfect adaptation to disturbances is through integral feedback control (Åström and Murray 2008; Del Vecchio and Murray 2014). With integral control, the error between the measured output and set point is summed up over time, with the strength of the feedback actuation being proportional to the summed (integrated) error. Thus, as the controller applies a correcting action to reduce the error, it can determine whether to increase or decrease its correcting action depending on the persistence of the error. This contrasts most relevantly with proportional control, in which the correcting action is proportional to the current magnitude of the error. The memory of past error critically enables an integral controller to eliminate steady-state error and thus achieve perfect adaptation. Proportional control cannot, alone, eliminate error, but is frequently used in combination with integral control to improve dynamics and minimize overshoot (Åström and Murray 2008). Here we will discuss different mechanisms by which integral control can be implemented in cells and specific implementation challenges.

In general, integrators are devices that compute the difference between the levels of two different molecular species in the cell and integrate it over time (Briat et al. 2016). Mathematically, integral controllers within reaction networks can take the following form:

$$\begin{aligned} \frac{dX}{dt} &= \alpha \cdot f(Z) - \delta \cdot X + d_1 \\ \frac{dZ}{dt} &= K \cdot (u - X) \\ \Rightarrow Z(t) &= K \cdot \int_0^t (u - X(\tau)) \cdot d\tau, \end{aligned}$$

where $K$ encapsulates the feedback gain, $u$ is a reference input (set point) that is compared to $X$, $d_1$ is a disturbance, and $\alpha$ and $\delta$ are the production and decay rates of $X$, respectively. Here, the error is the difference between $u$ and $X$, which is integrated over time, multiplied by the feedback gain $K$, and used to alter the concentration of $Z$. As long as partial $\partial f(Z)/\partial Z > 0$, the system's equilibrium is stable and at steady state we obtain that $X = u$, independent of $d_1$.

A key challenge to the biological implementation of an integral function is that the controller species $Z$ can decay

through either cell division or degradation:

$$\frac{dX}{dt} = \alpha \cdot f(Z) - \delta \cdot X + d_1$$
$$\frac{dZ}{dt} = K \cdot (u - X) - \gamma \cdot Z,$$

where $\gamma$ is the decay rate of $Z$. The decay causes the integrator to become "leaky" and thus no longer correctly sums the error over time (Qian and Del Vecchio 2018). Given the omnipresence of molecule dilution and degradation, this leakiness makes perfect integrators nearly impossible to achieve within a cell. To restore the performance of a leaky integral controller, all of the controller reaction rates should be made fast compared to the decay rate of the controller species, leading to "quasi-integral" control (Qian and Del Vecchio 2018; Huang et al. 2018). In our example, quasi-integral control is possible through a large feedback gain $K$, leading to $Z(t) \approx K \cdot \int_0^t (u - X(\tau)) \cdot d\tau$. However, if $K$ is too large, the controller can cause the output level to overshoot the set point and oscillate (Åström and Murray 2008; Del Vecchio and Murray 2014).

There are two main types of reactions that have been proposed to implement quasi-integral control (**Figure 7**): enzymatic (left) and sequestration (right) reactions carried out by RNA or protein regulators ($\hat{Z}$) on RNA or protein substrates ($Z$). In both of these designs, the steady state level of the output can be made practically independent of all the indicated parameters, which are functions of disturbance inputs ($R$). Under stability conditions, for which we require $\partial f(Z)/\partial Z > 0$, the output can adapt to changes in all parameters (and thus in $R$) for $K$ sufficiently large. In the enzymatic reaction implementation, to obtain the form of differential equations in **Figure 7**, it is necessary that both the forward and backward enzymatic reactions operate in the zero-order regime (Atkins and Paula 2006; Goldbeter and Koshland 1981; Del Vecchio and Murray 2014) (see derivation by Qian *et al.* (Qian and Del Vecchio 2018)). The need for a zero-order regime limits the function of the system as a quasi-integral controller to conditions where the concentrations of both $Z$ and $\hat{Z}$ are sufficiently high. For the sequestration reaction-based design, the mutual sequestration of $Z$ and $\hat{Z}$ can occur either through an irreversible binding reaction in which the complex formed is non-functional or through a reversible binding reaction where the complex is irreversibly converted to an inactive species (Briat et al. 2016; Huang et al. 2018; Aoki et al. 2019). In the latter case, the concentrations of neither $Z$ nor $\hat{Z}$ can be in excess of one another, such that the rate of complex formation is approximately proportional to the product $Z \cdot \hat{Z}$, leading to the form of equations in **Figure 7**.

In both implementations, to achieve robustness to perturbations $R$, it is not necessary that $X$ enters linearly the dynamics of $Z$, $\hat{Z}$, and $Z^*$. Indeed, $X$ could enter through an increasing function $g(X)$ and the same robustness property would hold. However, the quasi-integral feedback control fails to restore the output to the set point in regimes where the $X$ becomes insensitive to $Z$. This can happen, for example, if $Z$ activates the production of $X$ and approaches saturation.

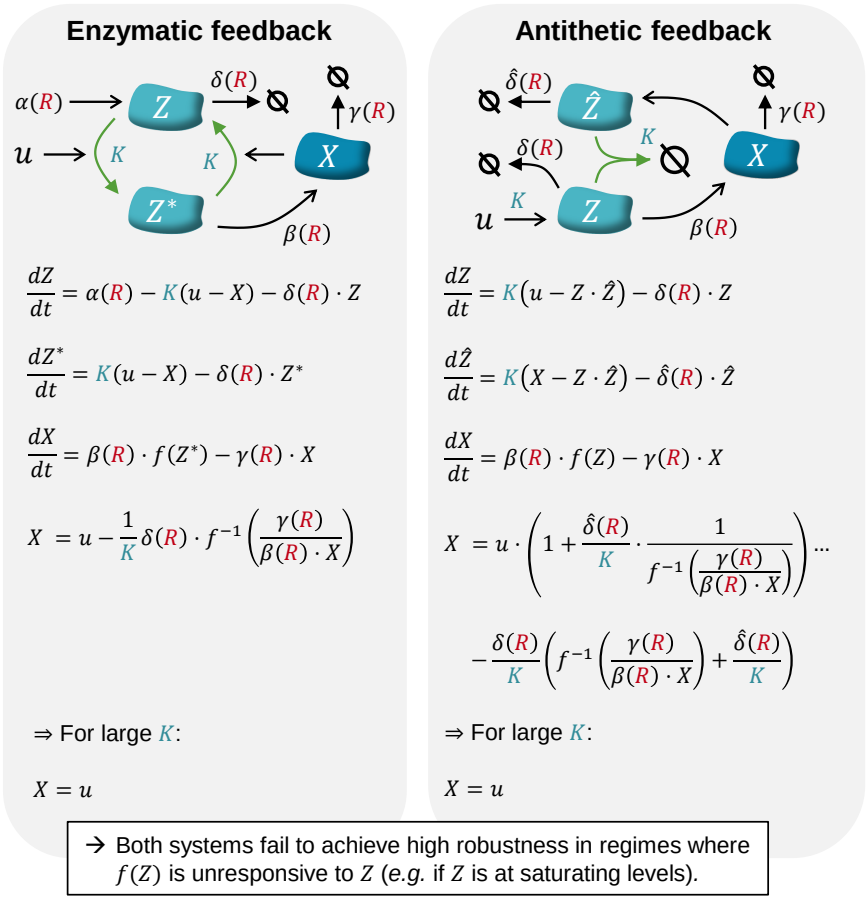



**Figure 7:** Basic topologies for quasi-integral feedback control

## Box 2: Example of solving context-dependence with feedforward control

To solve any problem of context-dependence in engineering cell behavior, it is first necessary to identify the source of context-dependence. For example, we identified that resource sharing, primarily at the transcriptional level, causes expression of simple genetic devices to unexpectedly change upon introduction of other genes into mammalian cells (Jones et al. 2020). More detailed analysis identified "squelching" to be the culprit. Squelching occurs when TFs sequester coactivator resources from other genes, regardless of whether the TF is actively bound to its target gene or not (Gill and Ptashne 1988; Berger et al. 1990). Thus, the effect of squelching by a TF is to reduce the transcription rate ($\alpha$) of other genes. Resource sharing can cause any rate in the expression of a gene to become coupled among genes competing for the shared resource. Examples of such resources acting as disturbance inputs ($R$) to a genetic device are given in **Figure 8A**.

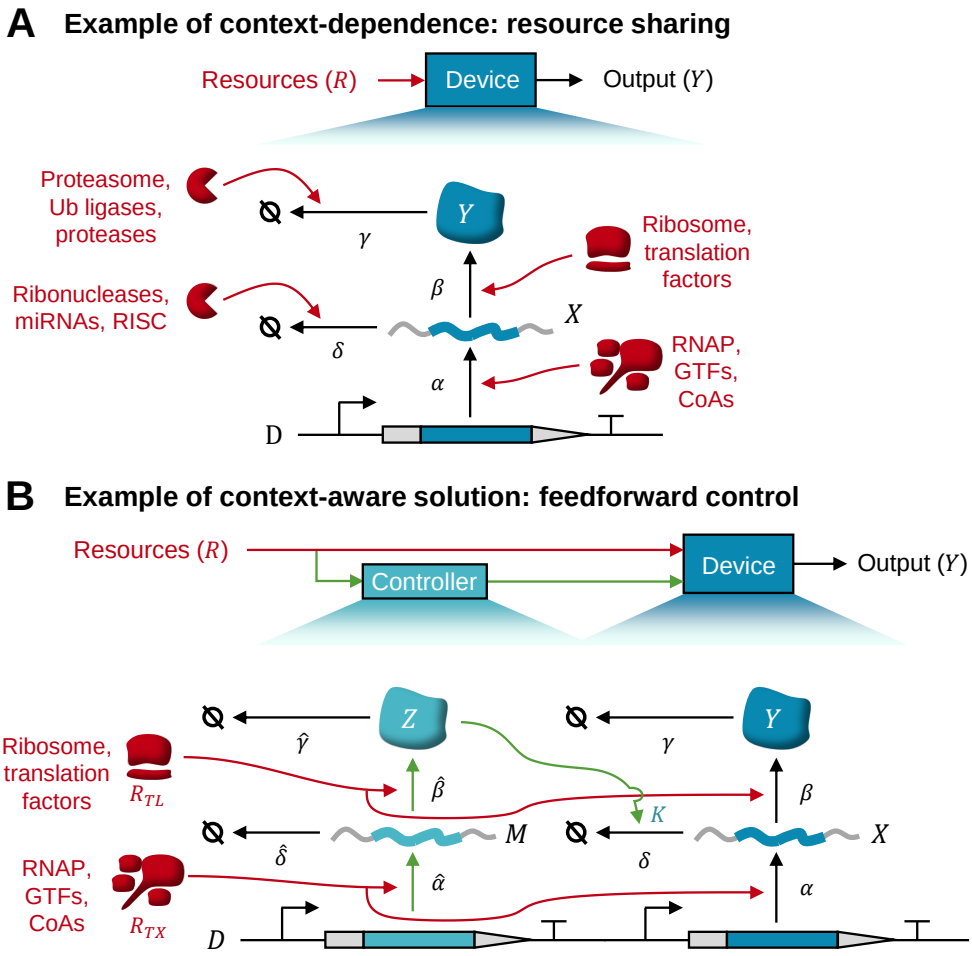
To decouple gene expression from transcriptional/translational resource loading, we designed a feedforward-controlled device in which the controller senses RNA and protein production resources, $R_{TX}$ and $R_{TL}$, respectively, and then actuates the genetic device through post-transcriptional repression (**Figure 8B**). In response to changes in production resources that are disturbance inputs to both the controller and the output, the feedforward controller actuates an offsetting signal to maintain a set level of output expression. In our design, the controller was implemented through

a protein endoRNase that enzymatically destroys the output mRNA (Jones et al. 2020), though a conceptually similar design with miRNAs instead of endoRNases can give similar robustness to loading of transcriptional resources (Frei et al. 2020a).

The ability of our feedforward controller to offset changes in resource availability can be seen by analyzing the differential equations describing the system (**Figure 8C**, left). Note that here we have assumed for simplicity of illustration that the concentration of $X$ is well below the point of saturating the action of $Z$, such that the degradation of $X$ depends linearly on both $X$ and $Z$.

Without the controller (*i.e.* when $K = 0$, or equivalently when $M$ and $Z$ are absent), the expression level of the output $Y$ is dependent on cellular resources used for both transcription ($R_{TX}$) and translation ($R_{TL}$) (**Figure 8C**, right). With the controller and for large values of $K$, the steady-state of $Y$ reduces to an equation that is independent of the transcription and translation resources $R_{TX}$ and $R_{TL}$, respectively (**Figure 8C**, right). Indeed, in our experimental results, we found that the controller could perfectly offset changes in resource availability caused by squelching (Jones et al. 2020).

An important note is that the adaptation enabled by the controller does not necessarily hold if the change in resource availability is accompanied by a change in the growth rate of the cell. The above analysis assumes that the decay rates are constant, but such a perturbation can differentially affect the decay rate ($\gamma$) of proteins that are relatively stable such that their decay is dominated by dilution from cell growth/division (Jones et al. 2020). Most mRNAs and unstable proteins have decay rates dominated by degradation (Schwanhäusser et al. 2011), and thus are less affected by changes in cell growth. If the controller ($Z$) and output ($Y$) species have substantially different degradation rates, then changes in cell growth rate resulting from changes in resource availability will cause the $\gamma$ and $\hat{\gamma}$ parameters to differentially change and perturb the observed level of output $Y$ (Jones et al. 2020). Thus, care should be taken when implementing a feedforward controller to ensure that the effect of the disturbance on the output can be properly sensed and offset accordingly.




## C Model analysis

**Generalized differential equations**:

$$\frac{dM}{dt} = \hat{\alpha} \cdot R_{TX} \cdot D - \hat{\delta} \cdot M$$

$$\frac{dZ}{dt} = \hat{\beta} \cdot R_{TL} \cdot M - \hat{\gamma} \cdot Z$$

$$\frac{dX}{dt} = \alpha \cdot R_{TX} \cdot D - K \cdot Z \cdot X - \delta \cdot X$$

$$\frac{dY}{dt} = \beta \cdot R_{TL} \cdot X - \gamma \cdot Y$$

Without controller ($K = 0$): $Y = \frac{\alpha \cdot \beta}{\delta \cdot \gamma} \cdot R_{TX} \cdot R_{TL} \cdot D$

With controller ($K > 0$):

$$Y = \frac{\alpha \cdot \beta \cdot R_{TX} \cdot R_{TL} \cdot D}{\left(\delta + K \cdot \frac{\hat{\alpha} \cdot \hat{\beta}}{\hat{\delta} \cdot \hat{\gamma}} \cdot R_{TX} \cdot R_{TL} \cdot D\right) \cdot \gamma}$$

For large $K$:

$$Y = \frac{U}{K} \quad | \quad U = \frac{\alpha \cdot \beta}{\hat{\alpha} \cdot \hat{\beta}} \cdot \frac{\hat{\delta} \cdot \hat{\gamma}}{\gamma}$$

→ The output is robust to all resources needed for transcription ($R_{TX}$) and translation ($R_{TL}$) of both $Z$ and $Y$. Note the tradeoff in robustness and output level imposed by increasing $K$.

**Figure 8:** Example of solving context-dependence with feedforward control

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