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Modular and signal-responsive transcriptional regulation using CRISPRi-aided genetic switches in *Escherichia coli*

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Abstract

Background Precise and dynamic transcriptional regulation is a cornerstone of synthetic biology, enabling the construction of robust genetic circuits and programmable cellular systems. However, existing regulatory tools are often limited by issues such as leaky transcription and insufficient tunability, particularly in high-expression or complex genetic contexts. This study aimed to develop a CRISPRi-aided genetic switch platform that overcomes these limitations and expands the functionality of transcriptional regulation tools in synthetic biology.

Results We established a versatile CRISPRi-aided genetic switch platform by integrating transcription factor-based biosensors with the Type V-A FnCas12a CRISPR system. Exploiting the RNase activity of FndCas12a, this system processes CRISPR RNAs (crRNAs) directly from biosensor-responsive mRNA transcripts, enabling precise, signal-dependent transcriptional regulation. To mitigate basal transcription and enhance regulatory precision, transcriptional terminator filters were incorporated, reducing leaky expression and increasing the dynamic range of target gene regulation. The platform demonstrated exceptional adaptability across diverse applications, including ligand-inducible genetic switches for transcriptional control, signal amplification circuits for enhanced output, and metabolic genetic switches for pathway reprogramming. Notably, the metabolic genetic switch dynamically repressed the endogenous *gapA* gene while compensating with orthologous *gapC* expression, effectively redirecting metabolic flux to balance cell growth.

Conclusions The CRISPRi-aided genetic switch provides a powerful and flexible toolkit for synthetic biology, addressing the limitations of existing systems. By enabling precise and tunable transcriptional regulation, it offers robust solutions for a wide array of biotechnological applications, including pathway engineering and synthetic gene networks.

Keywords CRISPR interference, Genetic switch, Transcriptional regulation, Signal amplifier, Metabolic genetic switch

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Background

Bacteria possess intricate signal transduction pathways that enable them to sense and respond to environmental fluctuations by modulating gene expression and cellular physiology through advanced regulatory mechanisms [1–3]. Transcription factors (TFs), as primary regulators, interact with specific promoter regions to activate or repress gene expression in response to chemical signals, thereby enabling dynamic metabolic adaptation [4, 5]. These attributes make TFs essential tools in synthetic biology, where they are utilized in the design of gene expression systems, biosensors, and genetic logic gates [6–11]. Despite their utility, TF-based systems face significant challenges, including limited scalability, labor-intensive engineering processes, and difficulties in reconfiguring ligand specificity or operator binding [12, 13]. Such limitations constrain their effectiveness in building complex and modular genetic circuits in synthetic biology.

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas) technology has transformed genome engineering, expanding its application far beyond its initial role as a prokaryotic immune defense mechanism [14–16]. Its adaptations encompass transcriptional and post-transcriptional regulation, diagnostics, and nucleic acid detection, highlighting its unparalleled versatility [17–22]. Within this field, Cas12a (formerly known as Cpf1), a type V-A CRISPR-Cas RNA-guided DNA endonuclease, has emerged as a valuable tool for genome editing and genetic regulation [23]. Variants such as *Acidaminococcus* sp. Cas12a (AsCas12a), *Lachnospiraceae* bacterium Cas12a (LbCas12a), *Eubacterium eligens* Cas12a (EeCas12a), and *Francisella novicida* Cas12a (FnCas12a) have been widely adopted for genome editing in various organisms [24–27]. The FnCas12a variant stands out due to its unique ribonuclease (RNase) activity, enabling direct processing of CRISPR RNAs (crRNAs) from precursor transcripts [28, 29].

This feature facilitates the creation of modular and signal-responsive genetic circuits with precise transcriptional control [27, 30]. Compared to Cas9-based systems, FnCas12a offers several advantages, including a shorter crRNA length, staggered DNA cleavage, innate RNA processing, and enhanced multiplexing capacity, making it particularly suited for designing complex genetic circuits [31, 32]. Despite these advancements, CRISPR-based systems often exhibit challenges such as leaky transcription, limited tunability, and difficulties in achieving proportional gene regulation, hindering their deployment in dynamic cellular contexts [33–35].

While numerous studies have focused on optimizing dead Cas9 (dCas9)-based CRISPR interference (CRISPRi) systems [36–39], research on dCas12a-based CRISPRi optimization remains relatively limited. Although dCas9-based CRISPRi has gained widespread popularity, its use in bacterial systems often faces limitations due to cytotoxic effects arising from non-specific DNA binding and extended transcriptional interference [40]. In contrast, dCas12a has demonstrated reduced toxicity, especially when expressed at moderate levels and in scenarios with minimal off-target effects [41–43]. These favorable attributes position dCas12a as a more appropriate choice for genetic regulation in bacterial systems, as it minimizes the burden on cells while still achieving effective gene repression. In dCas9-based CRISPRi, several strategies have been employed to improve repression efficiency and tunability, including reversible crRNA binding [36, 37, 39], decoy target sites [38, 39], and feedback control mechanisms [39]. These approaches have successfully enhanced transcriptional regulation and minimized leaky repression, leading to improved CRISPRi performance in dCas9 systems. Despite these advancements, the optimization of dCas12a-based CRISPRi remains an emerging area of research with significant potential for further development. Among the few existing strategies for dCas12a optimization, one approach involves employing antisense RNA (asRNA) to sequester guide RNAs (gRNAs), effectively suppressing leaky repression in dCas12a-based CRISPRi systems [33]. This strategy combines asRNA sequestration with regulatory feedback to minimize unintended repression, thereby improving the stability and accuracy of genetic circuits.

To address these challenges, innovative strategies include an integration of biosensors, transcriptional control systems, and RNA-processing methods to create robust genetic tools.

TF-based biosensors have shown promise for detecting metabolites and modulating cellular pathways, yet they require extensive optimization to function effectively across diverse host organisms and metabolic conditions [44]. Efforts to improve tunability and reduce basal transcription have included engineering genetically encoded biosensors to target specific metabolites and enable independent regulation of multiple genes [45]. However, these approaches often fall short in delivering consistent and versatile gene regulation across a variety of biological contexts.

In this study, we introduce CRISPRi-aided genetic switches that combine TF-based biosensors with FnCas12a-mediated CRISPRi systems. These switches exploit the RNase activity of FnCas12a to process crRNAs derived from biosensor-responsive transcripts, enabling precise and signal-dependent repression of target genes. By incorporating transcriptional terminator filters, the system minimizes basal transcription, thereby enhancing the dynamic range and reliability of gene regulation. The versatility of this platform is demonstrated

through applications in regulating both plasmid-encoded and chromosomally integrated genes, accommodating variations in gene copy number. Additionally, the system facilitates signal amplification in repressor-based circuits and enables metabolic genetic switches to reprogram cellular pathways, establishing a foundation for future biochemical production optimization.

These findings underscore the transformative potential of FndCas12a-based CRISPRi-aided genetic switches in synthetic biology. By addressing limitations inherent in existing tools, this study establishes a robust and flexible platform for advanced genetic circuit engineering. This approach not only enhances programmable cellular responses but also holds significant promise for applications in industrial biotechnology and beyond.

Methods

Strains and culture conditions

Cloning experiments were performed using *Escherichia coli* DH5α. Cultivation of *E. coli* DH5α was conducted in lysogeny broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl). For plasmid selection, ampicillin (100 μg/mL), kanamycin (25 μg/mL), or chloramphenicol (34 μg/mL) was added as required. Polymerase chain reaction (PCR) was carried out using high-fidelity KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and Gibson Assembly Master Mix were obtained from New England Biolabs (Ipswich, MA, USA).

Plasmid construction

Plasmids and oligonucleotides used for gene cloning are listed in Tables S1 and S2, while sequences for crRNA binding sites are summarized in Table S3. The names, strengths, and sequence information of the terminator filters used in this study are provided in Table S4.

To construct the pFnSECRVi plasmid, the pY002-FndCas12a plasmid containing a nuclease-deficient FndCas12a (D917A) mutant was derived from the pY002 plasmid (a kind gift from Feng Zhang) using the Gibson Assembly method. FndCas12a was then inserted into the pSECRVi backbone via Gibson Assembly. The crRNA region was subsequently removed through AgeI/XmaI digestion and ligation, resulting in the final pFnSECRVi plasmid. The pG-FncrRNA plasmid was constructed from the pG-crRNA plasmid by introducing a 19-nt FndCas12a direct repeat sequence upstream of the spacer region using reverse PCR and T4 DNA ligase.

To construct the RFP expression plasmid, the *lacI* gene and P_{TRC} promoter from the pT-GFP plasmid were assembled upstream of the *mCherry* gene on the pA-RFP plasmid using the Gibson Assembly method, generating the pTRC-RFP plasmid. To insert crRNA, double-stranded DNA (dsDNA) oligos containing FncrRNA were ligated into the HindIII-digested pTRC-RFP plasmid. For the IPTG-inducible promoter, the P_{TRC} promoter was replaced with the P_{BAD} promoter from the pBAD/His plasmid via Gibson Assembly.

To construct a tetracycline-inducible promoter system, the *rfp* gene was inserted into the pdCas9-bacteria plasmid (a kind gift from Stanley Qi) by transferring the *rfp* gene from the pTRC-RFP plasmid using Gibson Assembly. For GFP expression, the *rfp* gene in the pTET-RFP plasmid was replaced with the *gfp* gene from the pT-GFP plasmid through Gibson Assembly.

Cell growth and fluorescence monitoring of genetic switches

E. coli DH5α cells were transformed with the respective plasmids and selected on LB agar plates supplemented with the appropriate antibiotics. A single colony was inoculated into LB broth containing antibiotics and incubated overnight at 37 °C with shaking at 200 rpm. The overnight culture was subsequently transferred (1% v/v inoculum) to fresh LB broth containing antibiotics and inducers, followed by incubation at 37 °C with shaking. Cell growth and fluorescence were monitored using an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland). For cell growth measurements (OD_{600nm}), background absorbance from a blank media control was subtracted from all readings to ensure accurate cell density normalization. To analyze fluorescence data, ON-state and OFF-state fluorescence intensities were calculated based on time-dependent fluorescence measurements. ON-state fluorescence ($\Delta Fluorescence_{ON}$) was determined by subtracting the initial fluorescence intensity at 0 h from the fluorescence intensity measured at a given time point under inducer-present conditions ($\Delta Fluorescence_{ON} = Fluorescence_{t} - Fluorescence_{0h}$). Similarly, OFF-state fluorescence ($\Delta Fluorescence_{OFF}$) was calculated using the same approach in the absence of an inducer ($\Delta Fluorescence_{OFF} = Fluorescence_{t} - Fluorescence_{0h}$). To quantify the switch's dynamic range, both ON/OFF and OFF/ON ratios were calculated. The ON/OFF ratio was obtained by dividing the ON-state fluorescence by the OFF-state fluorescence (ON/OFF ratio = $\Delta Fluorescence_{ON} / \Delta Fluorescence_{OFF}$). Conversely, the OFF/ON ratio was determined by dividing the OFF-state fluorescence by the ON-state fluorescence (OFF/ON ratio = $\Delta Fluorescence_{OFF} / \Delta Fluorescence_{ON}$). These ratios provide complementary insights into the switch's performance under different conditions.

Results and discussion

Establishment of CRISPRi-aided genetic switches

To establish a CRISPRi-aided genetic switch, we constructed genetic circuits integrating TF-based biosensors for signal detection, red fluorescent protein (*rfp*)

gene expression under a biosensor-responsive promoter, and FndCas12a (DNase-inactive FnCas12a) for CRISPRi-mediated regulation of a target green fluorescent protein (*gfp*) gene (Fig. 1). A crRNA cassette, comprising a 36-nt direct repeat (DR) and a spacer sequence, was positioned downstream of the *rfp* gene to facilitate crRNA generation from the *rfp*-crRNA single transcript. Since plasmid copy number directly influences promoter-driven gene expression, we selected p15A as the replication origin for all sensor plasmids to maintain experimental consistency. Its medium-copy nature supports stable plasmid maintenance, ensuring reliable coexistence of multiple genetic circuits. Due to its compatibility with other commonly used plasmid backbones, p15A is a practical choice for applications requiring controlled gene expression and modular circuit design [46]. While plasmid copy number may vary with cellular growth state, employing a single replication origin across all constructs helped standardize expression conditions, allowing for more reliable comparisons. The DR sequence (5'-GUCUAAGAACU UUAAAUAAUUUCUACUGUUGUAGAU-3') corresponds to the processing site recognized by FnCas12a, enabling precise cleavage of the mRNA transcript and subsequent production of functional crRNA [47]. In the presence of an input signal, the responsive promoter induces *rfp*-crRNA transcription, which is processed by FndCas12a to generate crRNA capable of targeting specific DNA sequences for transcriptional repression. For effective CRISPRi functionality, the crRNA spacer sequence was designed to bind the template strand of target genes. FndCas12a-mediated transcriptional repression operates by blocking RNA polymerase elongation at the template strand (Fig. S1, a mechanism consistent with other Type V-A dCas12a variants, such as AsdCas12a and EedCas12a [48, 49]). To evaluate this system, a crRNA(T1) cassette targeting the *gfp* gene was introduced downstream of an L-arabinose-inducible *rfp* gene (Fig. 1A). In the absence of L-arabinose (OFF state), *rfp*-crRNA(T1) expression was minimal, allowing GFP production under the constitutive J23100 promoter from an episomal plasmid. Upon L-arabinose induction (ON state), *rfp* expression increased significantly, while GFP fluorescence was simultaneously repressed as L-arabinose concentration increased, indicating that crRNA expression is modulated by L-arabinose levels (Fig. 1A). Remarkably, significant GFP repression was observed even at a low concentration of 16 μM L-arabinose (Fig. S2, upper). This result demonstrates that *rfp*-crRNA(T1) mRNA was effectively processed by FndCas12a, forming a functional FndCas12a/crRNA(T1) complex that successfully repressed *gfp* expression. Notably, this genetic switch effect was absent when FndCas12a was not expressed, confirming the essential role of FndCas12a in crRNA processing and target gene regulation (Fig. 1A). To extend the versatility of this system, we utilized an IPTG-inducible promoter (P_{TRC}) to express *rfp*-crRNA(T1) mRNA (Fig. 1B). The P_{TRC} promoter exhibited increasing RFP expression with higher IPTG concentrations, reaching strong expression at 0.25 mM IPTG (ON state: 4,841 ± 70 a.u., $\Delta RFP_{(23\ h-0\ h)}$) and showing moderate basal expression even in its absence of IPTG (OFF state: 303 ± 10 a.u., $\Delta RFP_{(23\ h-0\ h)}$) (Fig. 1B, Fig. S2, bottom). However, basal expression levels in the OFF state from the P_{TRC} promoter exceeded those observed in the ON state of the L-arabinose-inducible P_{BAD} promoter (ON state: $\Delta RFP_{(23\ h-0\ h)} = 248 \pm 25$ a.u.). Consequently, basal *rfp*-crRNA(T1) mRNA expression from the P_{TRC} promoter caused significant GFP repression, even in the absence of IPTG induction. In the absence of FndCas12a, GFP fluorescence remained unaffected by IPTG, though a slight reduction in GFP signal was observed, likely due to the metabolic burden associated with RFP overproduction (Fig. 1B). These results demonstrate the feasibility of designing ligand-inducible CRISPRi-aided genetic switches capable of precise transcriptional regulation in *E. coli*. The system effectively integrates TF-based biosensors, robust promoters, and FndCas12a processing to enable programmable control of gene expression. Future efforts to mitigate basal expression, particularly for strong promoters like P_{TRC}, may further enhance the dynamic range and applicability of these genetic switches.

Terminator filters to tune crRNA expression for improved target gene regulation

To address the constitutive *gfp* repression caused by basal transcription from the P_{TRC} promoter, we initially attempted to adjust CRISPRi activity by lowering the expression of FndCas12a through varying L-rhamnose concentrations (Fig. S3) or by truncating the crRNA spacer sequence at the protospacer adjacent motif (PAM)-distal region (Fig. S4). However, these approaches failed to achieve proportional GFP recovery upon IPTG induction, as the reduced CRISPRi activity still resulted in ineffective regulation of *gfp* expression. This indicated that additional mechanisms were necessary to minimize leaky transcription while maintaining dynamic control. To overcome this limitation, we incorporated terminator filters upstream of the crRNA cassette to reduce basal transcription from the P_{TRC} promoter (Fig. 2). Terminators, both natural and synthetic, are well-characterized regulatory elements in *E. coli* that provide a practical means to fine-tune crRNA expression, thereby improving the dynamic range of target gene regulation [50, 51]. We tested a series of terminators (T9, T14, T25, and T48) [50] to evaluate their impact on *gfp* repression kinetics and OFF-state GFP recovery (Fig. 2, Fig. S5). As expected, the weak T9 terminator was insufficient to significantly reduce leaky transcription from the P_{TRC}

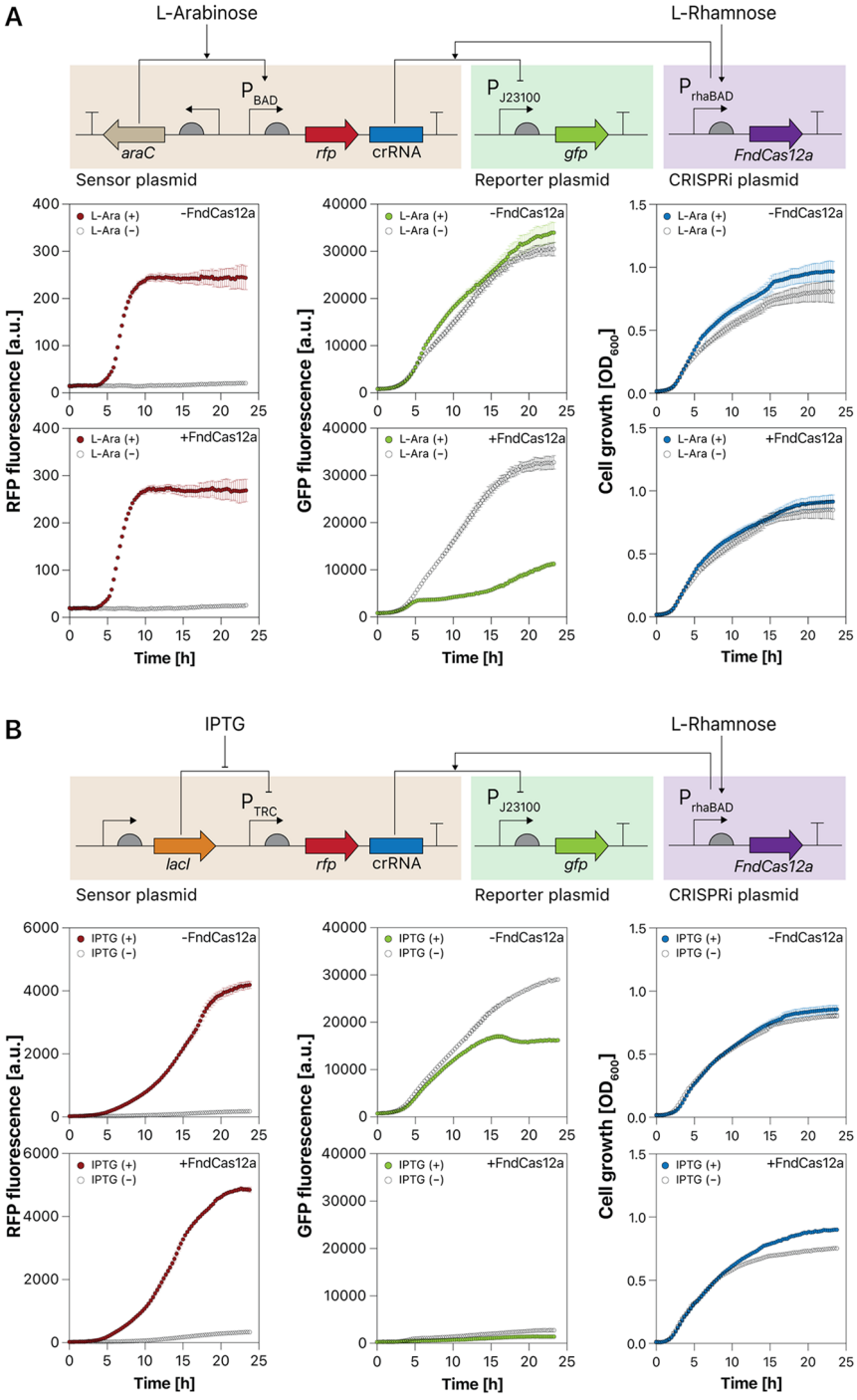

Fig. 1 Ligand-inducible CRISPRi-aided genetic switches targeting an episomal gene. (**A**, **B**) Schematic representations of an L-arabinose-inducible genetic switch (**A**) and an IPTG-inducible genetic switch (**B**). Both systems regulate the transcription of the *rfp* gene linked to a messenger crRNA cassette targeting the *gfp* gene (crRNA(T1)). FndCas12a expression was controlled by the P_{rhaBAD} promoter, induced with 1 mM L-rhamnose, to enable pre-crRNA processing and CRISPRi activity. The absence of FndCas12a was achieved by omitting L-rhamnose. *E. coli* DH5α strains harboring the genetic circuits were cultivated with specific inducers (4 mM L-arabinose for the P_{BAD} promoter and 0.25 mM IPTG for the P_{TRC} promoter). RFP fluorescence, GFP fluorescence, and cell growth (OD_{600}) were monitored using an Infinite 200 PRO microplate reader. Control values (cultures without inducers) are shown as open circles. Data are presented as the mean ± standard deviation (SD) from three biological replicates

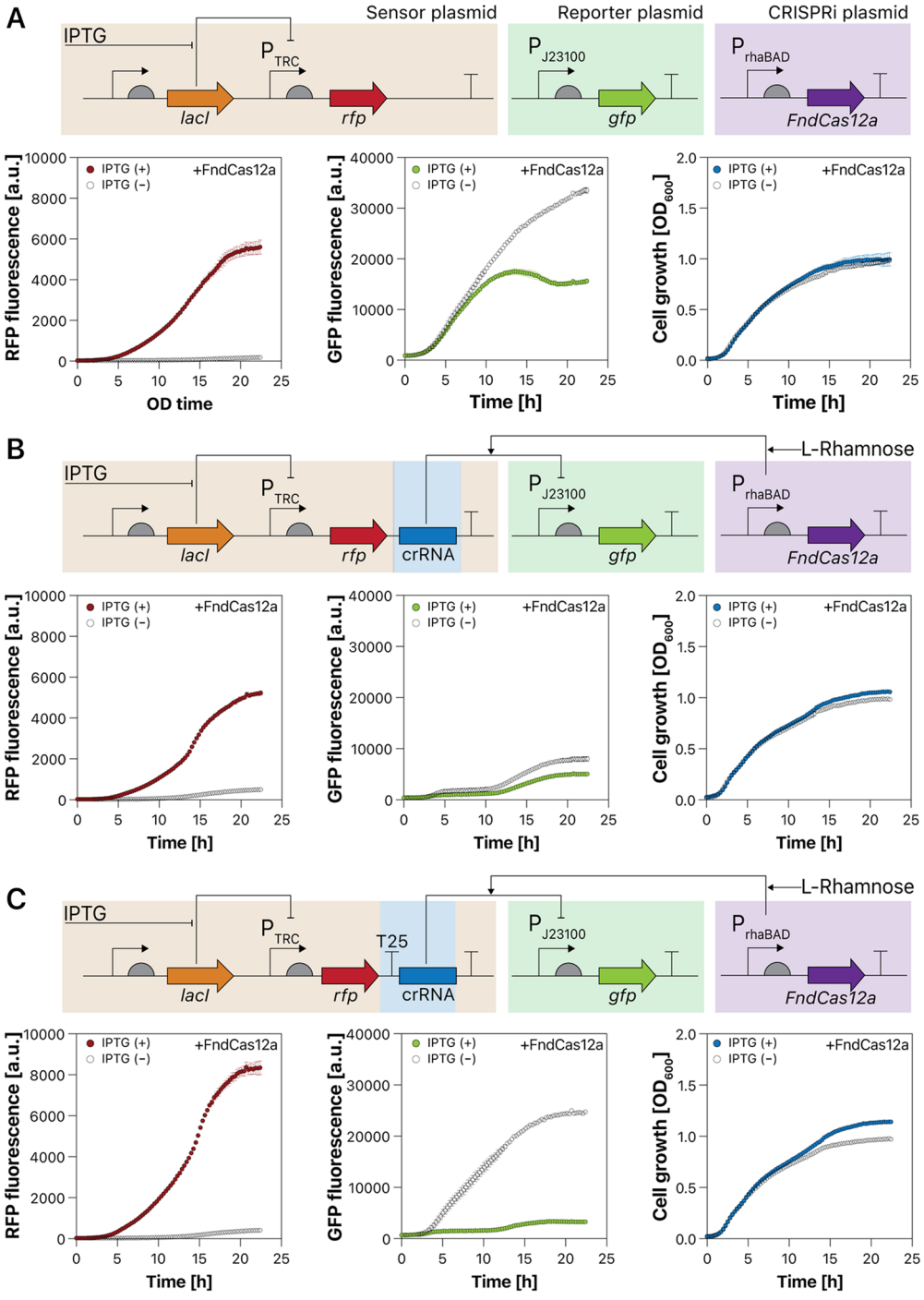

Fig. 2 Impact of terminator filters on the performance of IPTG-inducible CRISPRi-aided genetic switches targeting an episomal reporter gene. (**A**, **B**, **C**) Schematic representations of genetic circuits: (**A**) *rfp* gene under the P_{TRC} promoter, (**B**) IPTG-inducible *rfp*-crRNA(T1) circuit, and (**C**) *rfp* gene linked to a T25 terminator and crRNA(T1). FndCas12a expression was controlled by the P_{rhaBAD} promoter, induced with 1 mM L-rhamnose, to facilitate pre-crRNA processing and CRISPRi activity. *E. coli* DH5α strains harboring these genetic circuits were cultivated in the presence of 0.25 mM IPTG. RFP fluorescence, GFP fluorescence, and cell growth (OD_{600}) were monitored using an Infinite 200 PRO microplate reader. Control values (cultures without IPTG) are indicated as open circles. Data are presented as the mean ± standard deviation (SD) from three biological replicates

promoter, leading to minimal improvements in GFP expression in the OFF state (Fig. S5). In contrast, stronger terminators, such as T25 and T48, effectively minimized basal crRNA expression, leading to substantial recovery of GFP production in the absence of IPTG. Among the tested terminators, the T25 filter was the most effective, achieving a 77% recovery of GFP expression in the OFF state compared to the control (no crRNA, -IPTG)

at 10 h (Fig. 2C). Additionally, the T25 terminator enhanced the OFF/ON GFP ratio to 14.7 ± 2.0-fold [(OFF state $\Delta RFP_{(10\ h-0\ h)}$)/(ON state $\Delta RFP_{(10\ h-0\ h)}$)], reflecting improved dynamic regulation of GFP expression. These results underscore the critical role of terminator filters in mitigating basal transcription, enabling precise regulation of gene expression in CRISPRi-aided genetic circuits. By significantly improving the dynamic range of target gene regulation, terminator filters provide a robust strategy to address challenges associated with strong promoters like P_{TRC}. This approach broadens the applicability of CRISPRi systems in synthetic biology, particularly for constructs requiring stringent OFF-state control and high ON-state responsiveness.

We further evaluated the capacity of these CRISPRi-aided genetic switches to regulate chromosomally integrated target genes. For the P_{BAD} promoter, GFP expression in the OFF state from the chromosomal *gfp* gene was significantly lower without a terminator filter (Fig. 3B) compared to the control (no crRNA) (Fig. 3A). This result indicates that single-copy target genes are more susceptible to leaky P_{BAD} promoter activity compared to multi-copy plasmid-based genes (Fig. 1A). Initial attempts to address this leakage using moderate terminator filters (T14, T25, T48) showed slight improvements in OFF-state GFP expression but were insufficient to achieve effective repression (data not shown). To overcome this limitation, we employed stronger terminator filters (T240, T244, T312, T378) to enhance transcript regulation (Fig. 3C, Fig. S6). Among these, the T240 and T244 filters demonstrated the highest efficacy. Specifically, the T244 filter achieved a 72% recovery of GFP production in the OFF state compared to the control (no crRNA) at 10 h, with an OFF/ON GFP ratio of 8.7 ± 0.6-fold (Fig. 3C). For the P_{TRC} promoter, moderate terminator filters (T14, T25, T48) failed to mitigate OFF-state GFP repression due to the inherently high basal transcriptional activity of the P_{TRC} promoter (data not shown). However, the stronger T244 terminator slightly reduced *gfp* repression in the OFF state, indicating its potential to partially alleviate basal expression (Fig. S7). To further enhance the efficacy of terminator filters, we tested dual and triple terminator combinations positioned upstream of the crRNA cassette. These configurations consistently improved OFF-state GFP expression compared to single terminator filters (Fig. 3D and F, Fig. S7). Notably, the T25-T244 dual terminator combination demonstrated superior performance, recovering 64% of GFP expression in the OFF state relative to the control (no crRNA) at 10 h. This dual filter also increased the OFF/ON GFP ratio to 10.5 ± 4.4-fold (Fig. 3F). Interestingly, under L-arabinose or IPTG-induced conditions, the location of the reporter gene-whether on an episomal plasmid (Figs. 1 and 2) or integrated into the chromosome (Fig. 3)-did not result in a significant difference in RFP fluorescence [a.u.] values. However, a noticeable difference was observed in GFP fluorescence [a.u.] values. This discrepancy is likely due to the difference in *gfp* gene copy number between episomal plasmid-based expression and chromosomal integration. While the chromosomal reporter genes exist as a single copy, plasmid-based expression involves multiple copies, leading to relatively higher expression levels. Despite this, CRISPRi-mediated regulation remains effective, as the relative repression patterns align with those observed in the plasmid-based system. These findings highlight the versatility and efficacy of our CRISPRi-aided genetic switch system for modulating chromosomal target gene expression. The inclusion of terminator filters significantly enhances regulatory performance by mitigating promoter-specific leaky expression and accommodating variations in gene copy number. Consequently, this approach broadens the applicability of CRISPRi-based systems across diverse genetic contexts and establishes a robust framework for precision transcriptional regulation.

Signal amplification using CRISPRi-aided genetic switches

Transcriptional repressors are widely used as components of genetic circuits for applications such as recombinant protein production, metabolic pathway engineering, and biosensors for detecting small molecules. Their orthogonality and high predictability also make them suitable genetic parts for constructing synthetic promoters and complex Boolean logic gates [52, 53]. To investigate whether our CRISPRi-aided genetic switch could enhance the performance of repressor-based genetic circuits, we utilized a TetR-based inducible system as a model.

In this system, a crRNA cassette targeting *tetR* was inserted downstream of the *gfp* gene, and various terminator filters were incorporated upstream of the crRNA cassette to modulate its expression (Fig. 4A). The P_{TET} promoter drives the transcription of *gfp*-crRNA(TetR) mRNA upon anhydrotetracycline (aTC) induction (ON state). FndCas12a processes the crRNA(TetR), facilitating CRISPRi-mediated repression of the TetR repressor. As TetR levels decrease, the resulting derepression further increases *gfp*-crRNA(TetR) transcription, creating a positive feedback loop that amplifies GFP production. This feedback mechanism enables high output signals even at low inducer concentrations.

To further refine the CRISPRi-aided genetic switch, we replaced the L-rhamnose-inducible promoter with constitutive promoters for FndCas12a expression and examined the effect of FndCas12a expression levels on cell growth (OD_{600}) and GFP fluorescence (a.u.) (Fig. S8). For this purpose, the promoter driving FndCas12a expression in the CRISPRi plasmid was replaced with

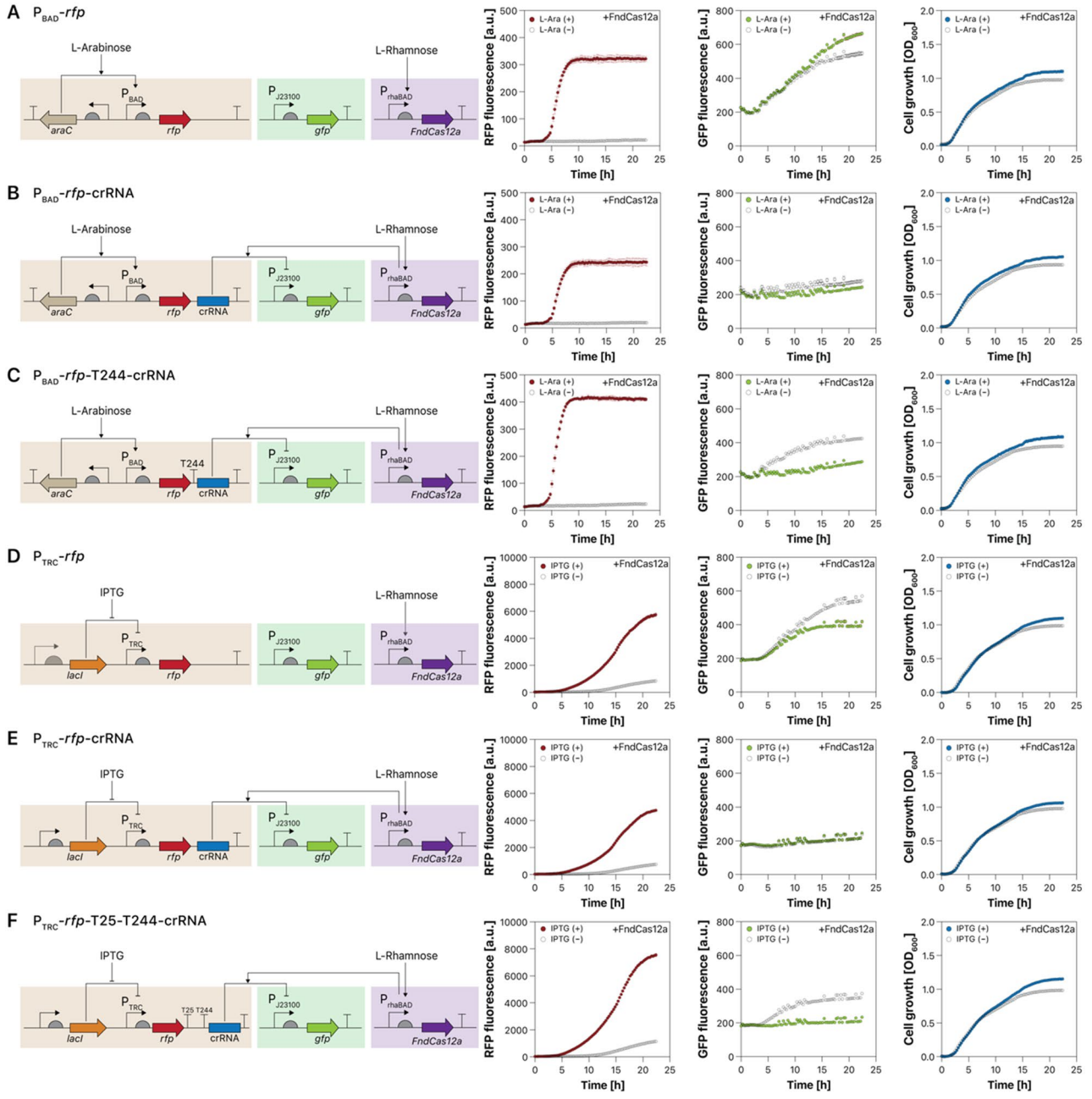

Fig. 3 Application of CRISPRi-aided genetic switches targeting a chromosomal reporter gene. (**A**, **B**, **C**) L-Arabinose-inducible genetic switches targeting a chromosomal reporter gene (P$_{J23100}$-*gfp*). (**A**) P$_{BAD}$-*rfp*, (**B**) P$_{BAD}$-*rfp*-crRNA(T1), and (**C**) P$_{BAD}$-*rfp*-T244-crRNA(T1) genetic circuits were tested. (**D**, **E**, **F**) IPTG-inducible genetic switches targeting a chromosomal reporter gene. (**D**) P$_{TRC}$-*rfp*, (**E**) P$_{TRC}$-*rfp*-crRNA(T1), and (**F**) P$_{TRC}$-*rfp*-T25-T244-crRNA(T1) genetic circuits were tested. FndCas12a expression was controlled by the P$_{rhaBAD}$ promoter, induced with 1 mM L-rhamnose, to facilitate pre-crRNA processing and CRISPRi activity. *E. coli* DH5α strains harboring the genetic circuits were cultivated with specific inducers (4 mM L-arabinose for the P$_{BAD}$ promoter and 0.25 mM IPTG for the P$_{TRC}$ promoter). RFP fluorescence, GFP fluorescence, and cell growth (OD$_{600}$) were monitored using an Infinite 200 PRO microplate reader. Control values (cultures without inducers) are shown as open circles. Data are presented as the mean ± standard deviation (SD) from three biological replicates

a constitutive promoter (P$_{CON}$), ranked in decreasing strength as J23100 (strong), J23105 (moderate), and J23114 (weak) (Fig. S8A). Both cell growth (Fig. S8B and S8C) and GFP fluorescence (Fig. S8D and S8E) were measured in the absence (0 nM) and presence (250 nM) of aTC. For cell growth, no significant differences were observed regardless of aTC presence or the strength of the FndCas12a expression promoter, showing patterns comparable to those of the empty vector (Fig. S8B and S8C). In contrast, among the J23100, J23105, and J23114

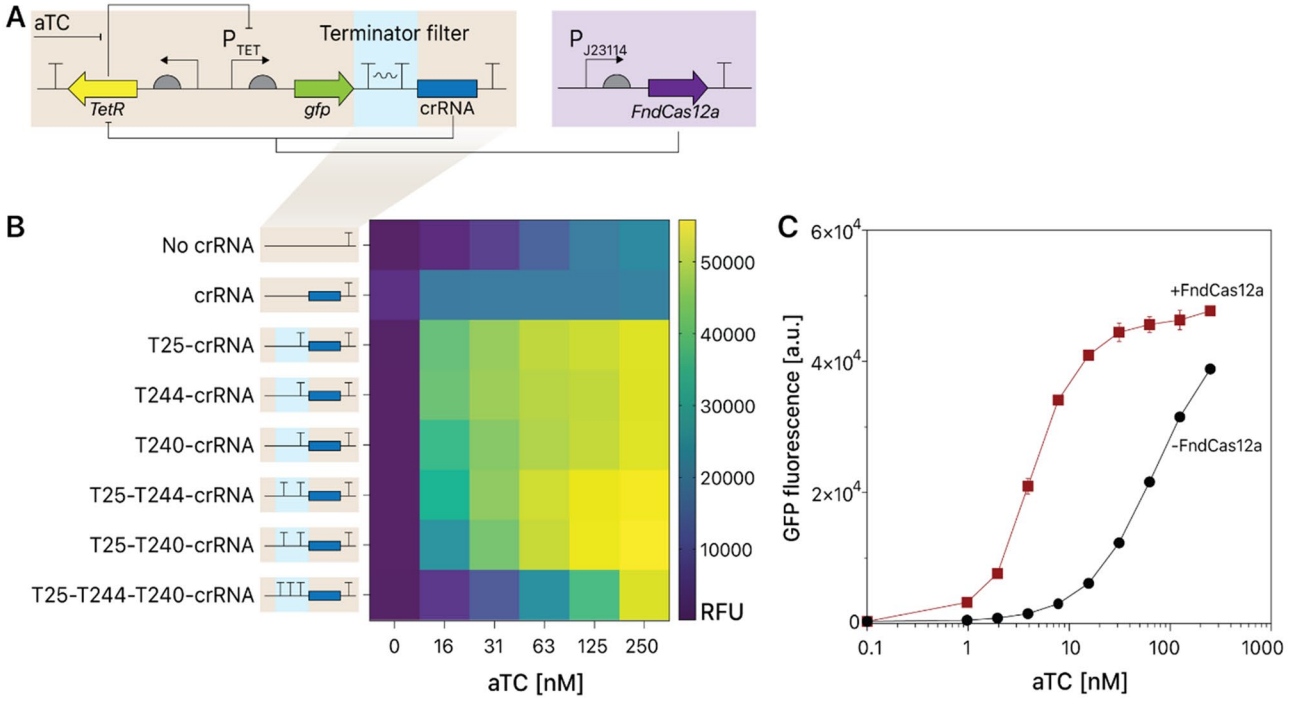

Fig. 4 Genetic signal amplification using CRISPRi-aided genetic switches targeting the TetR repressor. (**A**) Schematic representation of an aTC-inducible *gfp* gene linked to a crRNA cassette targeting the *tetR* gene (crRNA(TetR)). Various terminator filters (illustrated in panel **B**) were inserted upstream of the crRNA(TetR) cassette to regulate its expression. FndCas12a was constitutively expressed under the control of the P_{J23114} promoter. (**B**) Heat map showing GFP fluorescence (a.u.) as a function of aTC concentration (0, 16, 31, 63, 125, and 250 nM) for 24 h. The performance of the genetic signal amplifier was evaluated in *E. coli* DH5α strains harboring the respective circuits. ON/OFF ratios for all groups are provided in Table S5. (**C**) GFP fluorescence (a.u.) for the P_{TET}-*gfp*-T244-crRNA(TetR) circuit co-transformed with either P_{J23114}-FndCas12a (FndCas12a+) or the pSEVA221 control plasmid (FndCas12a-). Data are presented as the mean ± standard deviation (SD) from three biological replicates

promoters, GFP fluorescence was lowest in the absence of aTC when using the J23114 promoter (Fig. S8D). However, in the presence of aTC, all three promoters exhibited similar GFP fluorescence levels, regardless of their strength (Fig. S8E). These results indicate that constitutive FndCas12a expression does not affect cell growth, as its pattern closely resembled that of the empty vector, suggesting that FndCas12a does not induce cell toxicity. This observation is consistent with previous reports showing that dCas12a exhibits minimal toxicity, particularly when expressed at moderate levels with minimal off-target effects [41–43]. Based on these findings, the weak J23114 promoter, which resulted in the lowest fluorescence under aTC-free conditions, was selected for subsequent experiments.

Having optimized FndCas12a expression, we next investigated the impact of terminator filters on genetic circuit performance. The genetic circuit containing crRNA(TetR) demonstrated enhanced GFP expression upon aTC induction, except in configurations with triple terminator filters (T25-T244-T240) (Fig. 4B). In these cases, the terminators likely suppressed nearly all transcription from the P_{TET} promoter, preventing crRNA(TetR) production. Without terminator filters, leaky transcription from the P_{TET} promoter resulted in elevated GFP levels even in the absence of aTC (OFF state). However, single or double terminator filters effectively suppressed this background expression while preserving the ability to amplify GFP production in the ON state. The resulting ON/OFF ratios quantitatively demonstrate the effectiveness of terminator filters in optimizing signal amplification. These values were normalized to fluorescence at aTC = 0 nM (set as 1.0) and are summarized in Table S5.

Among the tested configurations, the T244 terminator filter demonstrated the most significant improvements in system performance. The EC_{50} value (half-maximal inducer concentration) for GFP production was reduced to 5 nM, approximately ten times lower than the control circuit lacking FndCas12a (EC_{50} = 54 nM). Additionally, OFF-state GFP fluorescence was reduced to 161 ± 4 a.u., closely approximating the control without FndCas12a (120 ± 7 a.u.), ensuring minimal background expression (Fig. 4C).

This CRISPRi-aided genetic switch approach is not limited to TetR-based systems and could be extended to other repressor-based circuits for signal amplification. This design strategy enhances the versatility and applicability of CRISPRi-based circuits across diverse synthetic biology applications, providing a reliable framework

for the development of high-performance genetic systems. Especially, this approach could be adapted for biosensors that detect important biological or environmental molecules. To validate this potential, we applied our CRISPRi-aided genetic switch to an indoleacetic acid (IAA)-responsive biosensor system [54]. IAA is a microbiota-derived metabolite that modulates gut immune responses and is associated with intestinal inflammation [54]. By constructing a P_{IacA}-GFP circuit regulated by the IacR repressor from *Pseudomonas putida* 1290 and introducing a crRNA targeting *iacR*, we achieved enhanced biosensor output (Fig. S9). Upon treatment with 16 μM IAA, the strain expressing the *iacR*-targeting crRNA exhibited significantly higher GFP fluorescence compared to the non-targeting control, demonstrating that CRISPRi-mediated repression of the IacR repressor effectively amplified the biosensor signal. These results demonstrate that CRISPRi-based genetic switches can be combined with ligand-responsive transcription factors to create highly sensitive and tunable detection platforms for various targets, including heavy metals [55], small metabolites [56], and disease biomarkers [57]. Moreover, the modular nature of this method allows for the design of dynamic regulatory networks, enabling precise control over gene expression in synthetic biology applications. By leveraging CRISPRi's accuracy in gene regulation, this technique paves the way for more flexible and programmable next generation biosensing technologies.

CRISPRi-aided metabolic genetic switches for reprogramming cellular pathways.

Metabolic flux redirection using a genetic switch is a powerful strategy to enhance biochemical production by balancing cell growth and target compound synthesis [58, 59]. Conventional metabolic genetic switches often rely on engineered strains in which endogenous genes encoding key metabolic enzymes are deleted. These designs are complemented by episomal plasmids that conditionally express both the deleted enzymes and pathways for biochemical production. While effective, such approaches require extensive genetic modifications and optimization, limiting their scalability and applicability. To simplify this process, we developed a CRISPRi-aided metabolic genetic switch capable of simultaneously repressing endogenous target genes and expressing metabolic enzymes without permanent modifications to the host strain. As a proof of concept, we targeted the *gapA* gene, encoding glyceraldehyde-3-phosphate dehydrogenase (GapA), a critical enzyme in *E. coli* glycolysis [60]. GapA functions as a metabolic valve to regulate the production of biochemicals such as 3-hydroxypropionic acid and isoprenoids [59, 61]. Additionally, substituting the NAD-dependent GapA with NADP-dependent orthologs has been shown to improve the synthesis of NADPH-dependent biochemicals including isoprenoids [62, 63]. To construct the metabolic genetic switch, we designed a crRNA cassette targeting the endogenous *gapA* gene downstream of a P_{TET}-driven *gfp* reporter gene (Fig. 5A). In the absence of terminator filters, leaky crRNA(GapA) transcription from the P_{TET} promoter in the OFF state (no aTC) caused slight delays in cell growth compared to the control circuit lacking FndCas12a (Fig. S10B). Incorporating a T244 terminator filter effectively mitigated this issue by reducing basal crRNA expression (Fig. S10C). Upon aTC induction (ON state), CRISPRi activity significantly delayed cell growth, increasing the ET_{50} (time to half-maximal cell growth) from 6.5 h to 13.7 h. Although the T244 terminator filter reduced this inhibitory effect, the ET_{50} in the ON state still increased to 11.3 h (Fig. S10C). Next, we replaced the *gfp* gene with the *gapC* gene from *Clostridium acetobutylicum* to compensate for the loss of GapA activity. As anticipated, repression of the endogenous *gapA* gene reduced cell growth (Fig. 5A and C). However, the expression of the orthologous *gapC* gene fully restored glycolytic function, resulting in growth rates comparable to the control (Fig. 5B and D). These results demonstrate that the central glycolytic pathway in *E. coli* can be effectively reconstituted using enzymes from different species. This study highlights the versatility of CRISPRi-aided genetic circuits as metabolic genetic switches, particularly for targeting essential genes that are challenging to manipulate with conventional genome engineering tools. The system offers precise modulation of gene expression and metabolic pathways without requiring permanent genomic modifications. Its broad applicability across various bacterial species with identified essential genes makes it a powerful tool for dynamic gene regulation and metabolic engineering. These findings underscore the transformative potential of this platform, paving the way for its integration into diverse synthetic biology and biotechnological applications.

The CRISPRi-aided genetic switch platform developed in this study represents a transformative advancement in transcriptional regulation, offering precise, modular, and dynamic control over gene expression. While the results demonstrate the system's versatility and effectiveness, there remain several limitations and challenges that highlight opportunities for further refinement and expansion.

One of the primary challenges encountered in this study is basal transcription, particularly when employing strong promoters such as P_{TRC}. Although the incorporation of transcriptional terminator filters, such as T244 and T25-T244 combinations, significantly reduced background expression, complete suppression of basal transcription remains elusive. This limitation was evident in the partial repression of *gfp* in the OFF state despite the inclusion of robust terminators (Fig. 3). This issue is particularly critical in systems requiring tight transcriptional

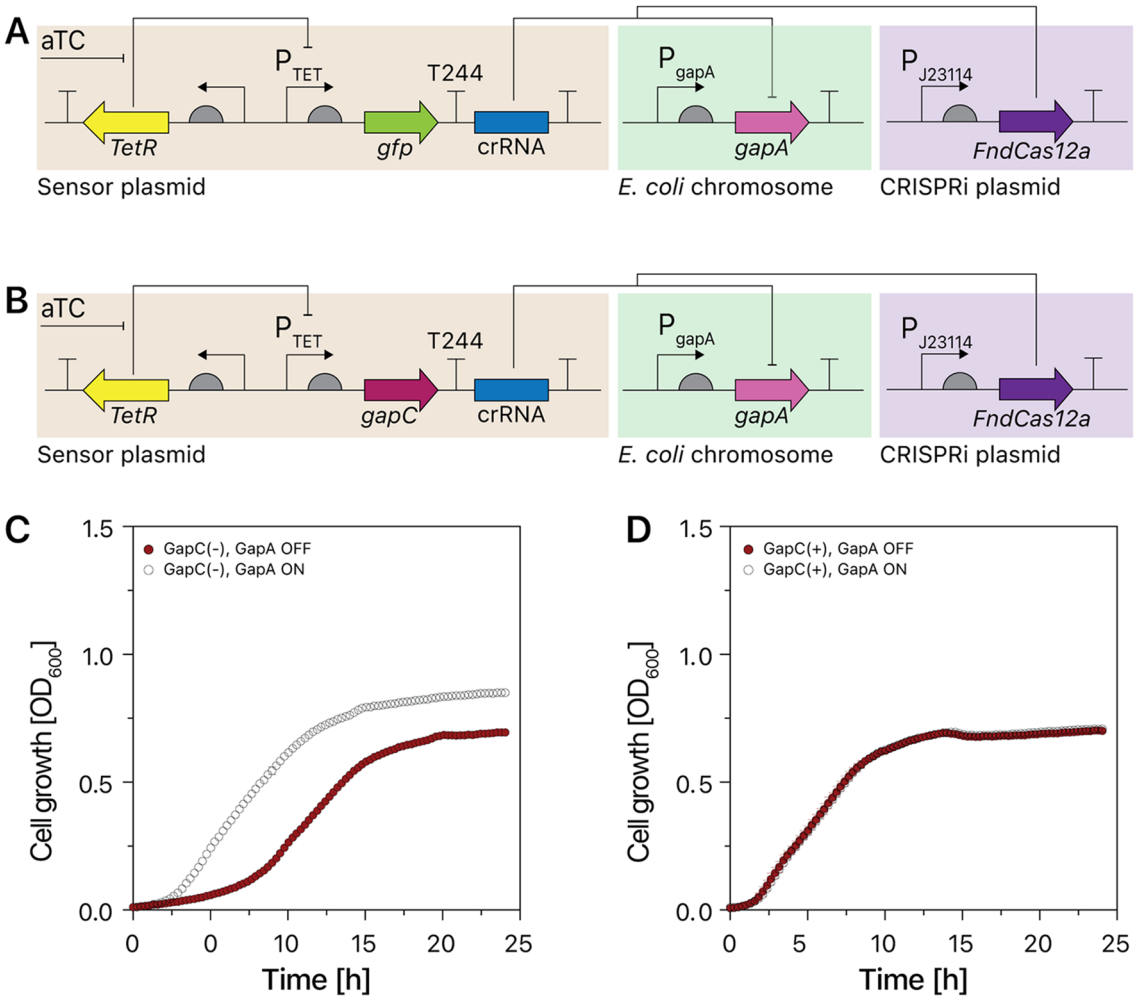

Fig. 5 Metabolic genetic switch targeting the endogenous *gapA* gene. (**A**) Schematic representation of an aTC-inducible *gfp* gene linked to a crRNA cassette targeting the *gapA* gene (crRNA(GapA)) with a T244 terminator filter. (**B**) Schematic representation of an aTC-inducible *gapC* gene linked to a crRNA cassette targeting the *gapA* gene (crRNA(GapA)) with a T244 terminator filter. FndCas12a was constitutively expressed under the control of the P_{J23114} promoter. (**C**) Cell growth (OD_{600}) of *E. coli* DH5α strains harboring the P_{TET}-*gfp*-T244-crRNA(GapA) circuit co-transformed with either P_{J23114}-FndCas12a (red circles) or the pSEVA221 control plasmid (open circles). (**D**) Cell growth (OD_{600}) of *E. coli* DH5α strains harboring the P_{TET}-*gapC*-T244-crRNA(GapA) circuit co-transformed with either P_{J23114}-FndCas12a (red circles) or the pSEVA221 control plasmid (open circles). Data are presented as the mean ± standard deviation (SD) from three biological replicates

control to prevent resource drain or metabolic crosstalk [64]. To further minimize transcriptional leakiness, genomic integration of genetic circuits could be considered as an alternative approach. Reducing gene copy number through genomic integration may help lower background expression, although it could also affect expression levels and system flexibility. Future studies could explore the impact of genomic integration on the performance of CRISPRi-aided genetic switches to assess its potential benefits and trade-offs.

Future efforts should explore the design of next-generation regulatory elements, such as synthetic terminators with higher termination efficiencies or engineered RNA-binding proteins that act as additional repression layers. Additionally, advancements in promoter engineering, including dynamic control of promoter activity using inducible or feedback-regulated designs, could further minimize unwanted background expression. Such improvements would greatly enhance the utility of CRISPRi-aided genetic switches in applications demanding stringent ON/OFF states, such as bioproduction and dynamic pathway optimization.

Furthermore, considering the importance of tunable gene regulation across diverse transcriptional regulators, incorporating our CRISPRi-aided genetic switches into *E. coli* Marionette [65] could offer a promising direction. As a well-characterized system equipped with a broad range of orthogonal biosensors, the Marionette strain provides a robust and modular framework for integrating signal-responsive regulatory elements. When combined with

our system, this compatibility could further strengthen gene regulation performance and facilitate the construction of advanced multi-input genetic circuits.

While *E. coli* DH5α served as a robust chassis for system characterization in this study, the platform's broader applicability to other bacterial hosts remains unexplored. Industrially relevant organisms, such as *Corynebacterium glutamicum* for amino acid production [66] and *Bacillus subtilis* for enzyme synthesis [67], possess distinct genetic architectures and regulatory landscapes that could impact system performance. For example, differences in promoter activity, RNA processing efficiencies, and CRISPR protein expression levels may necessitate host-specific optimizations.

Adapting CRISPRi-aided genetic switches to diverse microbial systems would not only validate their universality but also expand their utility in industrial biotechnology. For instance, the metabolic genetic switch targeting *gapA* demonstrated in *E. coli* (Fig. 5) could be re-engineered to target essential metabolic valves in other hosts, thereby enabling tailored metabolic flux redirection for diverse biosynthetic pathways. Future studies should focus on transferring the platform to non-model organisms, systematically investigating host-specific constraints, and engineering compatibility through synthetic biology tools such as modular plasmid backbones or orthogonal regulatory systems.

While this study highlights the effectiveness of FndCas12a, alternative CRISPR systems could offer complementary functionalities that enhance the versatility of CRISPRi-aided genetic switches. For instance, dCas9 derivatives with altered PAM requirements [68] could enable a broader range of target sites, overcoming constraints imposed by the narrow PAM specificity of FndCas12a. Additionally, systems like dCas12b [69] or engineered dCas13 variants [70], which target RNA rather than DNA, could provide novel opportunities for post-transcriptional regulation.

The potential benefits of exploring such alternatives include greater precision in transcriptional control, expanded genome-editing capabilities, and the ability to implement more complex genetic circuits. For example, RNA-targeting systems could be used to regulate the stability or translation of target mRNAs, enabling fine-tuned control over protein expression [71]. Integrating multiple CRISPR systems within the same circuit could also facilitate the design of multilayered genetic switches, supporting advanced functionalities such as adaptive feedback loops or spatiotemporal control [35].

Beyond addressing basal transcription and host-specific limitations, expanding the range of applications for CRISPRi-aided switches represents an exciting frontier. The platform's demonstrated ability to regulate chromosomal genes (Fig. 3) and its application as a metabolic genetic switch for *gapA* repression and *gapC* compensation (Fig. 5) underscore its potential for metabolic engineering. By targeting essential metabolic genes and dynamically redirecting flux, the system could enable the optimization of complex biosynthetic pathways, such as those involved in producing high-value biochemicals like isoprenoids [72] or biofuels [73]. However, this study demonstrated the feasibility of dynamic metabolic pathway reprogramming by balancing essential gene functions, future efforts should focus on directly linking CRISPRi-aided genetic switches to enhanced biochemical production. Establishing such connections will further validate the utility of this platform for metabolic engineering and industrial biotechnology applications.

In industrial settings, scaling up these systems would require engineering their stability and efficiency under high-density cultivation conditions. This could involve developing genome-integrated versions of CRISPRi switches to reduce plasmid loss or utilizing tunable CRISPRi systems responsive to environmental cues. Additionally, combining CRISPRi switches with advanced biosensors could facilitate real-time monitoring and dynamic regulation of metabolic pathways, further enhancing productivity.

The results presented in this study demonstrate the robustness and adaptability of CRISPRi-aided genetic switches while highlighting areas for future innovation. By addressing the challenges of basal transcription, host dependency, and CRISPR system diversity, this platform could be further optimized to meet the needs of synthetic biology and industrial biotechnology.

Conclusions

In this study, we developed a versatile CRISPRi-aided genetic switch platform that enables precise, signal-responsive transcriptional regulation without requiring permanent modifications to the host strain. By leveraging FndCas12a's RNase activity for dynamic crRNA processing and incorporating transcriptional terminator filters, our system effectively mitigates leaky transcription, achieving enhanced regulatory precision. This modular approach successfully regulated gene expression across various genetic contexts. The CRISPRi-aided genetic switch platform established in this study provides a robust and flexible framework for dynamic gene regulation. Its potential applications span synthetic biology, metabolic engineering, and biotechnological innovation, offering a transformative tool for addressing complex challenges in these fields. Future advancements in the platform's design and deployment are expected to unlock new opportunities for its integration into diverse biological and industrial systems.

Abbreviations

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRi	CRISPR Interference
FndCas12a	*Francisella novicida* DNase-deactivated Cas12a
crRNA	CRISPR RNA
TF	Transcription Factor
PAM	Protospacer Adjacent Motif
RFP	Red Fluorescent Protein
GFP	Green Fluorescent Protein
sfGFP	superfolder Green Fluorescent Protein
aTC	anhydrotetracycline
EC_{50}	Half-Maximal Effective Concentration
ET_{50}	Time To half-maximal cell growth
IAA	Indoleacetic Acid
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate

Supplementary Information

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Supplementary Material 1

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Author contributions

S.K.K., S.W., J.P. and carried out the experiments and contributed to drafting the manuscript. S.L. and D.L. supervised the project and provided critical revisions to the manuscript. All authors have reviewed and approved the final version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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