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# The sound of silence: transgene silencing in mammalian cell engineering

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DECLARATION OF INTERESTS

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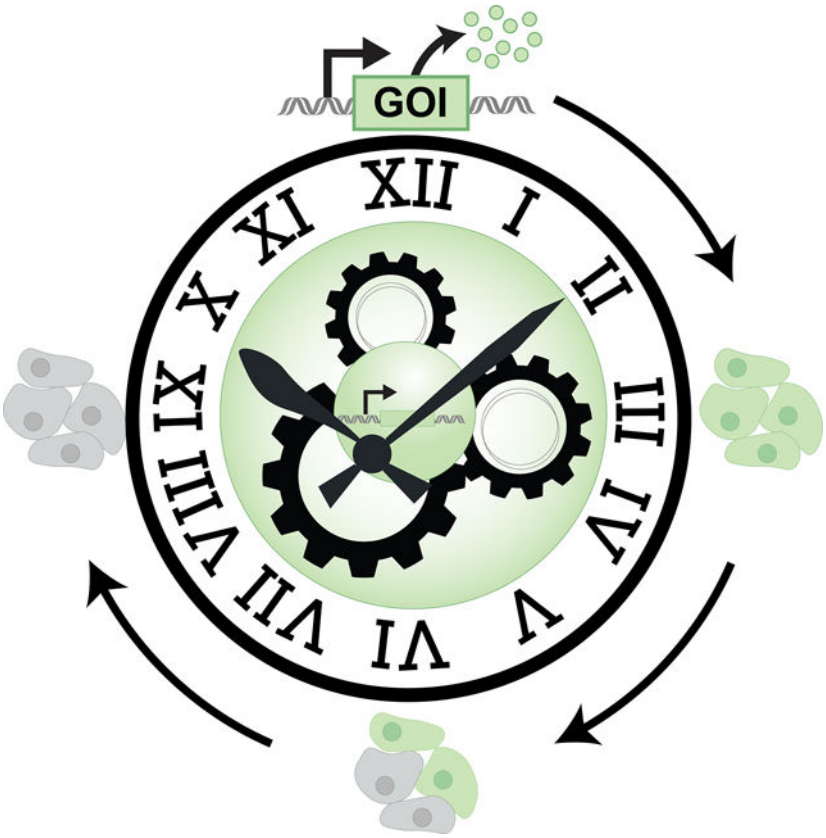
## Abstract

To elucidate principles operating in native biological systems and to develop novel biotechnologies, synthetic biology aims to build and integrate synthetic gene circuits within native transcriptional networks. The utility of synthetic gene circuits for cell engineering relies on the ability to control the expression of all constituent transgene components. Transgene silencing, defined as the loss of expression over time, persists as an obstacle for engineering primary cells and stem cells with transgenic cargos. In this review, we highlight the challenge that transgene silencing poses to the robust engineering of mammalian cells, outline potential molecular mechanisms of silencing, and present approaches for preventing transgene silencing. We conclude with a perspective identifying future research directions for improving the performance of synthetic gene circuits.

## eTOC

Transgene silencing, defined as the loss of transgene expression over time, persists as an obstacle for engineering mammalian cells and limits various applications of mammalian cell-based biotechnology. Diverse mechanisms contribute to transgene silencing, suggesting a variety of strategies may be needed to overcome these mechanisms. Furthermore, benchmarking of genetic components will be integral for designing and achieving roust transgene expression in mammalian cells.

## Graphical Abstract




## Introduction

Genome engineering within mammalian cells enables the stable expression of transgenes to support the design and implementation of custom genetic programs across a wide range of biotechnology applications [1–9]. Engineered cells must retain control over the expression of transgenes over many cell generations. However, stably integrated transgenes often experience silencing, or diminished expression over time, thus limiting the use of engineered cells for applications that require weeks or more of expression [10]. In the context of synthetic gene circuits, silencing interferes with circuit regulation, limiting the translation of engineered gene circuits for therapeutic and other applications. In this perspective, we highlight the challenge that transgene silencing poses to the robust engineering of mammalian cells, along with opportunities to mitigate this phenomenon.

Transgene silencing appears conserved across diverse organisms [11–13]. Host cell identity, sequence of the integrated transgene(s), its location of integration, and gene delivery methods all putatively contribute to the rate and degree of transgene silencing. Silencing can manifest as an all-or-nothing phenomenon in which a portion of cells do not express the transgene. Often the proportion of engineered cells that express the transgene decreases over time in culture [14–17] (Figure 1A). In some cases, transgene silencing can be observed

as a decrease in transgene expression levels in individual cells [18,19], and it often appears as a heritable change passed down through cell generations [14,15,20].

Transgene silencing represents a bottleneck for many mammalian cell-based biotechnology applications (Figure 1B). For instance, in industrial cell lines such as Chinese hamster ovary (CHO) cells, or human embryonic kidney (HEK) 293 cells, the silencing of integrated transgenes reduces the long-term production yield for biopharmaceutical manufacturing [21,22]. Similarly, silencing of sense-and-respond theranostic circuits lead to waning efficacy over time [23–25]. Additionally, more complex synthetic gene circuits with multiple transgenes may be more susceptible to performance failure as silencing of any individual transgene renders the whole circuit nonfunctional (Figure 1C). Furthermore, silencing can spread to neighboring genes through direct and indirect effects, resulting in silencing compounding over time [24,26]. Moreover, loss of expression of transgenes delivered via retroviruses has been well-documented in reprogramming, which may inhibit cell-fate transitions and lead to partially reprogrammed cells [27–30]. Thus, transgene silencing is a critical challenge to understand and overcome for effective cell-based technologies.

In this perspective, we provide an overview of known mechanisms for transgene silencing, provide practical guidelines on how to avoid transgene silencing, and offer a look into future efforts that can further expand our understanding and improve our ability to control transgene expression in synthetic gene circuits. In addition, we suggest that future publications include discussions of observed cases of transgene silencing to help move toward more predictable and reliable cell reprogramming (Box 1).

## MECHANISMS OF TRANSGENE SILENCING

Cells rely on transcriptional regulation to tune gene expression, respond to environmental stressors, and generate phenotypic diversity in complex multicellular organisms. Epigenetic regulation complements dynamic transcriptional control. Through the deposition, recognition, and erasure of covalent modifications to DNA and histones, epigenetic regulation confers stable memory and hysteresis within biological systems. Epigenetic regulation harmonizes with transcriptional control including the assembly of the preinitiation complex, double-stranded DNA melting at the promoter, initiation, or elongation [31]. In all, these can be affected by DNA modifications and influence the structure of the local chromatin and the protein-DNA complexes surrounding the gene of interest. Synthetic gene circuits must contend with native epigenetic and transcriptional regulatory mechanisms, which may support or impede transgene expression. As silencing often correlates with specific chromatin modifications profiles of transgenes, epigenetic regulation putatively supports and reinforces transgene silencing. Therefore, an understanding of the epigenetic mechanisms that silence transgenic cargoes in mammalian cells may facilitate the design of more robust genetic circuits and engineered transcriptional programs [32].

Cells engineered with multi-component circuits rely on the tight regulation of multiple transcriptional units to control cellular behavior. For example, cell-based therapies might employ circuits composed of multiple transgenic cassettes encoding biosensing and signal processing functions. Hence, one malfunctioning unit, or cassette, could result in the

breakdown of the entire circuit (Figure 1C). In one study, a genetic circuit consisting of four transcription cassettes in HEK293T cells was silenced at an estimated rate of ~2% of the population per week [24], whereas a more rapid shutdown of an 8 kb circuit was observed after approximately three weeks of culture in mouse embryonic stem cells [25]. Notably, in both examples the DNA circuits were integrated at genomic safe-harbor sites. While safe-harbor sites provide genomic regions that support transgene integration without adversely affecting normal cellular functions, cassettes integrated at these sites are still subject to silencing. These results highlight the need for a better understanding of how genomic context and composition of the synthetic gene circuit can influence transgene silencing.

## How do cells identify transgenes for silencing?

Over time, cells selectively silence integrated transgenes while maintaining endogenous genes at homeostatic levels [33,34]. Given that silencing appears to selectively impact transgenic elements, how do cells distinguish transgenes from other genomic elements to generate specific profiles of silencing? As recruitment of chromatin-modifying enzymes likely serves an essential step in epigenetic silencing, cells may recruit these enzymes through mechanisms that are dependent and independent of the transgene sequence. In sequence-dependent mechanisms, interactions at the exact site of transgene integration may prime transgenes for selective silencing. This may include protein recognition of specific DNA motifs, such as CpG islands, and the subsequent formation of complexes with chromatin-modifying activity [35]. Additionally, sequence-dependent formation of DNA, RNA, or hybrid structures may recruit chromatin-modifying enzymes. For instance, GC-rich sequences can induce G-quadruplexes, R-loops, and other DNA structures that may contribute to RNA polymerase (RNAP) stalling and recruitment of chromatin modifiers through factors that directly recognize these structures [36,37]. Furthermore, terminal repeat sequences that enable transposon-based insertion of transgenes into the genome can trigger RNAi-mediated silencing [38]. As the particular mechanism may vary based on the sequence of the transgene, we expect that interventions may show different efficacy across transgenic cargos.

Alternatively, sequence-independent silencing may result from passive loss of transcriptional activity which may be influenced by local genomic context effects. For instance, activity of chromatin modifiers near the locus of transgene integration may contribute to nonspecific silencing through spreading of heterochromatin. Additionally, chromatin-modifying enzymes may broadly survey the genome, actively silencing genes through reversible, transient modification [39]. Nascent chromatin remains inaccessible and transcriptionally inactive following DNA replication, and transcriptional reactivation is required to regain accessibility [40]. Potentially, gene activity is reestablished for endogenous genes through selective transcriptional reactivation by combinations of endogenous transcription factors after DNA replication. Lacking such mechanisms, transgenes may remain nonspecifically silenced.

In principle, sequence-dependent and -independent mechanisms may combine to induce transgene silencing. Whether recruited in a sequence-dependent manner or not, chromatin

modifying enzymes may prevent expression of synthetic circuits through heterochromatin formation, DNA methylation and post-translational modifications (PTMs) to histones. Processes that lead to silencing may be induced at the site of transgene integration via direct recruitment of chromatin regulators, encroachment of heterochromatin, activation of viral and transposon defense systems, or proliferation-mediated processes. Below, we discuss these mechanisms and their relation to transgene silencing.

## Proliferation-associated processes can mediate silencing

Transgene silencing increases over time in proliferating cells and correlates with the number of cell divisions. Both the fraction of cells that express the transgene and the mean expression level of marker-positive cells can decrease over time and with cell division [24,27,41]. Putatively, processes linked to the cell cycle provide a mechanism that enhances transgene silencing.

Transgene silencing may be accelerated by the inherent antagonism between transcription and the DNA replication necessary for proliferation, each increasing torsional strain and steric interference on the DNA polymer [42,43]. These processes can lead to the accumulation of positive and negative supercoiling (over- or under-wound DNA, respectively), which can in turn promote the formation of structures such as R-loops, interactions between DNA and nascent RNA [44,45]. R-loops have been shown to alter the binding of chromatin remodelers [36], suggesting a potential mechanism by which persistent changes in gene expression could arise (Figure 2A). Indeed, overexpression of transcription factors in reprogramming induces markers of genomic stress including increased negative DNA supercoiling, R-loop formation, and DNA replication fork stalling [27]. Thus, collisions between the transcription and replication machinery in proliferating cells may contribute to transgene silencing.

Proliferation and silencing are intimately linked in the process of stem cell reprogramming and differentiation. For example, proliferation has been shown to promote cellular reprogramming to induce pluripotent cells and to neurons [27,46]. The loss of transgene expression delivered via retroviruses is well-documented in reprogramming, and this loss of expression may inhibit cell-fate transitions and lead to partially reprogrammed cells [27–30]. On the other hand, proliferation can also drive transgene silencing while simultaneously increasing the probability that a cell will reprogram [27,46]. A tradeoff between transgene expression and proliferation emerges, leaving a narrow window of time for reprogramming. Notably, in a recent study, cells that sustain high transgene expression while undergoing rapid proliferation reprogrammed to neurons at high rates and display increased functional maturity [27]. While the loss of transgene expression may induce heterogeneity and reduce efficiency, the loss of reprogramming factors may improve differentiation of pluripotent stem cells to new cell fates [30]. Silencing of transgenes has been observed after they have been placed in various safe harbor loci during human pluripotent stem cell (hPSC) and mouse embryonic stem cell (mESC) differentiation into various lineages [47–50]. In some cases, transgene expression could be maintained by the introduction of a flanking chromatin insulator derived from the chicken β-globulin hypersensitivity site 4 (cHS4) [47]. In fibroblast conversion to induced pluripotent stem cells, Velychko *et al* found that retroviral silencing varied based on the reprogramming factors used and that silencing could

occur early in the reprogramming process, even before the loss of fibroblast identity [28]. In particular, inclusion of cMyc, which drives proliferation, increased transgene silencing. In reprogramming to neurons, loss of expression of retroviral transgenes occurs at higher rates in hyperproliferative cells [27]. In addition to these proliferation-associated phenomena, silencing also occurs in post-mitotic or slowly dividing cells [23,51]. Overall, silencing is often enhanced by proliferation but does not require proliferation.

## DNA methylation contributes to stable silencing

Cytosine methylation at CpG motifs contributes to epigenetic silencing (Figure 2B). The distribution of CpG dinucleotides delineates DNA with different states of methylation and plays a key role in epigenetic regulation [52]. When interspersed across genomic tracks including introns and exons, CpGs are canonically methylated and may contribute to transgene silencing. On the other hand, when CpGs cluster at promoters and enhancers to form CpG islands (CGIs), they are often hypomethylated [53].

Methyltransferases establish and maintain CGI methylation. During embryogenesis, DNA methyltransferase 3 Alpha, Beta, and Like (DNMT3A, DNMT3B, and DNMT3L) establish CpG methylation which is maintained during DNA replication by DNA methyltransferase 1 (DNMT1) [54–57]. Understanding CGI methylation and transcriptional repression may guide strategies to mitigate CpG-mediated transgene silencing [58–61]. In addition to a direct contribution to epigenetic silencing, DNA methylation can be involved in the recruitment of H3K9me3 to nucleosomes, which contributes to the formation of heterochromatin [62]. Heterochromatin is associated with limited DNA accessibility, nuclear reorganization, and silenced transcription, as discussed further below [63,64].

CGIs associate with ubiquitously expressed genes, but not with tissue-specific genes [65]. The presence of CpGs on promoters significantly impacts the silencing of downstream genes [55,57,66–71]. Furthermore, CGI hypomethylation and active transcription may reinforce one another through competition between transcription and methylase complexes, causing active promoters to remain hypomethylated and inactive genes to accrue methylation [65,72].

Native and synthetic CGIs may confer specific patterns of DNA methylation. Endogenous promoters ectopically inserted into the β-globin locus of mouse embryonic stem cells exhibited CGI methylation patterns that resemble their native counterparts [73]. Similarly, synthetic elements comprising CGIs and bacterial sequences recapitulate expected patterns of methylation [73]. However, CGIs do not effectively shield promoters from methylation if positioned beyond 100–200bp from the transcription start site [61,68,74]. Synthetic promoter-less CGIs may recruit histone methyltransferases and accrue H3K4me3, but the capacity to remain CpG-hypomethylated requires both high CpG density and high GC content, suggesting that AT-rich sequences act as DNA methyltransferase docking sites [71]. Overall, DNA CpG methylation is implicated in transcriptional silencing, whereas high GC-content CGIs associated with transcription sites remain hypomethylated.

## Heterochromatin-associated histone modifications are found at silenced transgenes

Histone modifications play a central role in epigenetic gene silencing through constitutive heterochromatin formation [75,76]. Heterochromatin is characterized by regions with relatively

high nucleosomal density, which may impede transcription initiation. DNA methylation can initiate formation of heterochromatin through the recruitment of histone deacetylase enzymes (HDACs), which remove histone acetylation, a feature typically associated with transcriptionally active chromatin (Figure 2B). Importantly, treatment of cells with the HDAC inhibitor sodium butyrate can restore inducible gene activation in mouse embryonic stem and transformed mammalian breast cancer cells [25,77]. To induce durable silencing, deacetylated histones are subsequently trimethylated by histone methyltransferases [78–80]. Silencing is facilitated by nuclear proteins such as heterochromatin protein-1 (HP1) which promote heterochromatin maintenance [81]. Targeted inhibitions of these processes at sites of transgene integration may prevent local heterochromatin formation without the global epigenetic perturbations induced by broad chemical inhibitors, potentially offering an approach to mitigate silencing due to heterochromatin formation.

## Spreading of heterochromatin silences proximal regions at the locus of integration

Although heterochromatin formation may initiate focally, proximal regions may be silenced through spreading of heterochromatin (Figure 2C). Silencing of proximal regions was identified in *Drosophila* and termed position effect variegation in 1930 by Muller et al. [82,83]. Although this phenomenon was initially described with genomic rearrangement of endogenous genetic elements, integration of transgenic payloads mimics these phenomena [84–86]. Encroachment of the surrounding heterochromatin can disrupt transcription. Spreading of H3K9me3 propagates via a feedback loop of chromatin regulators [75,87,88]. Combined with DNA methylation, spreading of H3K9me3 leads to heterochromatization and transcriptional repression. In the case of an integrated transgene heterochromatization of a nearby gene can spread to, and silence, the transgene [26]. Furthermore, spreading of heterochromatin has been observed using multicopy transcription arrays in hamster, mouse, and *Drosophila* cells showing that gene silencing correlates with appearance of repressive chromatin at transgene arrays [89–91].

Epigenetic silencing of transgenes depends on the specific locus and genomic context of integration [92,93]. Integration within topological associated domains (TADs) may impact transgene activity through TAD-specific determinants of chromatin state [94]. For example, H3K9me3 often spreads throughout a TAD [95]. Notably, integration near centromeres may influence the epigenetic silencing of nearby transgenes. In cases when random integration methods are used to engineer synthetic genetic circuits in mammalian cells, wide variability in gene expression and epigenetic silencing may result.

Site-specific genome engineering methods can be utilized to integrate transgenes at so-called safe-harbor loci, yet there remain associated complexities that require further examination. For example, silencing of safe-harbor loci is well-documented [25,47,48,96]. Furthermore, transgene insertion can alter the local chromatin state in a locus-specific manner and affect transgene expression [32]. A key open challenge is understanding why transgene insertion into safe-harbor loci confers stable, consistent gene expression in some scenarios (e.g. cell types, transgene sequences, and insertion conditions) but not others. For a comprehensive review of silencing of transgenes in safe harbor sites and discussions of criteria for identifying genomic safe harbors, we direct readers to the following perspective [97].

## Viral and transposon defense systems contribute to transgene silencing

Viral vectors and transposon systems provide powerful tools to integrate transgenic cargo into mammalian cells with high efficiency, but they confer specific challenges in maintaining transgene activity. Key gene therapy delivery agents such as lentiviral and gammaretroviral vectors are subject to transcriptional silencing upon integration into the mammalian genome [98–100]. Mechanistically, the silencing of lentiviral vectors is often associated with promoter methylation, especially during differentiation of stem cells [101]. Viral promoters appear to be more prone to epigenetic silencing compared to endogenous promoters [101], highlighting the need to choose an appropriate promoter for clinical gene delivery when viral vectors are used for transgene delivery. For example, DNA methylation and silencing was observed in murine hematopoietic stem cells (HSCs) following Moloney murine leukemia virus (MoMuLV)-based retroviral transduction in vivo following serial transplantation [102]. In these studies, both murine stem cell virus (MSCV) and human immunodeficiency virus type 1 (HIV-1) virus led to DNA methyltransferase activity independent of silencing in transgenic mice, murine embryonic stem cells, and Drosophila [103].

As a defensive adaptation against pathogens and transposon-mediated genomic instability, mammalian cells use epigenetic regulation mechanisms to specifically identify and repress virally integrated transgenes [104]. Mammalian hosts possess dedicated machinery that detects proviral sequences and recruits histone-modification complexes that mediate transcriptional repression (Figure 2D). One such proviral sequence is the primer binding site, an 18-bp element residing near the 5’ long terminal repeat (LTR) from which viral reverse transcription is initiated. The LTR sequence varies across viruses and is complementary to ribosomal tRNAs, allowing retroviruses to hijack tRNAs and prime reverse transcription of the minus strand [105,106]. Reciprocally, the host cell can use this site as a target for transcriptional repression. For example, pluripotent cells strongly repress gene expression from MoMuLV [107–109]. Biochemical analyses have shown that ZFP809 and TRIM28 (KAP1) bind the primer binding site and form a complex [110,111] that recruits the H3K9 methyltransferase SETDB1 [112,113] and components of the NuRD histone deacetylase complex [114]. Accordingly, ZFP809 and TRIM28 are enriched in an endogenous retrovirus sequence spanning the LTR, 5’UTR and beginning of gag, and are essential for H3K9me3 deposition, histone deacetylation, and repression of proviral genomes [113,115,116]. Additional native proteins have been implicated in reinforcing this epigenetic repression complex by acting as a scaffold for SETDB1 and NuRD components [117]. In this way, endogenous proteins recognize viral DNA motifs and induce epigenetic changes in a sequence-dependent manner.

Viral LTRs serve as prominent targets for CpG methylation. The DNMT-binding scaffold protein Daxx mediates repression of invading viruses and contributes to maintenance of LTR methylation [118]. Additionally, methylation of endogenous proviruses is facilitated by the ZFP809-TRIM28-SETDB1 complex [119]. Combining TRIM28 knockout with 5-azacytidine-induced CpG demethylation increases provirus transcriptional reactivation stronger than either treatment alone [115]. However, SETDB1 knockout reactivates endogenous retroviruses without affecting CpG methylation, and SETDB1-mediated H3K9me3 deposition is unaffected by co-deletion of *Dnmt3a*/*Dnmt3b*/*Dnmt1* [113]. Hence,

it appears that DNMT-Daxx-mediated CpG methylation and SETDB1-NuRD histone modifications act synergistically to robustly ensure retrovirus and transposon repression. It therefore seems plausible that engineering of viral vectors, e.g. modifying the ZFP809 recognition sequence in the LTR, could result in stealth variants that are less susceptible to epigenetic silencing.

Another defense mechanism involves the recognition of non-self macromolecules carrying pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs by host-expressed pattern recognition receptors (PRRs) triggers an innate immune signaling reaction [120,121]. The Toll-like receptor (TLR) 9 recognizes bacterial and viral DNA lacking methylated CpGs, triggering activation of NFκB and resulting in the production of cytokines in dendritic cells and macrophages [122,123]. An shRNA screen of baculovirus-infected A549 cells identified genes of the TLR, interferon, and interleukin families that silence transgene expression [124,125]. In another example of immune-mediated transgene silencing, influenza and Sendai virus infections of macrophages trigger IFN-α-mediated upregulation of TLR1–3 and TLR7 [126]. IFN-α reduces histone acetylation and transcription of hepatitis B viral mini chromosomes in HepG2 cells [127], and upregulates Daxx in HeLa cells [118]. PAMP-PRR reactions thus convert infected cells into cytokine hubs that signal an upregulation of repressive barriers against invading transgenes (Figure 2C). To ensure transgene expression, these reactions should be avoided through careful consideration and engineering of delivery vectors.

## PRACTICAL GUIDELINES FOR AVOIDING TRANSGENE SILENCING

Loss of transgene expression compounds with the myriad other challenges of cell engineering. The field needs improved methodological guidelines and data sharing of successes and failures alike across diverse systems to identify common, useful tools and frameworks. Here, we propose practical guidelines for stable engineering of mammalian cells.

### Choice of promoter influences the probability of transgene silencing

Promoters vary in their transcriptional activities and sensitivity to epigenetic silencing. Transgene expression is dependent on multiple factors that vary across genetically engineered clones (e.g. loci of integration, copy number, and target cell), which can obscure the role of the promoter in silencing. Thus, it is important to test the effect of the promoter while controlling for genomic context, for instance by comparing multiple clones with the transgene cassette integrated in the same location, differing only in the selected promoter. To our knowledge, there has not been a comprehensive comparison that systematically evaluated the long-term activity of all promoters commonly used in mammalian synthetic biology. Here, we surveyed the literature to assess promoter performance in terms of expression levels and stable activity over time in the context of their respective experimental details (Table 1).

Inducible systems provide extra safety by offering the ability to turn on and off expression of a transgene using external control, such as the addition of a small molecule, light, or other user-imposed or cell-sensed stimuli. Regulation by inducible promoters allows

stably integrated transgenes to be left in the inactive or OFF state for periods of time before induction. These periods of inactivity correlate with an increase in the proportion of cells that do not respond to induction. This phenomenon has been documented for tetracycline-inducible promoter systems, where continuous induction or higher basal activity of the promoter results in less silencing compared to versions with tightly regulated OFF states [77,128]. More recently, inducible expression systems have been developed that resist silencing over longer time periods compared to a tetracycline-inducible promoter, employing constitutive transgene transcription with post-transcriptional regulation to mitigate silencing [129].

Viral promoters such as cytomegalovirus (CMV), spleen focus-forming virus (SFFV), and Rous sarcoma virus (RSV) undergo CpG methylation resulting in silencing of the transgene within a few cell divisions, however, this silencing can be alleviated with 5-azacytidine, a DNA methyltransferase inhibitor, to partially restore the transgene expression [22,101,130,131]. A gene driven by the RSV promoter has also been shown to be silenced by polycomb repression complex 2 (PRC2) in CHO cells [26]. However, silencing of the cassette containing RSV and a downstream transgene, can be prevented by proximal cHS4 insulators, perhaps due to increased local histone acetylation and protection of the promoter from DNA methylation [132,133]. In mESCs, transgenes containing LTR and SV40 promoters can also be acutely silenced [134–136]. However, unlike in pluripotent stem cells, 5-azacytidine may not rescue transgene expression, as has been observed in undifferentiated pluripotent stem cells [25]. Additionally, while episome disappearance and provirus methylation occurred seven to ten days after infection, the viral cassette was silenced prior to that point, supporting a DNA methylation-independent mechanism of provirus silencing in pluripotent stem cells [108,109]. This mechanism appears to be histone methylation (H3K9me3 and H4K20me3) catalyzed by SETDB1 in complex with TRIM28 and ZFP809. A mutation in the LTR-associated primer binding site, demonstrated improved long-term transgene expression in bone marrow cells transplanted into irradiated recipient mice [137]. Together, these data show that viral promoters alone should be avoided for long-term transgene expression due to their propensity to be silenced, but engineered variants show promise.

Comparison of various promoters in their ability to drive GFP expression via lentiviral transduction of murine and human cell lines has shown that EF1α and CAG promoters consistently produce high fluorescence intensities; CAG exhibited the least variation between transductions, whereas the CMV promoter demonstrated fluorescent variability depending on the host cellular context (e.g. HEK-293T and human MRC5 fibroblasts) [138]. Similarly, high CMV activity in HEK-293T cells is corroborated by transient transfections when transgenes are not permanently integrated into the genome [139]. Non-viral Lipofectamine-mediated transgene delivery into HEK-293F cells showed that the top three promoters driving highest yields were, in descending order, CAG, EF1α and CMV, and the ranking remained consistent over time [140]. Therefore, the EF1α and CAG promoters are commonly used in workhorse mammalian cells because they seem to be the most suitable for long-term expression of high transgene levels.

The choice of promoter in stem cells appears to be more complicated. For example, EF1α, CAG and PGK promoters all have been successfully used to drive long-term transgene

expression in undifferentiated mESCs [101,141,142]. However, their performance is influenced by context-specific factors such as viral elements or locus of integration [141,143]. With respect to differentiation, EF1α promoter activity has been observed to suffer the least silencing during early-stage embryoid body differentiation [101,141], however, during neuronal maturation, the CMV promoter outperformed EF1α and CAG promoters in mESC-derived neurons, illustrating lineage-dependent promoter performance [141]. In hESCs transduced with lentiviral vectors, fluorescence intensity was highest when the EF1α, β-actin, and PGK promoters were used to drive GFP expression [33]. Moreover, promoters can exhibit different behaviors based on the type of silencing considered. For example, β-actin remained the most active during long-term hESC maintenance, however, during differentiation it drove the highest overall GFP intensity. In contrast, EF1α retained the largest percentage of GFP positive cells and overall activity across a number of lineage markers [33]. Overall, EF1α, CAG, and β-actin promoters efficiently drive transgene expression in stem cells, however, it is important to consider the context of reprogramming stem cells and whether the goal is for long-term stem cell maintenance, differentiation, or both.

Additionally, beyond stability of the mean expression level, consideration of the expression dynamics may also be relevant for some applications. Future work to characterize these parameters of common promoters across contexts is therefore needed. Ultimately, promoter choice should be considered in concert with other factors including insulating elements, locus of integration, and cell type.

## Insulators can block transgene silencing

One commonly adopted strategy to counteract transgene silencing is to include insulating DNA elements in the expression cassette. Two types of DNA insulator functions exist: barrier activity that blocks the spreading of heterochromatin from nearby repressive regions, and enhancer-blocking activity that prevents enhancer-dependent gene activation [144]. In the context of mitigating transgene silencing, the prevention of heterochromatin spreading is important. Various insulating elements have been reported and tested in mammalian cells, including the prototypic insulator, cHS4 [145,146], scaffold/matrix attachment regions (S/MAR) [147], ubiquitous chromatin opening elements (UCOE) [148,149] and human tRNA gene tDNA [150] (Table 2). Readers are directed to other reviews for more detailed discussions on this topic [151–153]. These barrier elements typically function by recruiting proteins (e.g., histone modifying enzymes, chromatin remodelers) that prevent the spreading of repressive heterochromatin and thus establish a local transcriptionally permissive environment [151]. More specifically, the core region of the cHS4 insulator has protein binding sites for VEZF1, CTCF and USF1/2, which protect against DNA methylation, help form chromosomal loops, and recruit histone modifying enzymes associated with active expression states, respectively [154]. Accumulating evidence has shown the significant role that chromatin insulators play in regulating the 3D genome architecture [155]. For instance, the binding of CTCF, the primary insulator protein in mammals, is essential to establish the boundaries of TADs [156]. The role of CTCF as an enhancer blocker has been well characterized, leading to the discovery of highly potent enhancer-blocking insulators from the high affinity CTCF-binding sites in the human genome [157]. Interestingly, genome-wide analysis of CTCF-binding sites in chromatin barrier regions indicate that CTCF may also play an important role in the barrier activity of

insulators [158]. Hence, future studies on chromatin boundary regions will likely contribute to the discovery of novel insulators beneficial for mammalian synthetic biology.

The insulating DNA elements identified so far face various challenges that limit their use in mammalian cell engineering. Incorporating the cHS4 sequence into the transgene cassette can significantly reduce the titer of packaged lentivirus carrying the transgene [159,160]. In addition, the relatively large size of S/MAR elements (e.g. the S/MAR 1–68 element is ~3.6kb) renders them unfavorable when using vectors with limited cargo capability. Although UCOE-type insulators have been shown to prevent silencing when used with numerous promoters in stem cells [149], their potential bidirectional promoter activity may lead to transcriptional activation of nearby genes upon integration, which poses a safety concern in gene therapy [161]. Interestingly, a recent study screened candidate UCOEs with various truncations, demonstrating their potential to function as barrier-type insulators without intrinsic promoter activity [162]. However, there is a lack of systematic comparison of the barrier activity of different insulating elements under the same conditions (e.g., cell line, chromosome context, and copy number). Therefore, both direct comparison of existing insulators within the same context and identification of other novel insulators with better features (e.g. compact size, broad tissue compatibility, and no intrinsic promoter activity) would be beneficial to the synthetic biology community. Overall, the choice of promoter and insulator combined with exclusion of transcriptional repression target elements is important to stabilize high levels of transgene expression (Figure 3A).

## Genomic locus of integration affects stable transgene expression

One major driver of transgene silencing and the instability of expression over time is the local repressive environment of the integration site in the genome. Viral vectors (e.g., retroviruses or lentiviruses) or transposase/transposon systems (e.g. piggyBac or Sleeping Beauty transposase) can deliver synthetic DNA cargo into the mammalian genome with high efficiency in a semi-random manner, as different vectors and systems have their own integration biases [163,164]. However, due to the uncontrolled integration, there is often minimal to no regulation over the integrant copy number or the integration site(s), which may result in concatemer-induced epigenetic silencing [21] or silencing caused by the existence or spreading of local repressive chromatin at the integration site [165]. More importantly, because transgenes exhibit different levels of expression when integrated into different chromosomal sites, random insertion is often unfavorable when systematic comparison or characterization of multiple DNA elements (promoter, enhancer, insulator, etc.) is desired.

One strategy to avoid these drawbacks associated with uncontrolled integration is to insert transgenic DNA at a predefined, transcription-permissible locus in the genome. Often the empirically determined genomic safe-harbor regions were chosen for this purpose [166]. Currently, the popular choices of safe harbor loci include AAVS1, CCR5, and hRosa26 in the human genome, and Rosa26 and Hipp11 in the mouse genome. These commonly adopted safe harbor loci have been validated in various models including human iPSCs and ESCs, human CD34+ T cells, HEK293T cells, CHO cells as well as transgenic mice [167–171]. Interestingly, a recent study by Aznauryan et al. identified two novel safe harbor sites (Rogi1

and Rogi2) that are capable of stable and safe expression of transgenes [172]. These two sites were first tested in HEK293T and Jurkat cells for long-term transgene expression, and were further validated in primary T cells and dermal fibroblasts, thus offering more target choices in the human genome (Figure 3B). However, safe-harbor regions may remain vulnerable to epigenetic silencing for reasons discussed above. Potentially, a combination of strategies may most effectively reduce the probability of transgene silencing.

These loci can be targeted with programmable genome editing tools such as the CRISPR-Cas9 system to achieve targeted insertion of relatively short DNA sequences (e.g., single gene cassettes) with high efficiency. However, considering that synthetic gene circuits typically consist of multiple transcription units, the inevitably large size (e.g. greater than 15 kb) of the circuit makes it challenging for CRISPR-based genomic insertion [25,173,174]. A serine integrase, on the other hand, is capable of integrating large DNA cargos with high specificity in mammalian cells [25,173,174]. Recently, the serine integrase-based landing-pad strategy has been widely adopted for various applications involving large DNA constructs, with examples including the rapid prototyping of synthetic DNA circuits [175], the parallel assessment of large human gene variants library [176], and the integration of up to nine copies (~100 kb) of a monoclonal antibody-expressing gene cassette to improve antibody production in mammalian cells [177]. Although serine integrases can be advantageous in their high specificity and large cargo capability, one caveat is that they require a landing pad (namely, an att recognition site) to be previously inserted at the chosen site to create a chassis cell line, which limits the ability to multiplex such a strategy. Therefore, recent development in novel genome editing tools combining CRISPR and integrases for targeted insertion of large DNA sequences [178] as well as the discovery of novel integrases with better activities at both landing pads and directly targeting the human genome [179], could enable the synthetic biology community to more rapidly test locations in the genome to characterize synthetic gene circuits in mammalian cells.

## Cell-type choice influences the stability of transgene expression

It is important not to assume that the transgene-silencing factors discussed here are present at the same levels in all cell types. Data downloaded from the Human Protein Atlas (HPA) show that one or more transgene-silencing–associated factors are expressed at high levels in cell lines that are often used as test beds for cell engineering, and levels vary across cell lines (Table 3). The wealth of available epigenomic data for these widely used cell lines (e.g., ENCODE [180] and 4DN, https://www.4dnucleome.org/cell-lines.html) should be leveraged to investigate context-dependent transgene behavior. For instance, chromatin immunoprecipitation (ChIP) signals can be used to compare levels of transcriptional silencing or activating chromatin features at genomically mapped safe harbor loci. Additionally, RNA-seq data can be used to identify factors that are expressed at high levels, and their impact on transgene silencing can be tested by genetic knock-down or chemical inhibition. For example, high HDAC1 expression may contribute to transgene silencing in T cells, as indicated by the HPA data for Jurkat cells. In T cells that showed lentiviral and retroviral transgene silencing after four weeks of passaging, treatment with HDAC inhibitors was used to restore transgene expression [181]. Future work could similarly identify context-specific methods to mitigate transgene silencing.

For cancer-derived cell lines components of the SWItch/Sucrose Non-Fermentable (SWI/SNF) ATP-dependent chromatin remodeling complex frequently show loss of function mutations or low expression [182,183]. This needs to be taken into consideration when attempting to modify histone marks via HDAC inhibition or epigenome editing to restore transgene expression because SWI/SNF is often required to act in concert with chromatin modifications such as histone acetylation to activate transcription.

Pluripotent stem cells allow for unlimited self-renewal and the ability to contribute to all germ-layers that give rise to the adult body. The pluripotent state entails unique epigenetic properties as unveiled through MoMuLV infection experiments of mouse embryos and pluripotent cells [107–109]. First, ESCs express Zfp809 that mediates Setdb1-catalyzed H3K9 and H4K20 methylation of retroviral sequences through the Zfp809-Trm28-Setb1 complex, allowing the cells to efficiently repress expression of transgenes delivered and integrated through viral vectors [110,111,113,184]. Second, mouse preimplantation stem cells abundantly express tRNA-derived fragments that inhibit translation of retroviral transcripts by competing for the primer binding site [185]. Third, mouse embryos carrying a human β-globin gene regulated by a Cre-excisable methylation-resistant CGI methylate and silence the transgene only if the island is excised before implantation [61]. These findings illustrate that pluripotent cells possess unique mechanisms that could silence transgene expression, and exit from pluripotency concomitant to implantation involves *de novo* methylation that is associated with transcriptional silencing [54,55].

During mouse development, high-density CpG promoters and CGIs are resistant to *de novo* methylation, most of which occurs during implantation at the E4.5-E5.5 transition catalyzed by DNMT3A and DNMT3B [55]. Given the comparability of naïve pluripotent ESCs to preimplantation E4.5 epiblast cells [186], it is conceivable that differentiation recapitulates passage through the developmental stage of implantation and the surge of *de novo* DNA methylation. Indeed, mESCs exiting naïve pluripotency exhibited increased DNMT3A/DNMT3B expression and genome-wide CpG methylation after 24 hours of differentiation triggered by PD0325901/CHIR99021 withdrawal from the media (with notable resistance of CGI promoters), although no correlation between promoter methylation and respective gene expression was determined [187]. Similarly, differentiating mESCs accrue DNMT3A and DNMT3B-dependent CpG methylation in the Oct4 promoter (curiously reduction of Oct4 mRNA preceded methylation [188], which has been described as a non-CpG promoter [68]. High-density CpG and CGI promoters might provide candidates for safeguarding promoter activity during pluripotent stem cell differentiation.

Differentiation encompasses dynamic chromatin state changes, with different loci changing from an open to a closed chromatin state and vice versa. This can lead to silencing of randomly integrated transgenes in a promoter-independent and locus-dependent manner. Constitutively active loci allowing ubiquitous transgene expression have been identified to tackle this problem: the Rosa26 locus in the mouse genome [189], and AAVS1, CCR5 and Rosa26 in the human genome [97,167]. It will be interesting to see how the two newly identified human genomic safe harbors [172] fare in ensuring ubiquitous transgene expression during human stem cell differentiation. Altogether, global changes in DNA methylation and

chromatin states are critical factors of transgene activity during stem cell differentiations and reprogramming (Figure 3C). CGIs and safe harbors may provide solutions to these barriers.

### Avoiding nutrient limitations supports active transgene expression

Engineered cell lines may encounter signals from the microenvironment that induce dramatic shifts in metabolic states that could impact epigenetic regulation of transgenes. A pool of metabolites that become depleted or replenished in response to environmental cues also provides substrates for the chromatin modification machinery. For instance, the free pool of acetyl-coA, the sole substrate for acetylation of histones in transcriptionally active chromatin, is heavily regulated by signals linked to nutrient availability and is primarily derived from extracellular glucose levels. Exposure to fatty acids or insulin can increase lipid storage and synthesis [190–192]. In yeast, when glucose becomes unavailable and cells enter the stationary phase, lipid synthesis outcompetes histone acetyltransferases (HATs) for acetyl-coA, and histone acetylation levels decrease [193,194], which favors the formation of closed chromatin. Human cancer cells show increased lipogenesis and broad reprogramming of gene expression in response to signals from adipocytes [195–197]. Thus, low levels of glucose in cell culture media could lead to transgene silencing through decreased availability of acetyl-coA, as well as high levels of fatty acids or insulin. Lactate can play a similar role to acetylation through lactylation of histones. Evidence thus far of this novel histone mark supports that lactylation promotes maintenance of active genes [198,199], so low levels of lactate could result in silencing. However, a delicate balance must be struck as high levels of lactate generally reduce cell growth and protein production [200]. Furthermore, inhibition of histone demethylases by D-2-hydroxyglutarate (D2HG), an “oncometabolite” produced by mutated IDH1/2, has been implicated in gene silencing in cancer cells, and has been mechanistically linked to elevated H3K39me3 and gene silencing in yeast [201]. Taken together, these observations suggest the importance of careful growth medium design and feeding strategies in order to reduce transgene silencing, focusing on providing sufficient glucose and reducing flux through the D2HG pathway.

## PROSPECTS FOR THE FUTURE

Despite the identification of abundant potential mechanisms and diverse strategies for mitigating transgene silencing, silencing persists as a challenge for cellular engineering, highlighting the importance of new descriptive studies and novel strategies for stable transgene expression. Basic research into the biology of silencing could elucidate its molecular and physical basis, identify the responsible host genes and pathways, and inform new strategies to address this challenge. Here, we propose future research directions that could propel the field of mammalian synthetic biology past the current challenges of transgene silencing. This research includes the application of publicly available data to investigate silencing at the epigenetic level or identify silencing-resistant promoters, CRISPR-based screens to identify genes associated with silencing and massively parallel reporter assays (MPRAs) to evaluate new circuit components to prevent silencing. Finally, alternative engineering solutions could be further developed to mitigate transgene silencing, including non-integrating methods of stable expression, post-transcriptional and translational regulation, and epigenetic modifying circuits.

### Mapping chromatin modifications in the transgene and at the integration loci

Mapping chromatin modifications both at endogenous integration loci and in silenced transgenes will be useful for better understanding the characteristics of effective safe-harbor loci as well as the mechanisms by which transgenes are epigenetically silenced (Figure 4A). Investigating chromatin modifications at integration sites across different cell types of interest could help identify any differences that affect silencing of a transgene upon integration. Several databases including ENCODE [180], 4DN [202] and Human Cell Atlas [203] contain data on chromatin modifications (ChIP-seq, CUT&RUN), chromatin accessibility (ATAC-seq) and gene expression (RNA-seq) in both human cell lines and primary cells. Using this epigenetic information to better characterize existing safe harbor loci will also aid the discovery of new integration sites that are less prone to transgene silencing. Similar epigenetic profiling of silenced transgenes for different chromatin modifications such as DNA methylation, H3K27me3, H3K9me3, and H3K27ac will provide insights into how transgenes are silenced, including which chromatin complexes are involved and how transgenes are recognized or potentially targeted for silencing by the cell. Mapping these chromatin modifications will help inform larger screens to determine genes that are responsible for transgene silencing.

### Identifying constitutive endogenous promoters by harnessing publicly available data

Identification of additional stable constitutive promoters could also be useful for maintaining transgene expression, as these elements may be silencing-resistant. This could be accomplished by scanning the mammalian transcriptomes for ubiquitously expressed housekeeping genes, mapping their respective promoters in the genome, and utilizing highly conserved candidates for stable transgene expression. The FANTOM5 database lists such housekeeping gene promoters for mouse and human cells, making it an attractive tool for identifying species-conserved stable promoter sequences [204]. Indeed, the ubiquitous-uniform promoter category contains β-actin and elongation factor-family genes (e.g. EF1α), along with p53 and members of the ribonucleoprotein processing machinery. On the other hand, promoters of non-coding RNAs showed the least cross-species conservation, while non-TATA and CGI-based promoters demonstrated non-ubiquitous expression [204]. CGIs are thought to confer resistance to DNA methylation-dependent gene silencing, so the non-ubiquitous expression associated with CGI promoters possibly reflects differential methylation: most CGIs are ubiquitously unmethylated, about 25% are ubiquitously methylated, and a few thousand exhibit tissue-specific methylation [68]. Therefore, the subcategory of housekeeper-associated unmethylated CGIs might hold attractive candidates for safeguarding transgene expression. Alternatively, novel CGI-promoter hybrid combinations could create synthetic promoters with desired properties. In support of this notion, fusion of the CGI of theCHO-K1 β-actin promoter to the CMV promoter improved long-term transgene expression and antibody production yields in CHO-K1 cells, compared to the original CMV promoter [205]. A high-throughput synthetic biology approach utilizing databases listing CGIs, transcription start sites [68] and mammalian promoters [204] could facilitate the computational design and genetic engineering of novel CGI-promoter variants optimized to ensure stable transgene expression.

## CRISPR screens to map genes responsible for transgene silencing

CRISPR screens can provide a better understanding of mechanisms that have evolved to avoid silencing and will inspire new synthetic biology strategies [206–208]. Endogenous cellular pathways are responsible for the silencing of transgenic payloads. Therefore, an identification of such pathways could facilitate the prevention of these silencing mechanisms. In order to map the cellular pathways involved in transgene silencing, one could perform high-throughput loss-of-function screens, using gene-perturbation strategies to avoid unintended consequences associated with the use of small-molecule inhibitors, such as effects on native gene expression and potential cellular toxicity [209,210]. Pooled CRISPR screens are a promising strategy to identify the host genes required for silencing (Figure 4B). One could use a genome-wide or epigenetics-focused sgRNA library to knockout host genes and then measure sgRNA enrichments in cells where a synthetic gene circuit is silenced versus maintained. Identification of the required host genes could be immediately useful. CRISPR screens have been used to identify genes involved with drug resistance, influenza A virus infections, and cellular reprogramming [211–213]. For example, one could generate stable knockout or knockdown cell lines that are silencing-incompetent, or transiently inhibit the host silencing machinery with small molecules or siRNAs to prevent or reverse silencing [125,214–216]

Aside from the practical outcomes of stable transgene expression, a deeper investigation of these host silencing pathways could expand our understanding of how the host machinery evolved to silence synthetic genes. Additionally, studies of the cGAS-STING sensing [217], and chromatinization of episomal transgenes indicate that DNA (e.g., linear DNA donor template for CRISPR editing and plasmid DNA used in Sleeping Beauty transposon system) can be sensed by the cell and modified prior to integration. Therefore, CRISPR screens prior to, and post, integration can provide insights into the pathways involved in silencing. One possible outcome is the identification of new mechanisms that have evolved to defend the cell against other foreign DNA including viruses and transposable elements.

## Massively parallel reporter assays (MPRAs) as a method to find novel elements and understand their optimal deployment

Advances in technologies for genetic screening, epigenomics, and synthetic gene circuit design are creating new opportunities to characterize and prevent transcriptional silencing in mammalian cells. Furthermore, high-throughput screening of endogenous genetic elements could facilitate the rational design of new construct components and aid in the understanding of epigenetic regulation with the ability to rapidly screen synthetic gene circuit stability after integration into the genome. Massively parallel reporter assays (MPRAs) including CapSTARR-seq, a high-throughput method to quantify enhancer activity [218,219] and Functional Identification of Regulatory Elements Within Accessible Chromatin (FIREWACh) [220] for mammalian cells have primarily been used to measure enhancer and promoter activity; however, most studies probe enhancers with the same minimal promoter that is decoupled from genomic locus as most reporters are not integrated into the genome [221,222]. Chromosomal domains have long been shown to affect transgene expression [223], and lentivirus based MPRA has shown that genomically integrated reporters have different expression than their episomal counterparts [224]. A method termed

Thousands of Reporters Integrated in Parallel (TRIP), allows high-throughput investigation of the influence of genomic integration loci on transgene expression [165]. Analysis of two promoters, mPGK and tet-Off, at 27,000 loci in mESCs showed 1,000-fold variation in expression levels, where chromatin state is related to expression level and lamina-associated domains attenuated transcription, while nearby enhancers increased expression [165]. Additionally, MPRA of enhancers have also shown cell-type specificity [225]. While there are numerous screens on the effect of enhancer-promoter interactions, promoters and insulators have been less well characterized. Screening human promoters at AAVS1 safe harbor locus in K562 cells found that core promoters drive unidirectional transcription [226]. Screening more broadly across loci, promoter activity scales across regions of integration suggesting that integration context provides a factor over the promoter-intrinsic properties [227]. However, there remain many human promoters to characterize for their variance in expression levels in different cell types and for their propensity of transgene silencing (Table 3). Using MPRAs to better characterize promoters in different genomic contexts and cell types would help identify which loci and promoter combinations in specific cell types reduce transgene silencing, as well as help further understand the mechanisms that drive silencing (Figure 4C). In addition to testing different cell types, promoters should also be tested in different cell states such as under various metabolic conditions or in the presence of immune simulation. High-throughput screening may facilitate identification of novel insulator elements that resist transgene silencing. When encoded proximal to transgenes, existing insulators such as UCOE and cHS4 promote stable transgene expression. Candidate UCOE elements vary in their ability to limit transgene silencing and depend upon the choice of the promoter [162]. Further efforts to screen and characterize diverse insulators will help add more reliable insulators to the synthetic biology toolbox.

New genetic elements should be benchmarked against current gold-standard regulatory elements to define their effectiveness. As noted above, genetic elements function differently between cell types and lines, so the performance of these elements will likely require a systematic comparison within the relevant cellular context. MPRAs have immense potential to discover new parts and optimize circuit configuration, but researchers ought to begin standardizing currently available genetic elements. For instance, alternate sequences of related genetic elements such as EF1α and EFS promoters can have drastically different performance and properties [228]. Therefore, a systematic comparison of currently available elements is a necessary step towards standardization of best practices to inform optimal construction, enhance circuit robustness, and minimize systemic inefficiencies.

## Inspiration from evolved solutions to transgene silencing

Viruses have evolved many mechanisms to avoid identification and silencing of viral elements. Therefore, there is significant potential in repurposing evolved viral defense mechanisms to design novel stable transgene expression strategies. For instance, incorporation of the S/MAR element was shown to enhance nuclear transport of transfected episomal DNA [229]. Engineering of this phenomenon may eventually yield robust expression of transgenes and synthetic circuits. As another example, viral covalently closed circular DNA (cccDNA) persistence is a hallmark of Hepatitis B virus (HBV) infection and there are non-integrated HBV elements that are believed to interact with host chromatin related

proteins to regulate viral gene transcription. Additionally, the persistence of these foreign elements is believed to be the cause of relapse after viral infection clearance [230]. The production of recombinant cccDNA has been employed in the search for drugs to treat and remove these persistent HBV elements [231]. Although these tools will need to be further explored and engineered, recombined cccDNA may be leveraged for stable transgene expression. Another viral defense mechanism involves the suppressors of RNA silencing (VSRs), a mechanism used by viruses to interfere with host RNA interference following infection of plant, insect, and mammalian cells [232] Engineering elements inspired by these and newly discovered viral defense mechanisms have the potential to result in the design of robust genetic circuits.

## Managing silencing in extrachromosomal vectors

Our understanding of the challenges associated with silencing in extrachromosomal vectors is informed in part by substantial experience with adeno-associated virus (AAV) vectors, which exist as episomal DNA. Adeno-associated viruses (AAVs) have attracted a significant amount of attention for use as a gene therapy vector to deliver DNA *in vivo* because they can have low immunogenicity and low rates of insertional mutagenesis [233]. Nevertheless, two early-phase clinical trials of gene therapy for inherited vision loss reported only short-term vision improvement following the treatment of patients with recombinant AAV [234,235]. Although the underlying mechanism of the decline in improved vision in the long-term remains unclear, transgene silencing was proposed as a potential cause in one of the studies [234]. The hypothesis that transgene silencing was the major cause of the poor robustness of AAV gene therapy approaches has yet to be proven, especially since cellular turnover and immune responses could also play a significant role. Determining the mechanism(s) involved in the durability of AAV gene therapy *in vivo* presents a challenge, and this challenge becomes increasingly complicated as studies focus on more complex and therapeutically relevant systems. For example, a preclinical study of liver-targeted AAV found a strong correlation between liver vector DNA copy number and transgene protein expression level in mice, but that there was very little protein expression from non-human primate liver despite DNA levels of approximately 1–100 vector copies per cell. The authors proposed vector silencing as one possible culprit [236]. A recent preprint describes a study on the loss of AAV transgene expression in the primate liver that used *in situ* hybridization and found a disproportionate loss of transgene RNA relative to DNA over time [23]. At day 14 there was high transgene expression and AAV DNA was found dispersed throughout the nucleus, whereas by day 77, the expression was largely lost and AAV DNA was found in a few distinct foci, which may be transcriptionally inactive. Since AAV rarely integrates into the genome and is not rapidly diluted in non-dividing cells, the use of AAV is viewed as one of the safest and most practical approaches for gene therapies [233]. However, with a positional bias towards transcriptionally active regions [237–239], it has been proposed that the few integration events may drive what is left of transgene expression after the virus wanes through cell divisions. Further, SETDB1, the H3K9 methyltransferase, has been identified by several groups as a host factor that can reduce both the percentage of transgene-expressing cells and the level of expression among those transduced cells when using AAV, lentivirus, and adenovirus delivery methods [240–242]. These findings further implicate chromatin-mediated transgene silencing as a mechanism with significant influence

over long-term AAV expression. In summary, the loss of AAV expression is an important area of active investigation, and transgene silencing of gene therapy vectors may be a major barrier to achieving long-term high treatment efficacy.

Introducing artificial chromosomes may avoid silencing mechanisms inherent to integration in endogenous loci. Human artificial chromosome (HACs) are a potential solution to limitations regarding transgene size limits, positional regulation effects and silencing associated with viral elements [243,244]. So far, the complexity of assembling HACs has limited their construction as well as their benchmarking with other transgene delivery methods [245]. Ongoing efforts have resulted in more streamline assembly and delivery of HACs into human T cells and iPSCs [246]. However, transcriptional silencing does occur on current-generation HACs. Thus, while bottom-up engineering may provide silencing-resistant HACs in the future, further characterization is required for HACs to become a viable method for preventing transgene silencing.

## Post-transcriptional and post-translational mechanisms as alternative regulatory strategies

Genetic circuit designs that robustly resist epigenetic silencing may not be compatible with common methods of transcriptional control, meaning alternative regulatory strategies are needed. For instance, synthetic circuits that are exclusively composed of active promoters may silence less than transcriptionally regulated circuits. In order to achieve the regulatory function of the genetic circuit, post-transcriptional or post-translational mechanisms could be employed instead. Post-transcriptional control can be achieved by including regulatory elements in untranslated regions of mRNA. These parts include microRNA and microRNA binding sites, RNA binding protein motifs, and ribozyme switches. For a more detailed review on this topic, see [247]. Recently, CRISPR Cas-binding motifs [129,248] and toehold switches [249] have also been used to engineer post-transcriptional or translational control. Alternatively, regulation can be implemented at the post-translational level using protein domains responsive to external inputs (such as small molecule- or light-inducible degrons) or by engineering protein-protein interactions. Complex logic has been achieved entirely post-translationally using proteases [250–252]. Because these circuits act independently from transcription, they are compatible with an array of promoters and expression methods. Thus, post-transcriptional and post-translational regulatory strategies could facilitate the decoupling of functional modules from transcriptional components that resist epigenetic silencing, allowing each to be optimized separately.

## Engineered epigenetic modulation to counteract silencing

Synthetic biology tools for epigenetic modulation can be potentially used in genetic circuits to directly counteract epigenetic silencing. Previously developed tools include engineered synthetic chromatin regulators, which typically consist of a DNA binding domain fused to an epigenetic effector domain [253]. The DNA binding domain can be programmable, including Zinc-finger proteins, TALEs and CRISPR proteins [254,255]. This enables epigenetic modifications to be targeted to synthetic genetic circuits, such as removal of silencing modifications (e.g., demethylases) or addition of activating modifications (e.g. acetyltransferases) [256,257] (Figure 4D). Besides using these approaches to study and

modulate epigenetic silencing [14], engineered synthetic chromatin regulators can be used as part of synthetic gene circuits to counteract transgene silencing. A potential approach involves the use of feedback, which is already employed in synthetic gene circuits and natural epigenetic regulatory pathways. For instance, in a positive autoregulatory synthetic gene circuit, synthetic chromatin regulators could be used to constitutively remove repressive epigenetic marks and maintain an open chromatin structure or maintain epigenetic marks associated with active transcription. This approach can be complemented with control theoretic systems biology strategies that mathematically model and study the role of feedback in epigenetics [258,259]. In another approach, a circuit could employ mechanisms to detect epigenetic silencing and subsequently activate an effector to remove the repressive marks. In this case, VP64-based transcriptional activators have been engineered to specifically bind repression-associated histone marks such as H3K27me3 [260] and ChIP data from the epithelial cell line, U2OS, suggest a change in chromatin state from silenced to active perhaps through Mediator recruitment (MED25, MED17) [261]. To use this approach for transgenes, a DNA sequence recognition module would need to be incorporated to achieve transgene-specific regulation and avoid off-target activation elsewhere in the genome. Finally, synthetic chromatin regulators can be used to engineer efficient transitions between closed and open chromatin states. Linking the establishment of an epigenetic state to an input of interest would enable the use of epigenetic memory as a form of information storage.

## Conclusion

In conclusion, although transgene silencing poses significant challenges for mammalian cell engineering, efforts are underway to elucidate the molecular mechanisms responsible for this phenomenon and develop solutions to mitigate it. The field of mammalian synthetic biology can overcome the challenge of transgene silencing by sharing silencing data with current elements discovering new regulatory elements and delivery approaches identifying and intervening with problematic pathways, and employing additional layers of transcriptional and post-transcriptional regulation. The design of more robust tools for mammalian cell engineering will undoubtedly accelerate the fields of cell and gene therapy, biomanufacturing, and basic biology research.

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## Glossary

**Transgene**
A gene that is delivered and expressed in a cell to produce a desired phenotype. A transgene can refer to a native gene that is introduced artificially (for example, for high expression), a gene from another organism, or an entirely synthetic gene. A transgene is often implemented as a transcriptional unit with other components that regulate its expression such as a promoter, a Kozak consensus sequence, and poly-adenylation signal

**Transgene silencing**
Loss or down-regulation of expression of a transgene in a cell despite its encoding DNA remaining present in the cell's nucleus

**Hysteresis**
a property or phenomenon in a system, where the state of the system depends on its history or prior events

**Cassette**
A single unit consisting of a transgene that has yet to be integrated into a genome

**Synthetic gene circuit or genetic program**
An assembly of cassettes that encode RNA or protein molecules that interact with each other to perform one or more biological functions

**Transcriptional unit**
The DNA sequence necessary to produce a single, unified RNA transcript. The transcriptional unit supports expression of one gene or unit of genes. A transcriptional unit often includes a promoter sequence, coding sequence and poly-adenylation sequence. Transcriptional units may include additional elements within the coding sequence such as a splice site or other regulatory elements within the untranslated regions (UTRs)

**Safe-harbor site or safe-harbor locus**
A locus in the genome with an open chromatin state that is amenable for stable transgene expression without adversely affecting normal cellular functions (e.g. the activation of nearby oncogenes)

**Insulator**
DNA elements that serve as barriers to transcriptional and epigenetic regulation of surrounding genes. Specifically, DNA insulators can exhibit barrier activity to block the spreading of heterochromatin or repressive epigenetic modifications. Alternatively, DNA insulators may function as enhancer blockers to prevent the acting of enhancers on the promoters of neighboring genes. Certain DNA insulators such as the prototypic cHS4 exhibit both functions

**Enhancer**
DNA elements that interact with target promoters to amplify initiation of transcription

**Epigenetic effector**

A protein that can modulate the addition or removal of epigenetic modifications on histones or DNA

**Poly-adenylation signal (pA)**
DNA sequence that signals the transcription complex to poly-adenylate the RNA being transcribed. This stabilizes RNA molecules by preventing their degradation to enable their export from the nucleus to the cytoplasm to be translated into protein by ribosomes

**Long terminal repeat (LTR)**
One of a pair of DNA sequences that form a retrotransposon, retrovirus or previrus. LTRs flank retroviral genomes and are common in lentiviral vectors and lentiviral integrations

**CpG islands (CGIs)**
Large DNA segments with high content of CpGs, particularly as compared to other regions of DNA

**H3K9me3**
A heterochromatin associated mark that is associated with the downregulation of nearby genes

**Viral vectors**
Engineered or modified viruses that serve as a vehicle for efficient delivery of nucleic acids to cells. Examples include lentiviral vectors and adeno-associated virus (AAV) vectors

**Topologically associating domains (TADs)**
TADs are self-interacting chromosomal domains identified by chromosome conformation capture technologies. DNA sequences located within the same TAD interact more frequently with each other than with DNA sequences outside the TAD. TADs are proposed as the fundamental regulatory units of the genome three-dimensional architecture

**Serine Integrases**
Single protein systems, derived from mobile genetic elements, that can integrate a large DNA sequence into the genome by mediating recombination between attachment sites (DNA motifs of ~30 bp) on the genome and donor. Examples include the BxB1, PhiC31, and Pa01 large serine recombinases

**Transposon systems**
Systems for transgene integration in the genome that utilize transposition as a mechanism for genomic integration. Examples include the piggyBac system and the sleeping beauty system

**EF1α and EFS promoters**
EF1α is the promoter sequence derived from the human *EEF1A1* gene that expresses the alpha subunit of eukaryotic elongation factor 1. EF1α is known as one of the strong promoters in various mammalian cell lines. The EFS promoter is the short, intronless form of the EF1α promoter

**cHS4 and cHS4 core insulators**

The cHS4 (chicken hypersensitive site 4) insulator is the prototypical chromatin insulator derived from the chicken β-like globin gene cluster. It possesses both the enhancer-blocking and barrier activities and has been adopted for transgene insulation in various mammalian cell lines. The cHS4 core usually refers to the 5’ 250 bp of the full-length cHS4 insulator

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**Box 1.**

### Publishing transgene silencing - making the invisible discoverable.

A large swath of valuable information generated by routine cell engineering goes unreported due to the lack of a system to share findings that are typically not incentivized for publication. Customarily, only the best-performing transgenic lines or clones take center stage in the final manuscript. We encourage scientists to include in figures, supplemental data, or materials and methods the frequencies of transgene silencing (e.g. rates of expressing and non-expressing clones). For example, a succinct description of "transgene performance" accompanied by tabulation of sub-optimal and misbehaving clones will not only provide discoverable data for meta-analyses, but also give authors an opportunity to highlight the magnitude of effort behind their work. For this reporting, we recommend inclusion of: cell type, special culturing conditions (if applicable), promoter type, transfection/transduction vector, number of passages since delivery when the transgene exhibited undesirable behavior, a description of the misregulation, and whether attempts were made to alleviate the silencing with their outcomes. Collectively, this reporting will help move the field forward to predictably and successfully engineer cells.

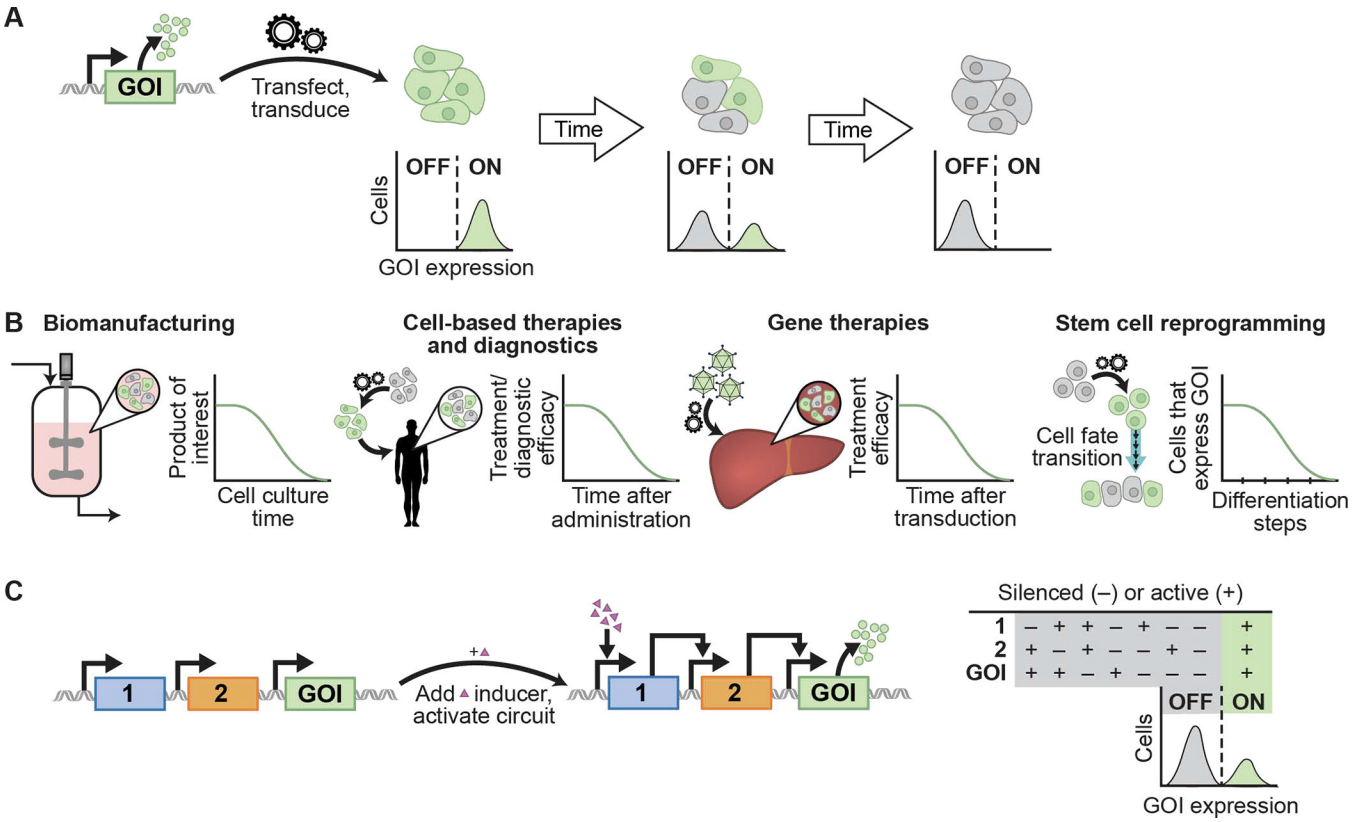



**Figure 1. Definition and impact of transgene silencing in mammalian cells.**

**A.** Mammalian cells engineered to express a transgene often undergo silencing. A variety of host-cell mechanisms contribute to transgene silencing which correlates with changes in chromatin structure at the site of the integrated transgene. Over time, transgene silencing generates a proportion of the engineered cell population that does not express the gene of interest (GOI). Transgene silencing is often observed as a bimodal distribution of cells that express the transgene (are in the ON state) or do not express the transgene (are in the OFF state) as shown [14,15]. Transgene silencing may also be observed as a decrease in the relative levels of transgene expression rather than a complete loss of expression. **B.** Diverse applications in biotechnology rely on stable expression of transgenes in engineered mammalian cells. In biomanufacturing, silencing of mammalian cells engineered to produce a product of interest results in a decrease in product produced over time [21,22]. Similarly, silencing of theranostic circuits in mammalian cells engineered *ex vivo* or engineered *in vivo* via gene therapies leads to waning efficacy over time [23]. In cellular reprogramming and differentiation, cells engineered to express a gene or circuit of interest often undergo silencing as they change cell fates. In particular, differentiation of induced pluripotent stem cells into mature cell types often generates the desired cell type with a low proportion of cells that retain expression of the GOI [47]. **C.** Gene circuits often require robust expression of multiple transgenes. Silencing of any individual transgene may limit the performance of stably integrated genetic circuits [24]. In the example shown, a cascade of inducible transgenes regulates expression of the GOI. Silencing of any of the transgenes will result in failure to express the GOI.

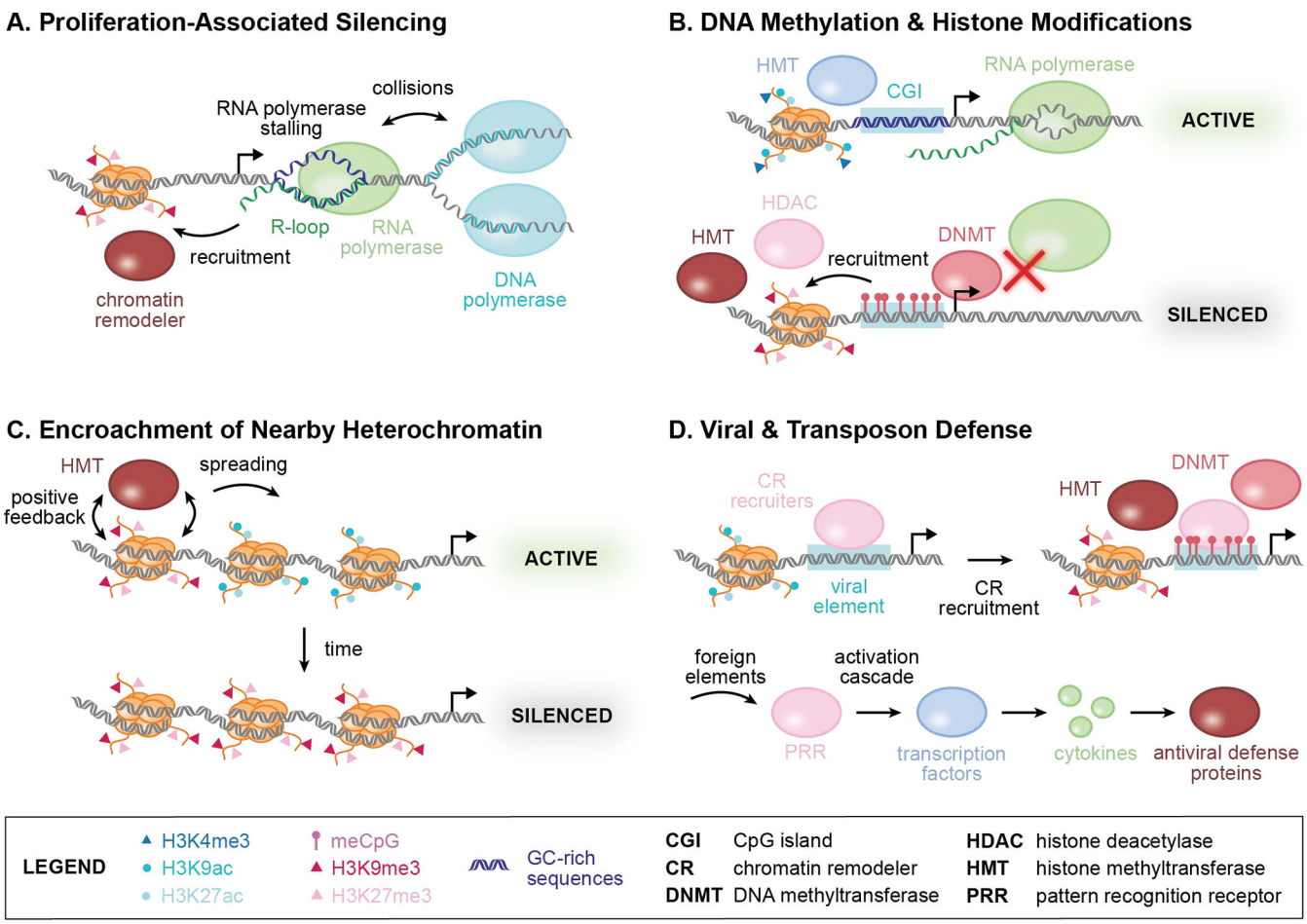



**Figure 2. Mechanisms of transgene silencing.**

**A.** Proliferation may contribute to gene silencing via antagonism between transcription and replication machinery. Increased strain on the DNA and collisions between RNA polymerases and DNA polymerases can promote the formation of structures such as R-loops, which can alter binding of chromatin remodelers and thus reshape epigenetic states at the sites of transgene integration. **B.** DNA methylation and histone modifications are associated with gene silencing. Top: Hypomethylated CpG islands (CGIs) can recruit histone methyltransferases (HMTs) and accrue H3K4me3, associated with active transcription by RNA polymerases. High GC content at promoter CGIs is correlated with resistance to silencing. Bottom: Hypermethylation of CpGs (meCpG) by DNA methyltransferases (DNMTs) can recruit histone deacetylases (HDACs) and subsequently HMTs, replacing active H3K9ac and H3K27ac marks with repressive H3K9me3 and H3K27me3 marks. Competition between transcriptional machinery and DNMTs may reinforce the association of DNA methylation and gene expression states (i.e., hypomethylation/active, hypermethylation/silenced). **C.** Heterochromatin spreading to neighboring regions may silence transgenes. H3K9me3 and meCpG can spread to neighboring genes via positive feedback supported by HMT recruitment to methylated sites, establishing a repressive chromatin state at nearby integrated synthetic circuits. **D.** Endogenous pathways that recognize viral and transposon elements may suppress transgene expression. Top: Proteins

that recognize viral DNA sequences, such as LTRs, recruit chromatin remodelers (CRs), including HDACs, HMTs, and DNMTs. Bottom: Recognition of foreign elements, such as unmethylated CpGs, by pattern recognition receptors (PRRs) activates transcription factors involved in innate immunity (e.g., NFκB). These factors promote cytokine production and lead to upregulation of antiviral defense proteins, which may result in transgene silencing.

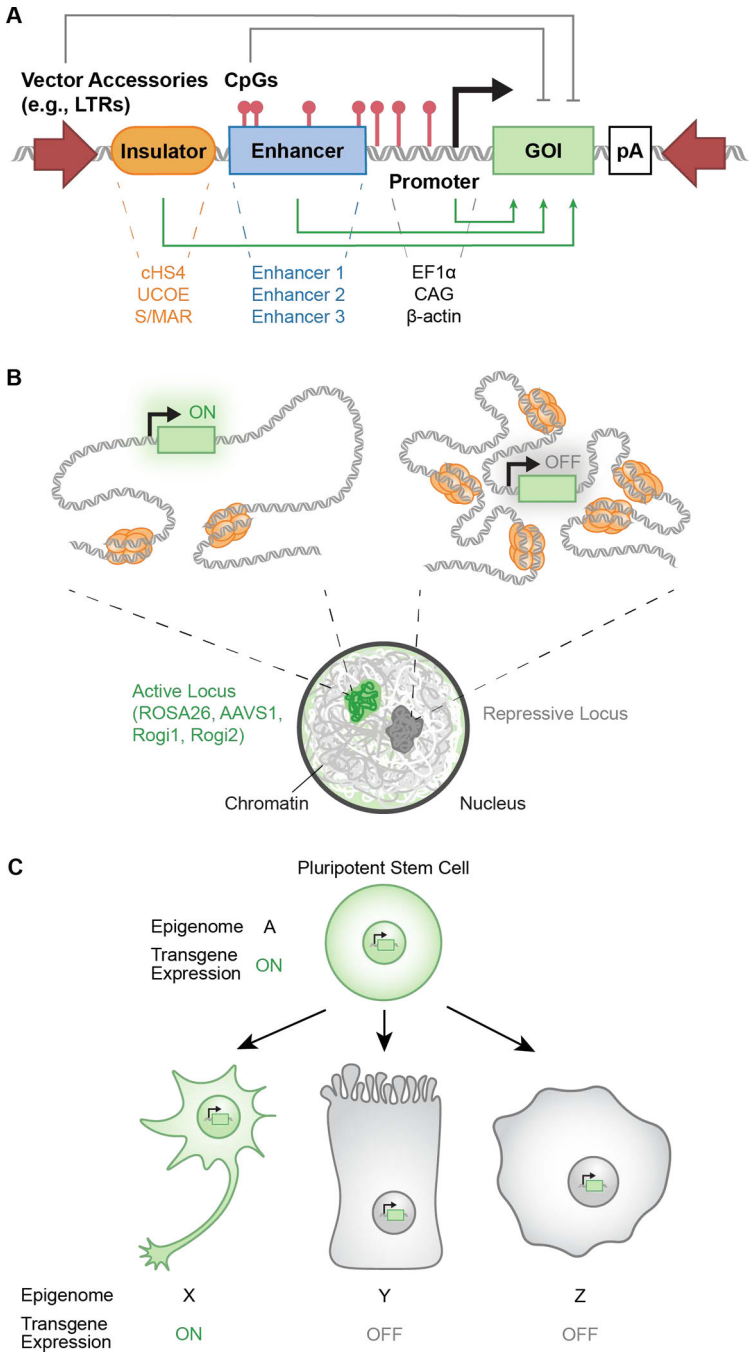



**Figure 3. Design considerations for preventing transgene silencing via selection of genetic elements, locus, and cell type of interest**

**A.** Genetic elements including insulators, promoters, and the combination of activating and repressive elements regulates gene expression. Promoters, enhancers, insulators, and CpG islands facilitate continuous gene expression. Elements such as low-density CpGs among GC-poor regions and viral sequences such as long-terminal repeats act as targets for transcriptional repression. Stability of transgene expression can be improved through the inclusion of activating elements, exclusion of repressive elements, and sequence-specific

parameter optimization. **B**. Transgene circuit integration into heterochromatic, repressive genomic loci increases the likelihood of silencing. Targeted integration of transgenes into genomic safe harbors that remain ubiquitously euchromatic may reduce silencing. **C.** Stem cell differentiation induces genome-wide changes across CpG methylation, histone modification, and chromatin remodeling landscapes. Transgene expression depends on the epigenome of the differentiated lineage; expression might be safeguarded through CpG islands, tissue-specific enhancers, and transgene integration into ubiquitously open genomic safe harbors. Abbreviations: Gene of interest (GOI); Poly-adenylation signal (pA); Long terminal repeat (LTR).

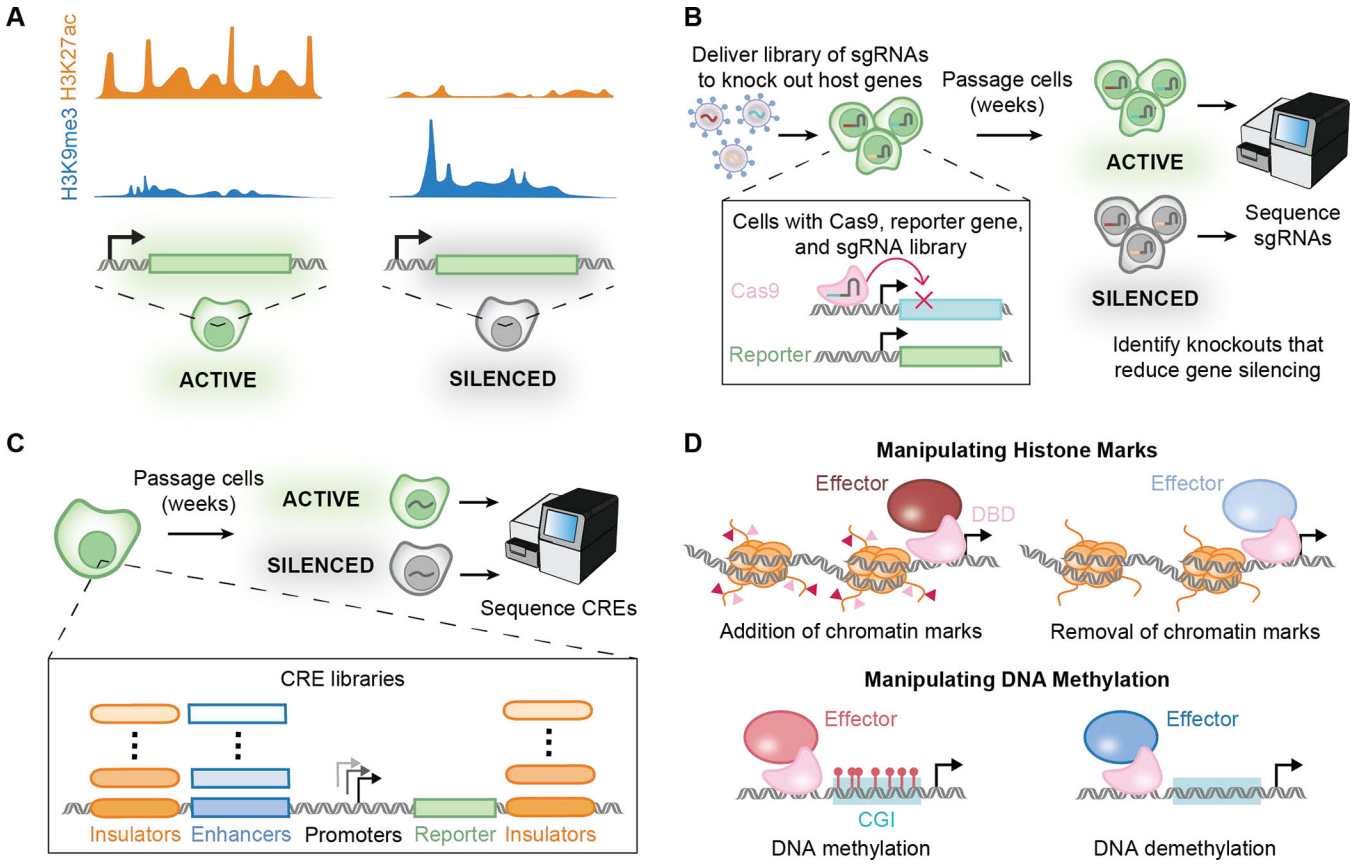



**Figure 4. Technologies being applied to better understand or manipulate transgene silencing.**

**A.** Measurement of chromatin marks and DNA methylation can inform the epigenetic state of a synthetic genetic element as well as the epigenetic modifications involved. The measurements can be performed using techniques such as ChIP-Seq. **B.** CRISPR screens can identify genetic dependencies for transgene silencing. A reporter gene is engineered in cells that also express Cas9. A library for sgRNAs is delivered to the cells. A cell receives a unique sgRNA that targets and knocks out a unique endogenous gene. The cells are passaged, and time is allowed for gene silencing to occur. Sequencing of the gRNAs is performed to identify knockouts that reduce epigenetic silencing. **C.** Massively parallel reporter assays (MPRAs) enable systematic identification of cis-regulatory elements (CREs), including promoters, enhancers, and insulators that can maintain transgene expression and prevent silencing. A library of CREs regulating a reporter gene is installed in a population of cells, the cells are passaged to allow time for gene silencing to occur, the cells are separated by reporter level, and the CREs are sequenced to identify library elements that are enriched in the population of cells with maintained gene expression. **D.** Synthetic chromatin regulators can be engineered by fusing DNA-binding domains (DBDs) to epigenetic modifying effectors, such as enzymes that catalyze specific additions or removal of methylation on histones or DNA. This and other technologies such as ChIP-qPCR, CUT&RUN, methylated DNA immunoprecipitation, and bisulfite conversion

enable synthetic biologists to understand the effect of different epigenetic effectors and to manipulate epigenetic silencing.

**Table 1.**

Summary of published studies on the effect of promoter choice in transgene yields ("Levels") and sustained expression ("Stability") over culture time.

| Transgene Delivery Method | Host Cell Type | Transgene Expression | Locus of Integration | Clone Information | Top Three Promoters | | Reference |
|---|---|---|---|---|---|---|---|
| | | | | | Levels | Stability | |
| Lentiviral vector | Various | Stable | Random | Polyclonal | CAG hEF1α CMV | *n.d.* | Qin et al 2010 |
| Lipofectamine 2000 | HEK-293F | Stable | Random or Episomal | Polyclonal | CAG hEF1α CMV | CAG hEF1α CMV | Dou et al 2021 |
| Lipofectamine 2000 | CHO-K1 | Stable | Random or Episomal | Clonal lines | hEF1α CMV CAG | Only hEF1α tested | X. Wang et al 2017 |
| Lentiviral vector | mESC (J1) | Transient | Random | Polyclonal | hEF1α CAG hPGK1 | Only hEF1α tested | Hong et al 2007 |
| Lentiviral vector | mESC (JM8.N4) | Stable | Random | Polyclonal | *n.d.* | hPGK hEF1α CMV | Herbst et al 2012 |
| φC31 integrase | mESC (IDG26.10–3) | Stable | *Rosa26* | Clonal lines | CAG hUbC hEF1α | *n.d.* | Chen et al 2011 |
| Electroporation | mESC (E14Ju09) | Stable | Random | Clonal lines | CAG; mPGK1 | *n.d.* | Malaguti et al 2022 |
| Lentiviral vector | hESC (SA121; Hues-4) | Stable | Random | Polyclonal | hEF1α hACTB PGK | hACTB PGK EF1α | Norrman et al 2010 |

For stability in stem cells, the list refers to robust transgene expression over prolonged stem cell maintenance (for differentiation, please see text).

Abbreviations: *CAG*: CMV early enhancer/β-actin synthetic hybrid promoter; *CMV*: cytomegalovirus promoter; *EF1α*: elongation factor 1-alpha promoter; *PGK1*: phosphoglycerate kinase 1 promoter; *UbC*: polyubiquitin-C promoter. *m* or *h* indicate *mouse* or *human* promoter origin and cells respectively; *n.d.*: not determined.

**Table 2.**

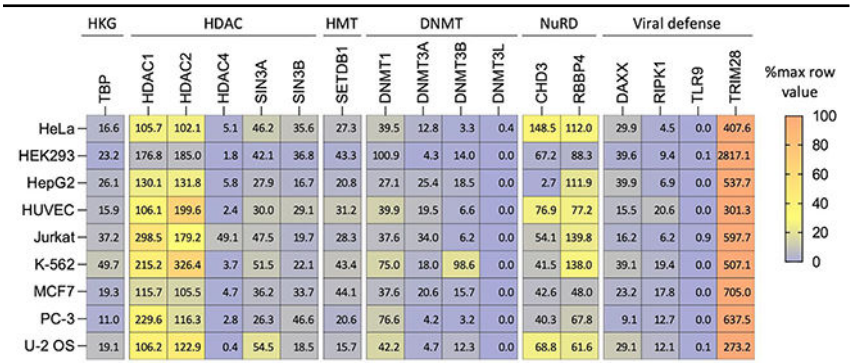
Summary of commonly used insulators in mammalian cells.

| Insulator | Size (bp) | Source | Chromosome coordinate | Reference |
|---|---|---|---|---|
| **cHS4** | 1200 (full) 250 (core) | Chicken beta-globin locus | Chr1:197,298,879–197,300,081 (galGal6) | [145] |
| **A2-UCOE** | 1500 | Human HNRPA2B1-CBX3 locus | Chr7:26,239,804–26,241,504 (hg19) | [262] |
| **CBX3-UCOE** | 700 | Human HNRPA2B1-CBX3 locus | Chr7:26,240,735–26,241,449 (hg19) | [149] |
| **MAR 1–68** | 3630 | Human Chromosome 1 intergenic between SPATA6 and AGBL4 | Chr1:48,947,776–48,951,409 (hg19) | [147] |
| **tDNA** | 1200 | Human tRNA genes | Chr17: 7,963,112–7,964,183 (hg19) | [150] |

Insulator elements are described in terms of their size and genomic source. cHS4: chicken hypersensitive site 4. UCOE: ubiquitous chromatin opening element. MAR: matrix attachment region. tDNA: tRNA gene.

**Table 3.**

Expression levels (normalized transcripts per million, TPM) from public RNA-seq data (Human Protein Atlas, proteinatlas.org [263] for key mediators of transgenes silencing.



| | HKG | HDAC | | | | | HMT | DNMT | | | | NuRD | | Viral defense | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TBP | HDAC1 | HDAC2 | HDAC4 | SIN3A | SIN3B | SETDB1 | DNMT1 | DNMT3A | DNMT3B | DNMT3L | CHD3 | RBBP4 | DAXX | RIPK1 | TLR9 | TRIM28 |
| HeLa | 16.6 | 105.7 | 102.1 | 5.1 | 46.2 | 35.6 | 27.3 | 39.5 | 12.8 | 3.3 | 0.4 | 148.5 | 112.0 | 29.9 | 4.5 | 0.0 | 407.6 |
| HEK293 | 23.2 | 176.8 | 185.0 | 1.8 | 42.1 | 36.8 | 43.3 | 100.9 | 4.3 | 14.0 | 0.0 | 67.2 | 88.3 | 39.6 | 9.4 | 0.1 | 2817.1 |
| HepG2 | 26.1 | 130.1 | 131.8 | 5.8 | 27.9 | 16.7 | 20.8 | 27.1 | 25.4 | 18.5 | 0.0 | 2.7 | 111.9 | 39.9 | 6.9 | 0.0 | 537.7 |
| HUVEC | 15.9 | 106.1 | 199.6 | 2.4 | 30.0 | 29.1 | 31.2 | 39.9 | 19.5 | 6.6 | 0.0 | 76.9 | 77.2 | 15.5 | 20.6 | 0.0 | 301.3 |
| Jurkat | 37.2 | 298.5 | 179.2 | 49.1 | 47.5 | 19.7 | 28.3 | 37.6 | 34.0 | 6.2 | 0.0 | 54.1 | 139.8 | 16.2 | 6.2 | 0.9 | 597.7 |
| K-562 | 49.7 | 215.2 | 326.4 | 3.7 | 51.5 | 22.1 | 43.4 | 75.0 | 18.0 | 98.6 | 0.0 | 41.5 | 138.0 | 39.1 | 19.4 | 0.0 | 507.1 |
| MCF7 | 19.3 | 115.7 | 105.5 | 4.7 | 36.2 | 33.7 | 44.1 | 37.6 | 20.6 | 15.7 | 0.0 | 42.6 | 48.0 | 23.2 | 17.8 | 0.0 | 705.0 |
| PC-3 | 11.0 | 229.6 | 116.3 | 2.8 | 26.3 | 46.6 | 20.6 | 76.6 | 4.2 | 3.2 | 0.0 | 40.3 | 67.8 | 9.1 | 12.7 | 0.0 | 637.5 |
| U-2 OS | 19.1 | 106.2 | 122.9 | 0.4 | 54.5 | 18.5 | 15.7 | 42.2 | 4.7 | 12.3 | 0.0 | 68.8 | 61.6 | 29.1 | 12.1 | 0.1 | 273.2 |



HKG: housekeeping gene (shown for comparison), HDAC: histone deacetylase, HMT: histone H3K9 methyltransferase, DNMT: DNA methyltransferase, NuRD: nucleosome remodeling and deacetylase complex.