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Small RNAs, big potential: Engineering microRNA-based synthetic gene circuits

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MicroRNAs (miRs) are small non-coding RNAs that regulate gene expression. Their dysregulation is closely associated with various diseases, positioning them as biomarkers of cellular state. Synthetic biology has leveraged these properties to engineer miR-based genetic circuits capable of sensing and interpreting endogenous miR levels. Early miR-OFF systems relied on reporter gene repression but were limited by ambiguous signal interpretation. Subsequent advances introduced miR-ON architectures, logic-based classifiers integrating multiple miRs, and layered regulatory strategies combining transcriptional, translational, and cleavage-based modules to enhance sensitivity and specificity. Recent innovations include CRISPR-associated miR-responsive systems and incoherent feed-forward loop (iFFL) architectures that stabilize gene expression amid cellular variability, shifting applications from passive sensing to therapeutic intervention. Despite challenges such as leakage, cellular resource resources, and delivery, progress in orthogonal miR toolkits, computational modeling, and RNA-based delivery platforms is rapidly driving miR-based circuits toward diagnostic and therapeutic applications.

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Introduction

miRs are small, non-coding RNA of approximately 18–25 nucleotides in length. They play a central role in post-transcriptional gene regulation by modulating the stability of the mRNA. Dysregulation of miR expression is strongly associated with a wide range of pathological conditions, including cancer, where altered miR profiles disrupt gene regulatory networks and cellular homeostasis. As a result, miR expression patterns inside the cell provide an informative snapshot of the cellular state. They can be formed in cells either in the canonical way, where they are transcribed as primary RNA transcripts with a size of about 150 nt, which is then processed into mature miR by a series of exoribonucleases, or can be synthesized as miRtrons, where the miRs are extracted from the intragenic segments or the introns of coding genes [1]. While various genetic and biochemical studies have shown that miRs act in most cases as suppressive modulators in the overall flow of genetic circuits [1–3], numerous studies have also highlighted their role as inducers of gene transcription [4–7].

As our understanding of miRs deepens over time, it is clear that they provide essential information about cellular health and can serve as potential biomarkers for a wide range of cellular conditions, particularly diseases [8–10]Synthetic biologists have leveraged this property by designing miR-based synthetic circuits that sense the cellular state by incorporating miR targeting sites within the genetic circuits, enabling understanding of the dynamic information about miR regulation.

In this review, we describe how miR-based synthetic sensor circuits have evolved, what limitations have been encountered in the development of these circuits, and how they are addressed in the next generation of circuits.

Architecture of the miR-based synthetic circuits

As the role of miRs have been primarily recognised as key regulators of gene silencing, most of the early synthetic circuits leveraged their repressive function for detecting their presence. The circuits were typically

designed so that a reporter gene contains completely complementary miR target sites in its 3′ untranslated region (3′ UTR), effectively placing the reporter under the control of miR regulation [11–16]. In the presence of miRs in the cell, and depending on the number of target sites, the miRs bind to these sites and repress reporter expression either by mRNA degradation or mRNA deadenylation [1,17]. Another way of designing these circuits is the incorporation of miR target sites in the 5′ UTR positioned immediately adjacent to the AUG start codon. In this design, reporter expression is repressed either by endonucleolytic cleavage mediated by the RISC complex or by sterically blocking the ribosome from accessing the initiating site for translation [17,18]. As a result, these architectures were termed as miR-OFF systems, as the signal from the reporter is downregulated due to the presence of miR [13–16] (Fig. 1a).

The problem arising from these types of reporters is the indirect correlation of miR expression through a loss of optical signal, which increases the likelihood of false positives. A decrease in reporter expression can not be confidently attributed to a direct correlation of miR dynamics, as it may also be caused by other unrelated factors, such as cell death, promoter inefficiency, epigenetic silencing, low imaging sensitivity, or equipment malfunction [19–21]. These variables cause an ambiguity in determining the reliability of the results. To overcome some of these limitations, circuit architectures have been designed that rely on the expression of the reporter in a direct correlation with the miR activity. These designs utilise a regulatory module under the negative influence of miR, which in turn positively activates the reporter gene in the presence of miRs, thus providing a direct correlation of the miR activity inside the cell by giving a positive optical output. Further, this system offers a spatio-temporal resolution of the miR dynamics at the single-cell level with a sensitivity that has never been confidently achieved in the miR-OFF systems [19,20,22,23]. These designs also enable the selection and segregation of target miR-positive cells for monitoring the “live” miR dynamics at a single-cell level [21]. Such circuits are called miR-ON systems, emphasizing their ability to produce a signal not only when the miR is expressed but also when it is functionally active (Fig. 1b).

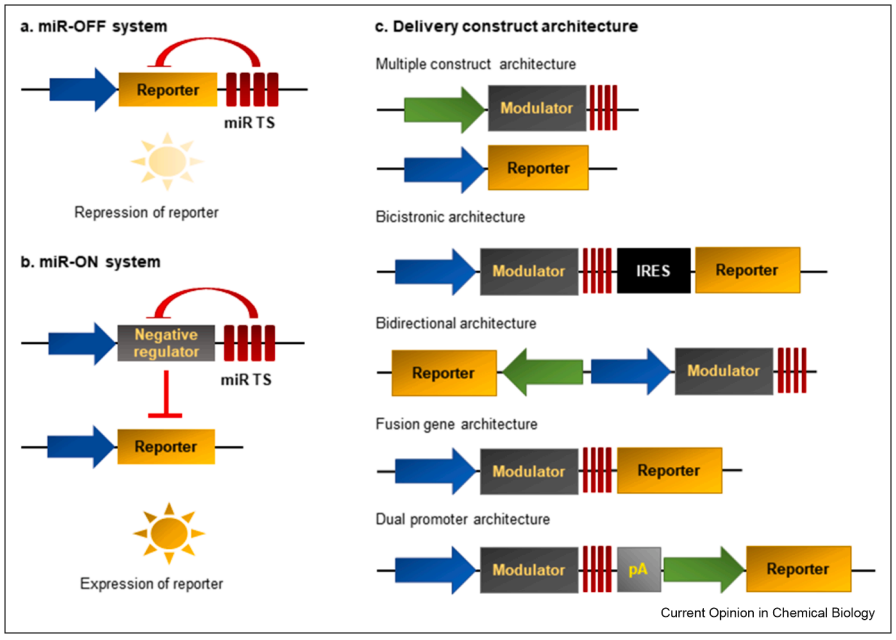
While the miR-ON circuit provides a measurable output for assessing the miR activity, it is important to understand the layers of regulation employed by these designs. Similar to all other synthetic circuits, their regulations can be either post-transcriptional-based, post-translational-based, or based on the cleavage of either RNA or protein. The architecture can be designed either as a two construct system, as a bicistronic system, a bidirectional promoter system, a fusion protein system, or with a dual promoter strategy (Fig. 1c).

Post-transcriptional regulation-based architecture

For transcription-based miR-ON systems, a lot of inspiration has been drawn from the prokaryotic repressor-based operons such as the Cumate gene switch inducible operon [20], the Tet R [23], Tet-Krab [21], or, in some cases, the yeast Gal4 binding domain [24]. These operons were successfully repurposed by placing their repressor proteins under the control of miRs, enabling miR-dependent transcriptional derepression resulting in activation (Fig. 2a). However, adapting prokaryotic modulators to mammalian systems requires careful optimisation, as uncontrolled dosing can significantly perturb the host transcriptome *in-vitro* [25], and similar concerns also arise regardng the risk of eliciting immunogenic responses in immunocompetent *in-vivo* models [26–29]. Incorporating KRAB-based reporters further introduces the risk of epigenetic silencing of over several tens of kilobases, potentially altering local chromatin states, along with the concern of inactivating endogenous genes flanking the reporter integration site in gene therapy applications [30,31]. In addition, another persistent limitation associated with these regulators is the leaky expression of reporters in the absence of target miR, often driven by promoter dysfunction and tissue-independent variability [19–21].

Among transcriptional-level miR regulators, the delivery strategy most often relies on DNA-based vectors, either for transient expression using plasmid DNA (pDNA) or for stable expression delivered through viral vectors [23,32]. However, viral vectors raise important safety concerns due to their potential for genomic integration, which can disrupt host genes or regulatory elements. A safer alternative is to deliver the genetic payload as mRNA rather than DNA, since mRNA remains cytoplasmic and therefore eliminates the risk of insertional mutagenesis [33]. To overcome such limitations, synthetic RNA-based, RNA replicon-based, and modified RNA (modRNAs)-based synthetic circuits have been designed [34–38]. These systems maintain similar expression levels compared to pDNA and offer a safer alternative. One such design encodes the RNA-binding proteins (RBPs), L7Ae and MS2-CNOT7 that cross-repress each other in a siRNA-dependent manner, enabling bistable switching and feedback regulation [*36]. L7Ae binds to the K-turn repeats present on the 3′ UTR of the MS2-CNOT7 in a siRNA-dependent manner, whereas MS2-CNOT7 inhibits the expression of L7Ae by binding to its target sites on the 5′ UTR of L7Ae and deadenylating its mRNA, thereby destabilising it (Fig. 2b). Further, additional RBP-based logic gates have also been engineered to demonstrate the application of these RNA-based miR sensors as cell-type classifiers [37], toggle switch designs combining TetR as small-molecule-responsive RBP along with L7Ae to generate dual-output regulatory switches [38].

Figure 1

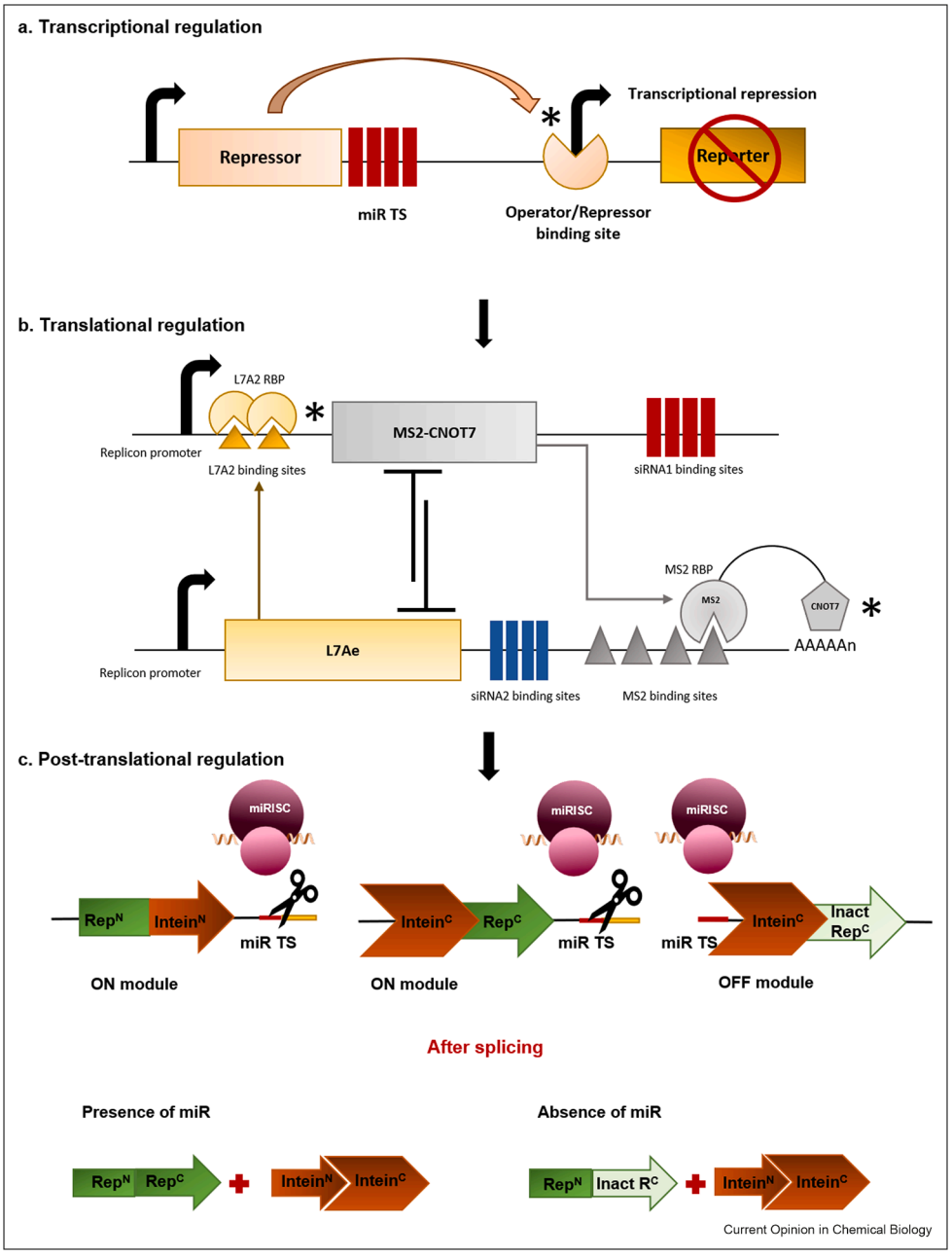


Major design principles underlying miR-based synthetic circuits and the different construct architectures used to implement them.
a. miR-OFF system: This panel illustrates how the miR-OFF construct is organized at the DNA level. The blue arrow represents the promoter, the yellow box denotes the reporter gene, and the red elements depict the tandem miR target sites (miR-TS) that are incorporated downstream of the reporter within the 3′UTR. Although these target sites are encoded in DNA, they become functional only at the RNA transcript level, where they form active miR-binding sites. When the cognate miR is present, it binds to these target sites and recruits the RISC, resulting in transcript degradation or translational repression of the reporter mRNA. This architecture therefore functions as a loss-of-signal or "miR-OFF" sensor, where reporter output inversely correlates with intracellular miR levels.
b. miR-ON system: This panel illustrates the DNA-level construct design used to achieve miR-dependent activation of the reporter. The blue arrow in the first construct represents the promoter that drives expression of a negative regulator gene (gray box). Downstream of this negative regulator, multiple miR-TS (red colour) are incorporated into its 3′ UTR. When the construct is transcribed, these miR-TS sequences become active binding sites for the cognate miR. In the absence of the target miR, the negative regulator is expressed and represses the reporter expression. However, in the presence of the cognate miR, the miR binds to the miR-TS on the negative regulator transcript, leading to its degradation or translational repression. As a result, the repression on the reporter is lifted. This indirect regulatory logic enables selective reporter expression in miR-positive cells, thereby functioning as an "miR-ON" sensor.
This configuration therefore converts miR activity into a gain-of-signal response.
c. Construct architectures for implementing miR-based sensors:
Various expression designs can be used, to co-express the modulator (e.g., repressor or regulator) and reporter components of the miR sensors: The blue and green arrows show promoter for individual DNA constructs.
1. Multiple construct architecture: The modulator with the miR-TS and the reporter are placed on separate vectors or genomic constructs. This offers flexibility in expression levels and delivery strategies but may introduce variability due to differences in copy number or transfection efficiency [23].
2. Bicistronic architecture: Both the modulator and the reporter are encoded on a single transcript, separated by an internal ribosome entry site (IRES). This ensures coordinated transcription while enabling independent translation of both proteins [76].
3. Bidirectional architecture: A bidirectional promoter or two promoters placed opposite to each other drives transcription outward in two directions, allowing simultaneous yet distinct expression of the modulator and reporter from the same locus [17]
4. Fusion gene architecture: The modulator and reporter are expressed as a continuous open reading frame, yielding a fusion protein. In the absence of miRs, the reporter is expressed, however, in its presence, the miR binds to the target sites, and the RISC complex cleaves the transcript, rendering it dysfunctional. [77].
5. Dual promoter architecture: A single construct contains two independent promoters, one regulating the modulator and one the reporter, allowing separate but co-localized expression modules [20].

Post-translational regulation-based architecture

As we climb the hierarchy of regulation of our reporter expression, architectures based on post-translational regulation emerged as a fascinating frontier, offering fine-tuning of the outputs. Existing single-input RNA switches that have been discussed often fail to achieve sufficient cell-type specificity because they suffer from two major limitations: leaky translational control [19–21], which causes undesired protein expression in the OFF state, and the lack of uniquely distinguishing biomarkers that can reliably separate target from non-target cells [39]. Although multi-input RNA switches can address the marker limitation, their increased

Figure 2



Detailed schematic representation of transcriptional, translational, and post-translational regulation used in miR-based synthetic circuits. Black arrows in the circuit represent promoters, and asterisks (*) denote the specific sites where the regulatory protein acts.

a. Transcriptional regulation: A transcriptional repressor is placed under miR control. In the absence of the cognate miR, the repressor protein is expressed and binds to its operator sequence adjacent to the promoter, blocking transcriptional initiation through steric hindrance or active repression. In the presence of miR, the repressor transcript is degraded or translationally suppressed, relieving promoter blockage and enabling reporter transcription. [20,21,23,24].

b. Translational regulation: The RNA-binding proteins (RBPs) L7Ae and MS2–CNOT7 are expressed from two separate promoter-driven constructs. L7Ae recognises and binds the K-turn RNA motifs placed in the 5′ UTR of the MS2–CNOT7 transcript and interferes with ribosomal loading, while MS2–CNOT7 binds MS2 hairpin structures engineered into the 3′ UTR of the L7Ae transcript and recruits the CNOT7 deadenylase complex, thereby destabilising the mRNA and repressing translation. By placing each RBP under miR control, only cells expressing the target miR reduce RBP levels, breaking this mutual inhibition and permitting translation of the corresponding partner protein [36].

c. Post-translational regulation: Intein-based miR-sensors operate at the level of protein assembly. The ON and OFF modules each contain split reporter fragments fused to inteins, expressed from promoters. The OFF module resembles a conventional miR-OFF system in which miR target sites are placed in the 5′ UTR of the transcript, causing RISC-mediated repression in the presence of the cognate miR. The OFF module expresses a dominant-negative split inactive reporter fused to one half of the split intein. The ON module contains the N-terminal fragment of the reporter fused to the complementary split intein. This transcript also includes an additional sequence located downstream of the poly(A) tail, which is selectively removed in the presence of

complexity often introduces noise and reduces the ON/OFF ratio [36,37,39]. One particularly clever design that tries to overcome this challenge uses splicing-based regulation to generate reporter activity. This design utilises miRs to assemble split inteins and to assemble a functional reporter protein, resulting in improved sensitivity. The circuit is divided into two complementary modules − the OFF and ON modules. The OFF module resembles the typical miR-OFF system, where the reporter transcript carries miR target sites on its 5′ UTR and is repressed in the presence of the cognate miR. However, instead of a full reporter, this module expresses a dominant negative version of the C-terminal fragment of the reporter fused to a split intein. The ON module contains a fusion of the remaining split intein along with the active split reporter protein, but flanked by an extra sequence downstream from the poly A tail, which gets cleaved in the presence of miR, allowing expression of the functional protein fragment. The split-intein NpuDnaE is fused to the N-terminal of the split reporter in one module, followed by the complementary intein fused to the dominant negative C-terminal OFF module as well as the functional C-terminal ON module, which in the presence of miR provides a functional correlation of the activity of miRs [**40] (Fig. 2c). This split-RNA switch system thus promises a sharp, multi-input RNA-based gene regulation capable of simultaneously sensing the combinations of different miRs and proteins. The original design was developed without incorporating RBPs, given that overexpression of L7Ae in mammalian cells has been associated with reduced growth rates and potential cytotoxicity. [38,41]. Finally, the design remains compatible with RNA-based delivery, further supporting its potential for modular and programmable gene-control applications.

While the split-RNA switch system provides a possibility to combine RBPs along with split inteins, however their regulation is still limited to a single layer. Another design to overcome the problem of leakiness is the introduction of a dual regulation, i.e., at both the transcriptional and translational levels as a fail-safe mechanism. In this approach, miR target sites are incorporated into the 5′ and 3′ UTRs of the transcriptional repressor LacI and the translational repressor, RBP L7Ae, respectively, where both genes are expressed from a single operon. As the LacI produces stronger transcriptional repression, L7Ae maintains the translational repression, and while both of them are under the same miR, the system remains tight and without background activity. This double-layered repression reduces leakiness and sharpens the fold change of reporter activity by improving a quantitative estimation of miR dynamics [42].

Beyond improving repression strength and reducing leakiness using dual regulation, miR-regulated modules can be further combined into higher-order circuits that perform multi-signal integration for complex decision-making. These advanced miR-based circuits, called cell classifiers, have been developed for discriminating between different cellular states based on miR profiles. Classifiers combine multiple endogenous miR target sites to compute logical operations like AND, OR, NOT, and AND NOT based on the presence or absence of specific endogenous miR. The double-inversion module design implements a two-layer repression strategy that enables output expression only when the miR is present at or above its characteristic level in HeLa cells, while efficiently repressing output when miR levels are low. In this design, the miR represses an activator of a downstream repressor protein, which is itself regulated by the same miR, thereby permitting expression only under the appropriate miR context. More advanced classifiers further integrate multiple endogenous “high” and “low” miRs to create cell-specific circuits, enabling precise distinction between different cellular identities and disease states [43−45] (Fig. 3a).

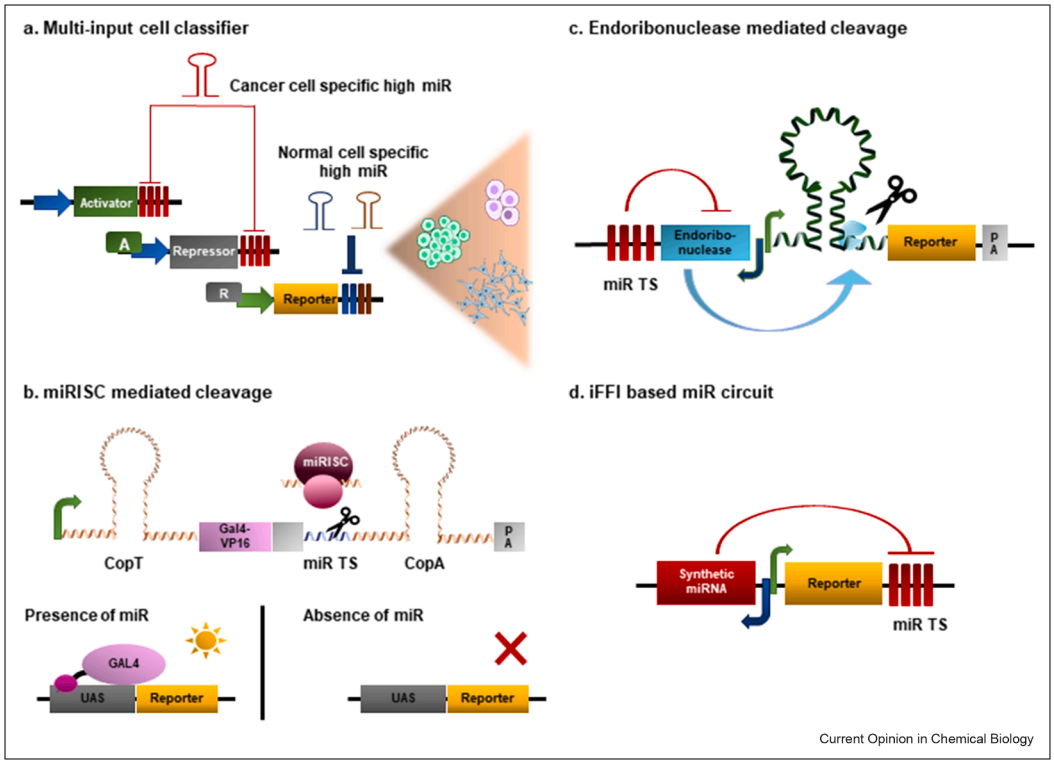
Cleavage-based regulation architecture

Another level of control, whose regulatory action is independent of participating in the competition of utilising endogenous resouces, are the cleavage-based systems, where regulation of modulators is independent of the host transcriptional or translational load and promise an orthogonal mammalian-compatible control. One prominent example is harnessing of a Csy4 bacterial endonuclease for targeted cleavage of the reporter RNA. When Csy4 is kept under the miR control, the presence of miRs indirectly allows the reporter expression [46], which is restricted by Csy4 in the absence of miR. Beyond RNA endonucleases, proteases can be similarly wired under miR control to regulate circuit behavior through conditional protein cleavage. Rather, they can be coupled with RBPs to obtain an orthogonal output in a siRNA dependent manner [47].

Another interesting design leverages the endoribonuclease activity of the miRISC to separate the steric inhibitory CopT-CopA RNA complex from the reporter transcript. The CopT-CopA RNA complex in the

the target miR. miR-dependent cleavage eliminates this inhibitory region, enabling proper expression and folding of the functional N-terminal reporter fragment. When both intein halves are present, NpuDnaE−mediated protein splicing reconstitutes the full reporter protein. In the absence of miR, the OFF-module dominant-negative fragment remains abundant and pairs with the ON-module fragment, yielding an inactive product. In the presence of miR, repression of the OFF module combined with activation of the ON module allows productive intein splicing that restores the full, functional reporter, thereby creating a post-translational output that correlates with miR activity [**40].

Figure 3



Schematic overview of advanced miR-based synthetic circuit architectures used for cell-type classification and gene therapeutics.
a. Multi-input cell classifier implementing a NOT−AND logic gate: This design integrates multiple endogenous miRNA inputs to distinguish cancer cells from normal cells based on their characteristic miRNA expression signatures. In cancer cells, high levels of cancer-specific miRNAs simultaneously repress both (i) the repressor and (ii) the upstream activator that normally enhances repressor expression. Because both the repressor and its activator are silenced, repression of the reporter is relieved, enabling reporter gene expression. In contrast, in normal cells that express normal-cell-specific miRNAs, these miRNAs target and suppress the reporter directly, keeping reporter output OFF despite the absence of cancer-specific miRNAs. The circuit therefore behaves as a NOT−AND gate, activating only when the cancer-specific miRNA pattern is present and normal cell specific miR are low. [44].
b. miRISC-mediated cleavage architecture using CopT−CopA translational repression: The CopT::CopA inhibits the translation of the gene present between them by forming a stable secondary hairpin structure, thereby sterically inhibiting the ribosome. However, with the presence of miR sites at the 3′UTR of the gene, miRISC complex cleaves the RNA at the target site and disrupts the inhibitory CopT−CopA RNA structure, relieving translational blockade and enabling downstream gene translation. Thus, reporter output becomes high only in the presence of the corresponding miRNA [**48].
c. Endoribonuclease-mediated cleavage-based architecture: A bidirectional promoter expresses (i) an endoribonuclease, Csy4 with 3′UTR miR target sites on one side and (ii) a reporter transcript containing a specific Csy4 cleavage site on the other side. In the absence of miR, Csy4 binds and cleaves the reporter mRNA at its cognate sequence, preventing reporter accumulation. However, in the presence of miR, Csy4 gets repressed and is unable to cleave the reporter [46]
d. iFFL-based miR circuit: This architecture is expressed from a single transcriptional unit that produces both (i) a synthetic miRNA and (ii) a reporter gene bearing complementary synthetic miRNA target sites in its 3′UTR. The synthetic miRNA represses the reporter post-transcriptionally while both components are co-expressed from the same promoter. This creates an iFFL motif in which the transcript activates reporter production at the transcriptional level but simultaneously represses it via miRNA targeting. The iFFL architecture is frequently used to attenuate noise, sharpen response thresholds, and improve robustness to fluctuations in expression levels [**69–71].

absence of miR forms a steric block of its functional genes, present between the CopT and CopA, which hinders the translation of Gal4-VP16, resulting in silencing of the reporter. This design exploits both the transcriptional and translational regulation without expression of any exogenous proteins or transcriptional repressors. It relies on the stability of bacterial-derived RNA secondary structures ensuring high biocompatibility with no observed toxicity [*48]. Although certain bacterial RNAs can activate innate immune receptors such as TLR7/8 or RIG-I when delivered exogenously, further studies n immunocompetent mouse models are required to fully assess the safety profile of this design [49–51] (Fig. 3b).

In the evolving story of miR-based synthetic circuits, CRISPR-associated tools have also stepped beyond their traditional roles, opening new avenues for engineering

miR-responsive gene circuits. Cas9, which is a well-known programmable endoribonuclease widely known for its precise DNA targeting and cleavage, is now engineered to regulate gene expression in a miR-dependent manner. Alongside Cas9 [52,53] and sgRNA [54], AcrIIA4, a natural inhibitor of Cas9 [55,56], provides an additional layer of control by modulating Cas9 activity, enabling sophisticated logic circuits (Fig. 3c). CRISPR-specific endoribonucleases (ERNs) under the control of miRs have been utilised to sense cell state transitions and guide cell-type-specific differentiation [57].

IFFL-based loops – posttranscriptional regulations

With the above-discussed synthetic circuits, we understand how the field of miR-based synthetic circuits has been developed for the detection of endogenous miRs for cell classification or cell state identification. These circuits operating as "smart identifiers", passively report cellular identity through reporter proteins or through coupled functional outputs like apoptotic cell death [44]. Eventually, as the field matured, the focus has reshifted from passive detection to active intervention, particularly for gene therapy. The reliance on the endogenous miRs sensing suffices when we want to identify a particular cell state, but it may not be sufficient for theranostic gene therapeutics because of the variable endogenous miR levels.

In terms of gene therapy, a recurring challenge has been unintended background activity arising from promoters behaving differently across cell types and the differences in output variability due to competition between endogenous resources, such as transcriptional and translational machinery components. Any synthetic circuit that relies on transcriptional and translational regulation inevitably competes for the available endogenous resources to obtain an output [17,58,59], leading to a unprecedented and counterintuitive behaviour [60]. For these reasons, such synthetic circuits cannot function as "plug and play" modules. This need gave rise to the implementation of a feed-forward loop (FFL) within the circuits, which allows various advantages over the traditional sensors, i) tight control of the expression of the gene despite fluctuations in the cellular environment [61], ii) maintains constant expression levels regardless of plasmid or vector copy number also called dosage compensation [61–63] iii) mitigate burden and improve robustness of gene expression by supressing biological noise [63–68] iv) allows tunable programming of the fully induced expression of the gene of interest [66,69] Among current architectures, iFFL-based circuits uniquely satisfy the combined requirements of dosage compensation, robustness, and therapeutic safetyby integrating a synthetic miR scaffold within a single transcriptional unit expressing the reporter gene containing complementary target sites for the corresponding miR (Fig. 3d).

A recent design has successfully combined all of these advantages into a single compact circuit, providing a standardized sequences and architectural rules for achieving precise, tunable, and dosage-invariant expression. This system supports multiplexing of independent regulatory modules within the same cell, functions robustly across diverse cell types and delivery modalities, and provides durable control in vivo, making it suitable for gene therapy applications [**69]. Further they also validated DIMMER for treating disorders with the "Goldilocks" constraints, where gene levels must be tightly maintained within a narrow optimal range. In the context of Rett syndrome, this synthetic circuit limited ectopic Mecp2 expression, buffered expression variability, and reduced mRNA levels *in-vivo* to values comparable to endogenous Mecp2. When delivered via an AAV vector, the regulated circuit significantly outperformed unregulated gene therapy, improving behavioral deficits over 24 weeks and demonstrating durable therapeutic benefit. The miR-based IFFL circuit compensates for fluctuations in viral copy number and other sources of expression variability, ensuring that total mRNA and protein levels remain within a defined therapeutic window. Overall, the simplicity, compactness, and robustness of the miR-based IFFL architecture make it broadly applicable to other AAV-based therapies that require tight dosage control. By enabling higher initial vector doses without risking overexpression toxicity, such circuits may also enhance the longevity of gene therapy in dividing tissues [**70]. These findings highlight the value of integrating miR-based synthetic gene circuits into therapeutic designs to improve safety, precision, and long-term efficacy across a wide range of genetic diseases. In addition, advances in orthogonal synthetic miR toolbox and computational modelling paved the road for streamlining the design and validation process of the iFFl-based miR synthetic circuits. This made the design of these circuits much easier, as well as very relevant to correcting disease pathologies [70–72].

Current consensus in the field suggests that transcription-only miR sensors are insufficient for therapeutic deployment due to variability, leakiness, and resource coupling. RNA-based and iFFL architectures now represent the most robust and clinically realistic solutions, although challenges remain in delivery, long-term stability, and miR network perturbation.

Discussion

The engineering of miR synthetic circuits originally started with identifying cellular conditions and diseases where a known miR already served as a biomarker. Building on toolkits from synthetic biology, these miR

circuits exploit the regulatory properties of miRs to generate a correlative, context-specific, and tuned output. Over the years, iterative design and understanding of these circuits have taught us key principles essential for their optimization and for their incorporation in therapeutics (Table 1).

miR-based synthetic circuits have evolved from simple miR-OFF [11–16] reporters that passively reflect endogenous miR levels into sophisticated, multi-layered regulatory systems capable of robust sensing, decision-making, and therapeutic intervention. Early architectures suffered from indirect readouts, prompting the development of miR-ON systems [19–23], with RBP-driven designs [36–38,41,42] and split-inteins [**40], and CRISPR-integrated modules [52–57] that offered direct optical readouts, sharper signal fidelity, orthogonality, and improved safety. The introduction of multi-input classifiers expanded circuit capability from sensing to cell-state discrimination [43–45], while the emergence of miR-based incoherent feedforward loops (iFFLs) [61–72] addressed fundamental challenges such as dosage variability, resource competition, and promoter heterogeneity by providing intrinsic noise buffering, dosage compensation, and tunable output control. iFFL platforms now demonstrate therapeutic-grade precision in vivo, enabling tightly regulated gene expression suitable for diseases requiring narrow expression windows [70–72].

An important lesson across generations of synthetic circuits is the necessity of fine-tuning dosage at three levels: input, expression of the circuit components, and the phenotypic output. For input, regulator selection influences most of the circuit performance, safety, and translational potential. While transcriptional repressors are traditional modules for downregulation [20,21,23], careful optimisation of dosage should be kept in mind, keeping the safety profile of using prokaryotic modulators in the circuits [25–29]. Similarly, the integration of KRAB domains poses a risk of long-term epigenetic silencing in the flanking regions of the genomic integration sites [30,31] and integration of prokaryotic endonuclease Csy4 within the genomic backbone can affect the overall transcriptome profile [73], potentially due to the endogenous cleavage sites within the host transcriptome.

With respect to expression of the circuit components, and the phenotypic output of the circuits, it is quite usual that any synthetic circuit imposes a burden on the host cell by competing for endogenous machineries and limited cellular resources during the processing of the circuit [47,58,59]. That is why, it is essential to titrate the circuit dosage under various cell settings and multiple cell lines. The second optimisation is the configuration of target sites, which directly influences the strength of repression. Depending on that, either a single complementary sequence or multiple partially

Table 1

List of reported genetic miR sensors and their regulatory components.

	Name of sensor	Modulator	Design of construct	Mode of regulation	Reference
1.	RILES	Cumate gene switch	Single construct - Subsequent dual promoter	Transcriptional repression	[20]
2.	Self-contained miR-ON system	iTR-KRAB	Single construct - Bidirectional promoter	Transcriptional repression	[21]
3.	Lenti RILES	Tet R	Two vector system	Transcriptional - steric hindrance	[23]
4.	Gal4DB circuit	Gal4 DB	Single construct - Subsequent dual promoter	Transcriptional repression	[24]
5.	Cell classifier	rtTA-LacI	Multiple vector system	Transcriptional repression	[44,45]
6.	RNA-only multi-input cell classifier	L7Ae and MS2	Multiple replicon plasmids	Translational repression	[36*]
7.	Split RNA switches	NpuDnaE split intein	Multiple mRNA circuits	Post-translational – splicing	[40**]
8.	miR-ON-D	LacI, L7Ae	Multiple vector system	Double regulation – Transcriptional, translational	[42]
9.	Csy4-miR-ON	Csy4	Single construct – Bidirectional promoter	RNA Cleavage	[46]
10.	miCU sensor	CopA-CopT-based Gal4-VP16	Single construct – single promoter	RNA cleavage	[48*]
11.	CRISPR-based sensor	Cas9, sgRNA, AcrIIA4, ERN	Multiple vectors	RNA cleavage	[52–57]

complementary sites can be incorporated. These choices determine whether the regulation is stringent or permissive [20,21,74,75]. However, incorporating an increasing number of target sites requires caution, as long-term expression of these circuits risks the potential scavenging of endogenous miRs in the cells, perturbing native regulatory networks [20,21,75]. Further, when multiple miRs are incorporated for logic-based sensing, it becomes vital to design orthogonal synthetic miR systems to minimise the crosstalk and ensure specificity, which can now be mitigated with the recent advances in computational modelling and orthogonal synthetic miR toolkits [44,69].

While modern synthetic circuits increasingly focus on therapeutics, it's worth remembering that even earlier generations often employed functional genes like Bax [44] or p21 [42], enabling classification to be tightly coupled with the action, such as killing cancerous cells, halting proliferation, or sensitizing cells to prodrugs. Yet, as applications grew more clinically relevant, the challenge shifted toward precision control, ensuring reliable, tunable expression across variable cellular environments. We now observe how the circuits have developed from sensors to cell state correctors, where the synthetic circuits have also competed against endogenous gene expressions and have delivered a similar output required for correcting various genetic conditions [70]. Finally, significant advances have also been made in addressing delivery challenges. Although DNA-based delivery remains prevalent, RNA-based approaches are gaining popularity for their transient expression profiles and reduced risk of genomic integration, a crucial consideration for clinical safety[34–38]. Altogether, miR synthetic circuits are evolving into sophisticated, therapeutically relevant tools capable of precise gene regulation with growing translational potential.

Authorship contribution statement

Archismita Kundu: Conceptualization, writing – original draft, review, and editing. Roman Jerala: Conceptualization, writing – review and editing and funding acquisition.

Declaration of Generative AI and AI-assisted technologies in the writing process

After preparation of the text, the authors used Grammarly and ChatGPT to correct grammar and improve readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Declaration of competing interest

There are no competing interests to disclose.

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Data availability

No data was used for the research described in the article.

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. O'Brien J, Hayder H, Zayed Y, Peng C: **Overview of MicroRNA biogenesis, mechanisms of actions, and circulation**. *Front Endocrinol* 2018, **9**.
2. Wilczynska A, Bushell M: **The complexity of miR-mediated repression**. *Cell Death Differ* 2015, **22**:22–33.
3. Huntzinger E, Izaurralde E: **Gene silencing by microRNAs: contributions of translational repression and mRNA decay**. *Nat Rev Genet* 2011, **12**:99–110.
4. Jame-Chenarboo F, Ng HH, Macdonald D, Mahal LK: **High-throughput analysis reveals miR upregulating α-2,6-Sialic acid through direct miR–mRNA interactions**. *ACS Cent Sci* 2022, **8**:1527–1536.
5. Place RF, Li L-C, Pookot D, Noonan EJ, Dahiya R: **MicroRNA-373 induces expression of genes with complementary promoter sequences**. *Proc Natl Acad Sci* 2008, **105**:1608–1613.
6. Dharap A, Pokrzywa C, Murali S, Pandi G, Vemuganti R: **MicroRNA miR-324-3p induces promoter-mediated expression of RelA gene**. *PLoS One* 2013, **8**, e79467.
7. Henke JI, Goergen D, Zheng J, Song Y, Schüttler CG, Fehr C, Jünemann C, Niepmann M: **microRNA-122 stimulates translation of hepatitis C virus RNA**. *EMBO J* 2008, **27**:3300–3310.
8. Azam HMH, Rößling RI, Geithe C, Khan MM, Dinter F, Hanack K, Prüß H, Husse B, Roggenbuck D, Schierack P, *et al.*: **MicroRNA biomarkers as next-generation diagnostic tools for neurodegenerative diseases: a comprehensive review**. *Front Mol Neurosci* 2024, **17**.
9. Wang J, Chen J, Sen S: **microRNA as biomarkers and diagnostics**. *J Cell Physiol* 2016, **231**:25–30.
10. Wu Y, Li Q, Zhang R, Dai X, Chen W, Xing D: **Circulating microRNAs: biomarkers of disease**. *Clin Chim Acta* 2021, **516**:46–54.
11. Ko HY, Hwang DW, Lee DS, Kim S: **A reporter gene imaging system for monitoring microRNA biogenesis**. *Nat Protoc* 2009, **4**:1663–1669.
12. Ko MH, Kim S, Hwang DW, Ko HY, Kim YH, Lee DS: **Bioimaging of the unbalanced expression of microRNA9 and microRNA9* during the neuronal differentiation of P19 cells**. *FEBS J* 2008, **275**:2605–2616.

13. Kato Y, Miyaki S, Yokoyama S, Omori S, Inoue A, Horiuchi M, Asahara H: **Real-time functional imaging for monitoring miR-133 during myogenic differentiation**. *Int J Biochem Cell Biol* 2009, **41**:2225–2231.

14. Kato Y, Sawata SY, Inoue A: **A lentiviral vector encoding two fluorescent proteins enables imaging of adenoviral infection via adenovirus-encoded miRs in single living cells**. *J Biochem* 2010, **147**:63–71.

15. Weitz SH, Gong M, Barr I, Weiss S, Guo F: **Processing of microRNA primary transcripts requires heme in Mammalian cells**. *Proc Natl Acad Sci* 2014, **111**:1861–1866.

16. Seo Y, Kim SS, Kim N, Cho S, Park JB, Kim JH: **Development of a miR-controlled dual-sensing system and its application for targeting miR-21 signaling in tumorigenesis**. *Exp Mol Med* 2020, **52**:1989–2004.

17. Cella F, Perrino G, Tedeschi F, Viero G, Bosia C, Stan G-B, Siciliano V: **MIRELLA: a mathematical model explains the effect of microRNA-mediated synthetic genes regulation on intracellular resource allocation**. *Nucleic Acids Res* 2023, **51**: 3452–3464.

18. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R: **Control of translation and mRNA degradation by miRs and siRNAs**. *Genes Dev* 2006, **20**:515–524.

19. Pichard V, Aubert D, Boni S, Battaglia S, Ivacik D, Nguyen TH, Arbuthnot P, Ferry N: **Specific micro RNA-regulated TetR-KRAB transcriptional control of transgene expression in viral vector-transduced cells**. *PLoS One* 2012, **7**, e51952.

20. Ezzine S, Vassaux G, Pitard B, Barteau B, Malinge J-M, Midoux P, Pichon C, Baril P: **RILES, a novel method for temporal analysis of the in vivo regulation of miR expression**. *Nucleic Acids Res* 2013, **41**, e192.

21. Amendola M, Giustacchini A, Gentner B, Naldini L: **A double-switch vector system positively regulates transgene expression by endogenous microRNA expression (miR-ON vector)**. *Mol Ther* 2013, **21**:934–946.

22. Rossetti S, Anauo MJ, Sacchi N: **MiR-221-regulated KIT level by wild type or leukemia mutant RUNX1: a determinant of single myeloblast fate decisions that – collectively – drives or hinders granulopoiesis**. *Oncotarget* 2017, **8**:85783–85793.

23. Simion V, Loussouarn C, Laurent Y, Roncali L, Gosset D, Reverchon F, Rousseau A, Martin F, Midoux P, Pichon C, *et al*.: **LentiRILES, a miR-ON sensor system for monitoring the functionality of miR in cancer biology and therapy**. *RNA Biol* 2021, **18**:198–214.

24. Haynes KA, Ceroni F, Flicker D, Younger A, Silver PA: **A sensitive switch for visualizing natural gene silencing in single cells**. *ACS Synth Biol* 2012, **1**:99–106.

25. Hackl H, Rommer A, Konrad TA, Nassimbeni C, Wieser R: **Tetracycline regulator expression alters the transcriptional program of mammalian cells**. *PLoS One* 2010, **5**, e13013.

26. Markusic DM, de Waart DR, Seppen J: **Separating lentiviral vector injection and induction of gene expression in time, does not prevent an immune response to rtTA in rats**. *PLoS One* 2010, **5**, e9974.

27. Richard P, Pollard H, Lanctin C, Bello-Roufaï M, Désigaux L, Escande D, Pitard B: **Inducible production of erythropoietin using intramuscular injection of block copolymer/DNA formulation**. *J Gene Med* 2005, **7**:80–86.

28. Guiner CL, Stieger K, Toromanoff A, Guilbaud M, Mendes-Madeira A, Devaux M, Guigand L, Cherel Y, Moullier P, Rolling F, *et al*.: **Transgene regulation using the tetracycline-inducible TetR-KRAB system after AAV-mediated gene transfer in rodents and nonhuman Primates**. *PLoS One* 2014, **9**, e102538.

29. Stieger K, Belbellaa B, Le Guiner C, Moullier P, Rolling F: **In vivo gene regulation using tetracycline-regulatable systems**. *Adv Drug Deliv Rev* 2009, **61**:527–541.

30. Wolf G, Yang P, Füchtbauer AC, Füchtbauer E-M, Silva AM, Park C, Wu W, Nielsen AL, Pedersen FS, Macfarlan TS: **The KRAB zinc finger protein ZFP809 is required to initiate epigenetic silencing of endogenous retroviruses**. *Genes Dev* 2015, **29**:538–554.

31. Groner AC, Meylan S, Ciuffi A, Zangger N, Ambrosini G, Dénervaud N, Bucher P, Trono D: **KRAB–zinc finger proteins and KAP1 can mediate long-range transcriptional repression through heterochromatin spreading**. *PLoS Genet* 2010, **6**, e1000869.

32. Simion V, Henriet E, Juric V, Aquino R, Loussouarn C, Laurent Y, Martin F, Midoux P, Garcion E, Pichon C, *et al*.: **Intracellular trafficking and functional monitoring of miR delivery in glioblastoma using lipopolyplexes and the miR-ON RILES reporter system**. *J Contr Release* 2020, **327**:429–443.

33. Tavernier G, Andries O, Demeester J, Sanders NN, De Smedt SC, Rejman J: **mRNA as gene therapeutic: how to control protein expression**. *J Contr Release* 2011, **150**: 238–247.

34. Miki K, Endo K, Takahashi S, Funakoshi S, Takei I, Katayama S, Toyoda T, Kotaka M, Takaki T, Umeda M, *et al*.: **Efficient detection and purification of cell populations using synthetic MicroRNA switches**. *Cell Stem Cell* 2015, **16**:699–711.

35. Fujita Y, Hirosawa M, Hayashi K, Hatani T, Yoshida Y, Yamamoto T, Saito H: **A versatile and robust cell purification system with an RNA-Only circuit composed of microRNA-responsive ON and OFF switches**. *Sci Adv* 2022, **8**:eabj1793.

36. * Wroblewska L, Kitada T, Endo K, Siciliano V, Stillo B, Saito H, Weiss R: **Mammalian synthetic circuits with RNA binding proteins for RNA-only delivery**. *Nat Biotechnol* 2015, **33**: 839–841.

This study developed the first RNA-based miR-synthetic circuits in mammalian cells enabling precise regulatory control while limitations associated with DNA vector delivery.

37. Matsuura S, Ono H, Kawasaki S, Kuang Y, Fujita Y, Saito H: **Synthetic RNA-based logic computation in mammalian cells**. *Nat Commun* 2018, **9**:4847.

38. Wagner TE, Becraft JR, Bodner K, Teague B, Zhang X, Woo A, Porter E, Alburquerque B, Dobosh B, Andries O, *et al*.: **Small-molecule-based regulation of RNA-delivered circuits in mammalian cells**. *Nat Chem Biol* 2018, **14**:1043–1050.

39. Endo K, Hayashi K, Saito H: **High-resolution identification and separation of living cell types by multiple microRNA-responsive synthetic mRNAs**. *Sci Rep* 2016, **6**, 21991.

40. ** Abe I, Ohno H, Mochizuki M, Hayashi K, Saito H: **Split RNA switch orchestrates pre- and post-translational control to enable cell type-specific gene expression**. *Nat Commun* 2025, **16**:5362.

This paper employs miRs to assemble split inteins, restoring a functional reporter with enhanced sensitivity. The circuit comprises complementary OFF and ON modules: the OFF module uses a dominant negative C-terminal reporter fragment fused to a split intein, repressing activity in the presence of miR, while the ON module contains the active split reporter and complementary intein, released by miR-mediated cleavage, to permit functional reporter assembly. Using NpuDnaE split inteins, this system links miR activity to reconstituted reporter expression.

41. Ono H, Kawasaki S, Saito H: **Orthogonal protein-responsive mRNA switches for mammalian synthetic biology**. *ACS Synth Biol* 2020, **9**:169–174.

42. Shu W-J, Lee K, Ma Z, Tian X, Kim JS, Wang F: **A dual-regulation inducible switch system for microRNA detection and cell type-specific gene activation**. *Theranostics* 2023, **13**: 2552–2561.

43. Rinaudo K, Bleris L, Maddamsetti R, Subramanian S, Weiss R, Benenson Y: **A universal RNAi-based logic evaluator that operates in mammalian cells**. *Nat Biotechnol* 2007, **25**: 795–801.

44. Xie Z, Wroblewska L, Prochazka L, Weiss R, Benenson Y: **Multi-input RNAi-Based logic circuit for identification of specific cancer cells**. *Science* 2011, **333**:1307–1311.

45. Mohammadi P, Beerenwinkel N, Benenson Y: **Automated design of synthetic cell classifier circuits using a two-step optimization strategy**. *cels* 2017, **4**:207–218.e14.

46. Sano M, Morishita K, Oikawa S, Akimoto T, Sumaru K, Kato Y: **Live-cell imaging of microRNA expression with post-transcriptional feedback control**. *Mol Ther Nucleic Acids* 2021, **26**:547–556.

47. Cella F, Wroblewska L, Weiss R, Siciliano V: **Engineering protein-protein devices for multilayered regulation of mRNA translation using orthogonal proteases in mammalian cells**. *Nat Commun* 2018, **9**:4392.

48. * Shi X, Hu C, Guo B, Zhang C, Xue Y, Yang Z, Wang F: **Programmable transcriptional–translation active sensors for miR-Responsive gene imaging and theranostics in mammalians**. *ACS Sens* 2025, **10**:4646–4657.
This paper utilises miRISC endoribonuclease activity to relieve steric inhibition by the CopT–CopA RNA complex. In the absence of miR, the complex blocks translation of Gal4-VP16, thereby silencing reporter expression

49. Ostendorf T, Zillinger T, Andryka K, Schlee-Guimaraes TM, Schmitz S, Marx S, Bayrak K, Linke R, Salgert S, Wegner J, *et al*.: **Immune sensing of synthetic, bacterial, and protozoan RNA by toll-like receptor 8 requires coordinated processing by RNase T2 and RNase 2**. *Immunity* 2020, **52**:591–605.e6.

50. Eberle F, Sirin M, Binder M, Dalpke AH: **Bacterial RNA is recognized by different sets of immunoreceptors**. *Eur J Immunol* 2009, **39**:2537–2547.

51. Uehata T, Takeuchi O: **RNA recognition and immunity-innate immune sensing and its posttranscriptional regulation mechanisms**. *Cells* 2020, **9**:1701.

52. Hirosawa M, Fujita Y, Parr CJC, Hayashi K, Kashida S, Hotta A, Woltjen K, Saito H: **Cell-type-specific genome editing with a microRNA-responsive CRISPR–Cas9 switch**. *Nucleic Acids Res* 2017, **45**, e118.

53. Hunt VM, Chen W: **A microRNA-gated thgRNA platform for multiplexed activation of gene expression in mammalian cells**. *Chem Commun* 2022, **58**:6215–6218.

54. Wang X-W, Hu L-F, Hao J, Liao L-Q, Chiu Y-T, Shi M, Wang Y: **A microRNA-inducible CRISPR–Cas9 platform serves as a microRNA sensor and cell-type-specific genome regulation tool**. *Nat Cell Biol* 2019, **21**:522–530.

55. Hoffmann MD, Aschenbrenner S, Grosse S, Rapti K, Domenger C, Fakhiri J, Mastel M, Börner K, Eils R, Grimm D, *et al*.: **Cell-specific CRISPR–Cas9 activation by microRNA-dependent expression of anti-CRISPR proteins**. *Nucleic Acids Res* 2019, **47**, e75.

56. Lee J, Mou H, Ibraheim R, Liang S-Q, Liu P, Xue W, Sontheimer EJ: **Tissue-restricted genome editing in vivo specified by microRNA-repressible anti-CRISPR proteins**. *RNA* 2019, **25**:1421–1431.

57. Wang L, Xu W, Zhang S, Gundberg GC, Zheng CR, Wan Z, Mustafina K, Caliendo F, Sandt H, Kamm R, *et al*.: **Sensing and guiding cell-state transitions by using genetically encoded endoribonuclease-mediated microRNA sensors**. *Nat Biomed Eng* 2024, **8**:1730–1743.

58. Frei T, Cella F, Tedeschi F, Gutiérrez J, Stan G-B, Khammash M, Siciliano V: **Characterization and mitigation of gene expression burden in mammalian cells**. *Nat Commun* 2020, **11**:4641.

59. Jones RD, Qian Y, Siciliano V, DiAndreth B, Huh J, Weiss R, Del Vecchio D: **An endoribonuclease-based feedforward controller for decoupling resource-limited genetic modules in mammalian cells**. *Nat Commun* 2020, **11**:5690.

60. Borkowski O, Ceroni F, Stan G-B, Ellis T: **Overloaded and stressed: whole-cell considerations for bacterial synthetic biology**. *Curr Opin Microbiol* 2016, **33**:123–130.

61. Strovas TJ, Rosenberg AB, Kuypers BE, Muscat RA, Seelig G: **MicroRNA-Based single-gene circuits buffer protein synthesis rates against perturbations**. *ACS Synth Biol* 2014, **3**:324–331.

62. Bleris L, Xie Z, Glass D, Adadey A, Sontag E, Benenson Y: **Synthetic incoherent feedforward circuits show adaptation to the amount of their genetic template**. *Mol Syst Biol* 2011, **7**:519.

63. Yang J, Lee J, Land MA, Lai S, Igoshin OA, St-Pierre F: **A synthetic circuit for buffering gene dosage variation between individual mammalian cells**. *Nat Commun* 2021, **12**:4132.

64. Segall-Shapiro TH, Sontag ED, Voigt CA: **Engineered promoters enable constant gene expression at any copy number in bacteria**. *Nat Biotechnol* 2018, **36**:352–358.

65. Ceroni F, Boo A, Furini S, Gorochowski TE, Borkowski O, Ladak YN, Awan AR, Gilbert C, Stan G-B, Ellis T: **Burden-driven feedback control of gene expression**. *Nat Methods* 2018, **15**:387–393.

66. Lillacci G, Benenson Y, Khammash M: **Synthetic control systems for high performance gene expression in mammalian cells**. *Nucleic Acids Res* 2018, **46**:9855–9863.

67. Siciliano V, Garzilli I, Fracassi C, Criscuolo S, Ventre S, di Bernardo D: **miRs confer phenotypic robustness to gene networks by suppressing biological noise**. *Nat Commun* 2013, **4**, https://doi.org/10.1038/ncomms3364.

68. Osella M, Bosia C, Corá D, Caselle M: **The role of incoherent MicroRNA-Mediated feedforward loops in noise buffering**. *PLoS Comput Biol* 2011, **7**, e1001101.

69. ** Du R, Flynn MJ, Honsa M, Jungmann R, Elowitz MB: **miR circuit modules for precise, tunable control of gene expression**. *bioRxiv* 2024, https://doi.org/10.1101/2024.03.12.583048.
In this paper, the authors introduce DIMMERs (Dosage-Invariant miRNA-Mediated Expression Regulators). DIMMERs utilize an incoherent feed-forward loop (IFFL) motif: both the transgene mRNA and its inhibitory miRNA are co-expressed from divergent promoters, enabling the system to buffer against wide variations in gene dosage or transcription rates and maintain uniform protein output.

70. ** Flynn MJ, Mayfield AMH, Du R, Gradinaru V, Elowitz MB: *Synthetic dosage-compensating miR circuits allow precision gene therapy for Rett syndrome*. 2024, https://doi.org/10.1101/2024.03.13.584179.
This study develops synthetic miRNA-based dosage-compensating circuits to regulate MECP2, a dosage-sensitive gene implicated in Rett syndrome. The circuits use an incoherent feed-forward loop to buffer against copy number variation, maintaining MECP2 expression within the therapeutic window. This approach demonstrates a generalizable strategy for precision gene therapy of dosage-sensitive disorders.

71. Love KS, Johnstone CP, Peterman EL, Gaglione S, Birnbaum ME, Galloway KE: **Model-guided design of microRNA-based gene circuits supports precise dosage of transgenic cargoes into diverse primary cells**. *cels* 2025, **16**.

72. An C, Katz N, Gao XJ: **Designing quantitative gene therapy on ComMAND**. *cels* 2025, **16**.

73. Zhang L, Qiu X, Zhou Y, Luo Z, Zhu L, Shao J, Xie M, Wang H: **A trigger-inducible split-Csy4 architecture for programmable RNA modulation**. *Nucleic Acids Res* 2025, **53**. gkae1319.

74. Scherr M, Venturini L, Battmer K, Schaller-Schoenitz M, Schaefer D, Dallmann I, Ganser A, Eder M: **Lentivirus-mediated antagomir expression for specific inhibition of miR function**. *Nucleic Acids Res* 2007, **35**, e149.

75. Gentner B, Schira G, Giustacchini A, Amendola M, Brown BD, Ponzoni M, Naldini L: **Stable knockdown of microRNA in vivo by lentiviral vectors**. *Nat Methods* 2009, **6**:63–66.

76. Ning H, Liu G, Li L, Liu Q, Huang H, Xie Z: **Rational design of microRNA-responsive switch for programmable translational control in mammalian cells**. *Nat Commun* 2023, **14**:7193.

77. Song Y, Xu Z, Wang F: **Genetically encoded reporter genes for MicroRNA imaging in living cells and animals**. *Mol Ther Nucleic Acids* 2020, **21**:555–567.