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Trends in Genetics

Review

Transcriptional Silencers: Driving Gene Expression with the Brakes On

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Silencers are regulatory DNA elements that reduce transcription from their target promoters; they are the repressive counterparts of enhancers. Although discovered decades ago, and despite evidence of their importance in development and disease, silencers have been much less studied than enhancers. Recently, however, a series of papers have reported systematic studies of silencers in various model systems. Silencers are often bifunctional regulatory elements that can also act as enhancers, depending on cellular context, and are enriched for expression quantitative trait loci (eQTLs) and disease-associated variants. There is not yet evidence of a 'silencer chromatin signature', in the distribution of histone modifications or associated proteins, that is common to all silencers; instead, silencers may fall into various subclasses, acting by distinct (and possibly overlapping) mechanisms.

Highlights

Silencers are less well-studied than enhancers, but a recent spate of papers has begun to systematically explore these repressive regulatory elements.

Silencers are important for precise control of gene expression and are enriched for disease-associated regulatory variants.

Most newly discovered silencers are bifunctional regulatory elements that also act as enhancers in other contexts. The traditional distinction between enhancers and silencers may be an oversimplification.

Silencers appear to act by a variety of mechanisms, and may fall into various functionally distinct subclasses. This complicates the search for a predictive chromatin signature that would enable the rapid identification of silencers from high-throughput data.

Understanding silencer function will be key to predicting the regulatory impact of genomic variation.

Functional Role of Silencers

Transcriptional regulation, the gene-specific control of the rate of initiation of mRNA production, is critical to the establishment of cell state during differentiation or in response to environmental signals, and is encoded in the genome. In microbes, the information controlling transcription in '*cis*' is generally concentrated in close proximity (within hundreds of base pairs) to the transcriptional start site and is broadly termed the 'promoter'. In multicellular eukaryotes, by contrast, *cis*-regulatory sequences can be spread out over great distances (in extreme cases, more than a megabase) [1]. The core promoter in these organisms is understood to provide information on where RNA polymerase should begin transcribing and is necessary for transcription to occur. However, many core promoters are not sufficient to drive transcription unless activated by regulatory input from other noncoding sequences [2,3].

The distal sequence elements that activate transcription from a core promoter are termed '**enhancers**' (see Glossary), and can act on promoters independent of their relative orientation and spacing [4]. Enhancers comprise collections of binding sites for sequence-specific transcription factors (TFs), including **activators** that are key for driving gene expression in a spatially and temporally specific manner [5]. By contrast, some distal elements, termed '**silencers**' [6,7], can reduce the activity of a linked promoter, rather than increasing it [6,7]. Like enhancers, silencers are modular (in that they retain their patterned repressive activity when removed from their native genomic context) and can act in a position- and orientation-independent fashion [8,9]. Also like enhancers, silencers provide binding sites that recruit regulatory factors, in this case transcriptional repressors [7]. This suggests that repressors are actively involved in silencing by modifying chromatin state or occluding activating factors.

Silencers have a vital role in sharpening and fine-tuning gene expression patterns (Figure 1). As an example, immature T cells express both CD4 and CD8 cell surface glycoproteins, but express only one after they mature. CD8 expression appears to be controlled by a series of enhancers specific for CD8+ cell types, so CD8 is downregulated by a loss of positive regulatory activity.

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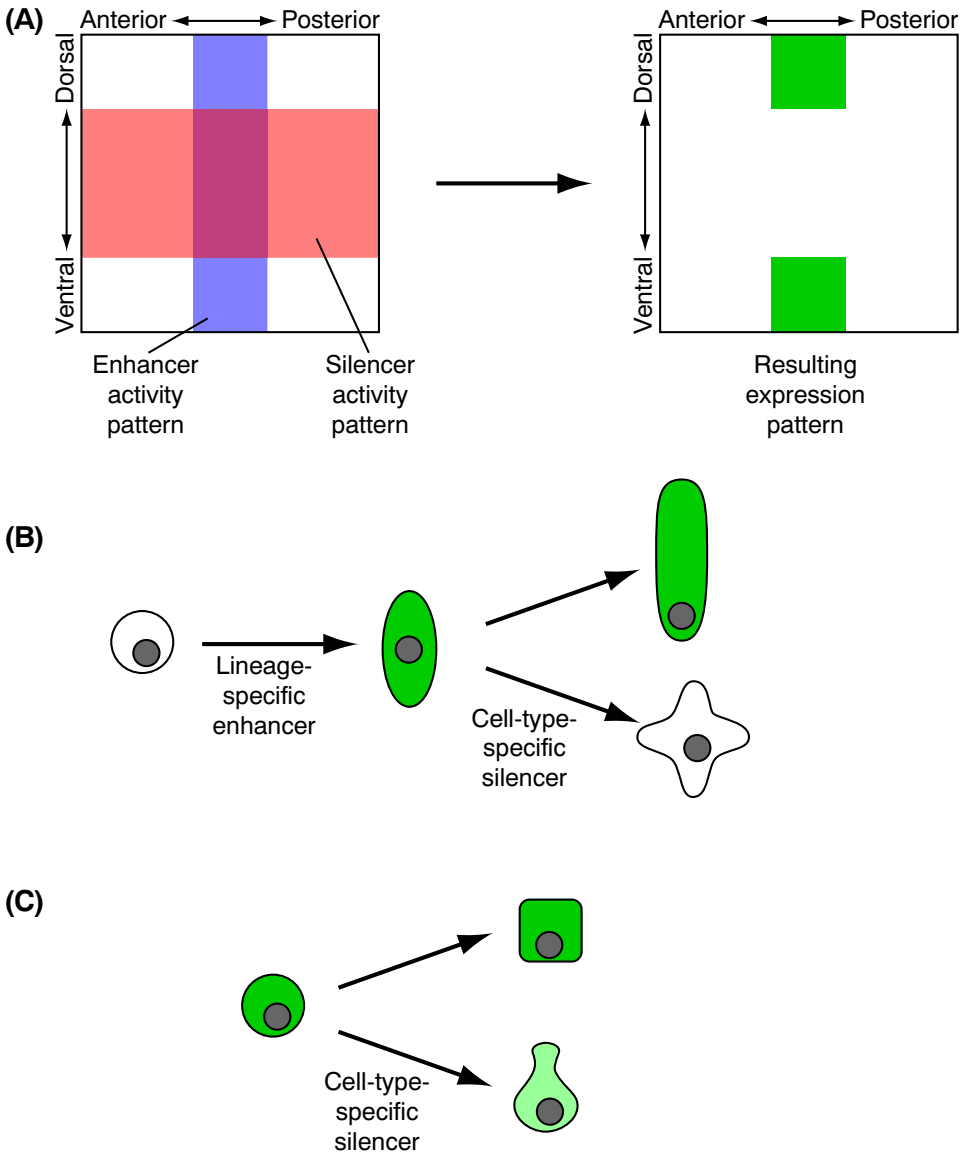


Figure 1. Silencers Can Fine-Tune Gene Expression Patterns. (A) Silencers can create complexity and specificity in spatial expression driven by more broadly acting enhancers. (B) Silencers can fine-tune temporal expression patterns by opposing the activity of enhancers activated in a parent cell lineage. (C) Silencers can reduce promoter activity to establish cell-type-specific expression levels.

The CD4 locus, by contrast, contains enhancer elements active in all mature T cells. Therefore, loss of CD4 expression, which is excluded from mature CD8+ T cells, must be achieved by the negative regulatory effect of a silencer element active in those T cells as they differentiate [10].

It is difficult to assess the range of effect sizes of silencers, because they are a relatively understudied class of genomic elements and many screens have used binary cutoffs to classify these elements. Gisselbrecht *et al.* [11] quantified the reduction in GFP expression in whole-mount *Drosophila* embryos when a silencer was placed in front of a mesodermal enhancer and

Glossary

Accessible ('open') chromatin: genomic regions enriched for nucleosomes with higher turnover rates [73], allowing for the DNA to be bound by TFs, RNA polymerase, and/or other DNA binding factors or enzymes [e.g., DNase I in DNase I hypersensitivity profiling, transposase in assay for transposase-accessible chromatin using sequencing (ATAC-seq), or micrococcal nuclease (MNase) in profiling nucleosome occupancy by MNase-Seq].
Activator: TF that promotes the transcription of a gene.
Antilooping: process of disrupting the looping of a distal enhancer to its target promoter so as to reduce gene expression. Refers to loss of a specific loop rather than a general architectural barrier presented by an insulator.
Bifunctional *cis*-regulatory element: transcriptional regulatory element that, depending on the cellular context, acts as both an enhancer and a silencer.
ChIP-Seq: method that uses antibody enrichment and high-throughput sequencing to map the occupancy of a specific protein or post-translational modification thereof across the genome.
Chromatin loop: 3D contact between two elements that are distal in linear space. Often refers to enhancer–promoter contacts.
Chromatin mark: transcriptional protein, architectural protein, or epigenomic feature (e.g., histone variant, histone post-translational modification, or DNA modification).
Compact ('closed') chromatin: regions of the genome that are densely occupied by linker histones and regularly spaced nucleosomes, making them inaccessible to most DNA binding factors.
Enhancer: *cis*-regulatory element to which TFs bind to upregulate the transcription of a gene, typically in a position- and orientation-independent manner.
Heterochromatin: highly compacted chromatin in which there is almost no gene expression. Constitutive heterochromatin occurs at repetitive genomic regions that are compacted in all cell types, whereas facultative heterochromatin refers to regions that are reversibly compacted to restrict gene expression in space and time.
Insulator: genomic element that reduces the interaction frequency of chromatin on opposite sides of itself.

observed significant but partial silencing effects, with spatial modulation of effect size. Additionally, Huang *et al.* [12] and Pang and Snyder [13], similarly validated and quantified several silencers using luciferase assays and found varying effect sizes. Overall, silencers appear to have a range of effect sizes, with some being quite modest. Furthermore, high-throughput screens are better powered to detect strong effect silencers, so many more weak silencers likely remain to be found.

A discussion of what silencers are must necessarily include a discussion of what they are not, and several important distinctions can be drawn. First, the *cis*-regulatory elements that direct silencing are, in principle, separate from the silenced genes which result from their activity. Indeed, silenced gene regions are typically characterized by **compact ('closed') chromatin** with the presence of linker histone [14], whereas silencers must provide access by **repressive TFs (repressors)** to their binding sites to facilitate context-dependent repression. Historically, repressive TFs have been classified as acting as 'short-range' versus 'long-range repressors' [15]. Short-range repression refers to factors binding near the binding sites of activating TFs and restricting their activity, while long-range repressors can act to silence distant promoters independent of their position relative to active enhancers. In this framework, silencers are thus understood as containing binding sites for long-range repressors, although several studies suggest that the distinction is not a sharp one [16]. Another class of negative regulatory elements are **insulators**, which present a physical barrier to enhancer action on a promoter [17,18]. This activity depends on the positioning of the insulator between the enhancer and the promoter to block their functional interaction, and thus is distinct from the position- and orientation-independence of silencers as traditionally defined. More information on gene silencing mechanisms that do not use silencer elements discussed herein can be found in Box 1.

Despite the importance of silencers in development and signal responses, they have generally been much less studied than enhancers, with more than 1.5 million PubMed hits for 'enhancers' versus ~100 000 for 'silencers'. The focus on identifying transcriptional enhancers has been consistent with a model that in eukaryotes, transcription is regulated primarily at the level of activation [19]. Together with technical challenges in detection of silencer activity in **reporter assays**

Polycomb repressive complex 2 (PRC2): and PRC1, are multisubunit complexes, that deposit H3K27me3 (PRC2) and recognize this chromatin mark (PRC1) to promote facultative heterochromatin formation.
Reporter assay: experiment to determine the gene regulatory activity of a DNA fragment by inserting the element near a reporter gene (e.g., *lacZ*, *GFP*) whose expression is measured by luminescence or mRNA quantification.
Repressive ability of silencer elements (ReSE) assay: massively parallel reporter assay employed by Pang and Snyder [13] to identify silencers in human cells. Silencers were identified by their ability to dampen expression of a toxic caspase selectable marker.
Repressive TFs (repressors): TFs that reduce the transcription of a gene.
Silencer: *cis*-regulatory element to which TFs bind, to reduce transcription of the associated target gene.
Silencer-FACS-Seq (sFS): reporter assay used by Gisselbrecht *et al.* [11] to identify silencers in *Drosophila* mesoderm. Fluorescence activated cell sorting (FACS) isolates a cell type of interest with reduced reporter gene (GFP) expression, followed by high-throughput sequencing of tested fragments.
TF binding site motif: collection of DNA sequences recognized by a sequence-specific TF.
Transposable elements (TEs): mobile genetic elements capable of inserting themselves into a host genome, with or without the use of an RNA intermediate. Most TEs in eukaryotic genomes are no longer actively transposable.

Box 1. Silencing Mechanisms That Are Not Silencers

The term 'silencing' is used to describe multiple modes of dampening gene expression that do not involve the silencer elements discussed in this review. Several types of gene silencing are carried out post-transcriptionally by small (~20–30 nt) noncoding RNAs. miRNAs and siRNAs are both loaded into Argonaute (AGO) proteins as part of the RNA-induced silencing complex (RISC). siRNAs are perfectly complementary to a single target and induce cleavage of the transcript, whereas miRNAs typically target multiple genes with imperfect complementarity and silence them by affecting mRNA stability or translation initiation [74]. Piwi-interacting (pi)RNAs are an orthogonal set of small RNAs that use PIWI proteins instead of AGO as an effector and have a major role in repressing transposable elements (TEs) [75]. In addition to short RNAs, long noncoding RNAs (lncRNAs) can interact with multiple transcriptional corepressors, and have profound silencing effects [76]. This has been extensively studied in X chromosome inactivation in therian female mammals. Mammalian XX cells silence one X chromosome randomly in order to modulate dosage of X-linked genes. The repression is mediated by the lncRNA *Xist*, which spreads in *cis* across the chromosome that expresses it and facilitates downstream chromatin modification, including monoubiquitination on Lys 119 of histone H2A (H2AK119ub1) and deposition of H3K27me3 by polycomb gene (PcG) complexes, which dramatically compact the inactive X [77,78]. This activity in *cis* makes some repressive lncRNAs act very much like silencers [79], but there is little evidence that most silencers are transcribed or that their silencing activity depends on an RNA product.

'Silencing' is also used to describe transcriptional repression by DNA methylation. In mammals, DNA methylation is deposited onto the cytosine of CpG dinucleotides by DNA-methyltransferase (DNMT) family members to generate a 5-methylcytosine (5mC) base [80]. Most of the mammalian genome is CpG poor, while methylation is concentrated in CpG-rich 'islands' commonly found in promoter regions [81]. The repression mechanism is not fully elucidated, but it has been suggested that methylation alters the affinity of DNA binding proteins or that methyl-CpG binding proteins, such as MeCP2, recruit repressive complexes [82]. While it has not been observed, it remains possible that a subclass of silencer elements recruit DNA methyltransferases as a silencing mechanism. However, *Drosophila* do not have methylated CpGs, so DNA methylation cannot explain any silencers in this system.

(Box 2 and Figure 2), this model of gene regulation has contributed to silencers, even decades after their first description, being much less well-studied than enhancers. Nevertheless, in 2008 Petrykowska *et al.* published a survey of 47 elements from the 1.8 Mb *CFTR* region for silencer activity, which they reported for 21 of these elements [18]. In the ensuing decade, a steady trickle of studies has increased the catalog of known silencers, typically one at a time by dint of careful dissection of a single regulatory locus. In the last 2 years, however, five new studies have been published that addressed silencers more systematically [11–13,20,21]. In this article we review silencer assays and common themes from these and other recent papers; discuss features of silencers, mechanisms of silencer activity, the potential evolutionary origins of silencers, and the potential role of silencers in human disease; and highlight some of the open questions that remain.

Identification of Silencers through Highly Parallel Screens

Five papers in the last 2 years have used a combination of experimental techniques to expand the catalogue of known silencers enormously. Huang *et al.* used a hypothesis-driven analysis of large-scale genomic data to identify candidate cell-type-specific silencers [12]. Reasoning that trimethylation of histone H3 lysine 27 (H3K27me3) is associated with transcriptional repression and that active silencers must be accessible to repressive DNA-binding proteins, they created a catalogue of DNase I hypersensitive sites (DHSs) that overlap with peaks of H3K27me3 identified by **ChIP-seq** in a panel of human cell lines. They then examined the correlation across cell lines between the presence or absence of this combined signal at a given element and the expression level of nearby genes. H3K27me3-DHSs that were negatively correlated with nearby gene expression were significantly enriched for several features consistent with silencers; the authors validated a small number of these experimentally by a reporter assay.

Gisselbrecht *et al.* used a parallelized reporter assay in whole *Drosophila* embryos to test hundreds of candidate elements for silencer activity *in vivo*, enriching for those that reduced GFP reporter expression by fluorescence-activated cell sorting (FACS) of dissociated primary

Box 2. Assays of Silencer Activity

Early studies of silencers, like those of enhancers, primarily relied on reporter assays (see Figure 2 in the main text). A reporter assay for enhancer activity typically uses a plasmid vector in which expression of an easily assayed ectopic gene (the 'reporter gene') is driven by a minimal promoter. This promoter usually comprises only core promoter elements which act (upon induction) to localize RNA polymerase to the transcriptional start site and is necessary but not sufficient for reporter gene expression. The sequence to be tested for enhancer activity is then cloned into the reporter vector. The resulting plasmid can be introduced into cultured cells or whole transgenic organisms. In cultured cells, reporter gene expression is typically assayed quantitatively, either by an enzymatic activity assay, if the reporter gene encodes an enzyme, or by measuring the level of reporter gene mRNA. These assays can be performed with or without some sort of stimulation and are thus particularly well-suited to studies of signal-responsive enhancers. Assays in transgenic organisms are more expensive in time, effort, and resources, but can provide additional spatiotemporal information on developmentally regulated enhancers.

The inclusion in a reporter vector of sequences that support robust expression, in addition to the test sequence, converts an enhancer assay into a silencer assay. An elegant study in *Drosophila* embryos [27] demonstrated this using an ectopic enhancer that drives expression in a stripe orthogonal to the domain of putative silencer activity, revealing repression of enhancer-driven expression where the two domains overlap. The requirement for a hypothesis about silencer activity pattern prior to such an experiment has been a challenge for silencer discovery more broadly.

The introduction of more efficient technologies for precise genome editing, primarily using a range of CRISPR-based methods, has allowed the exploration of *cis*-regulatory elements in their native context. Enhancers and silencers can be mutated or deleted, followed by comparisons of the resulting potential changes in target gene expression [83–85]. For all its recent advances, precise genome editing remains challenging in terms of efficiency and scalability [86], but these experiments offer compensatory advantages by avoiding possible artifacts caused by the use of ectopic promoters and/or genomic locations.

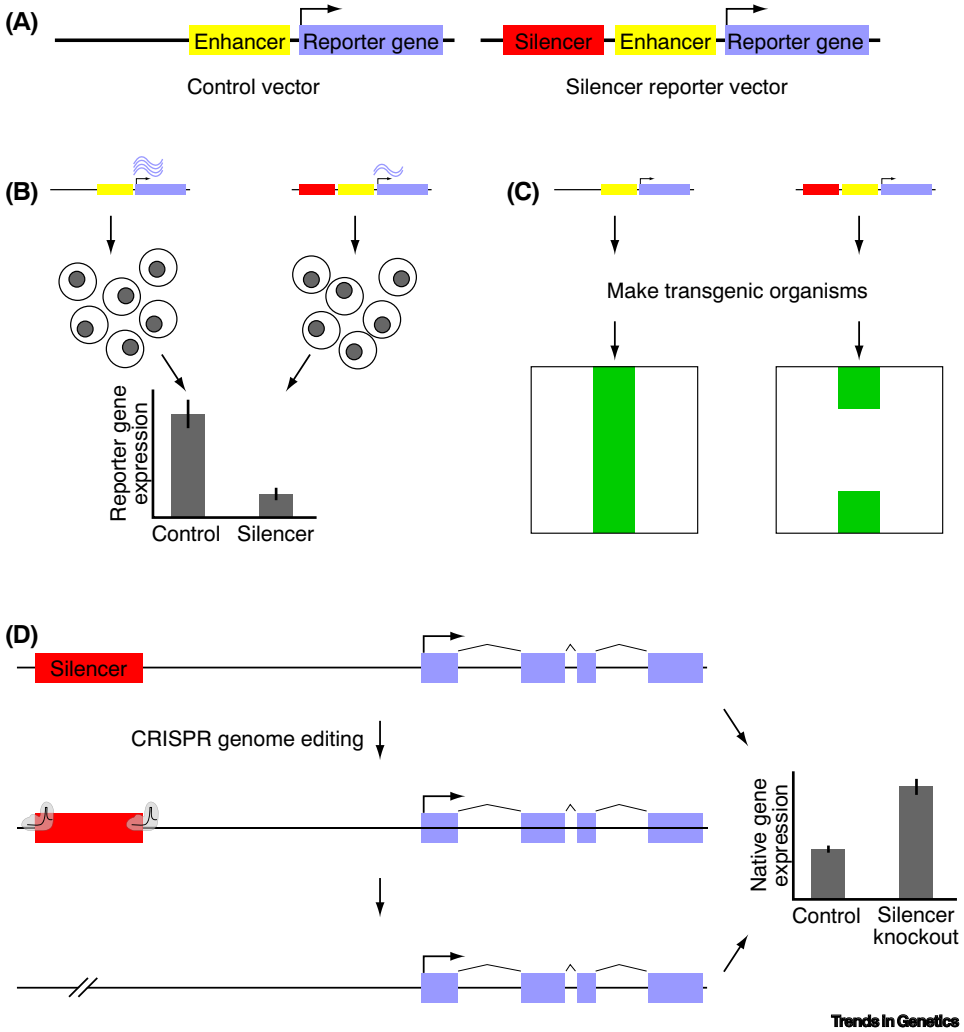


Figure 2. Techniques Used to Identify Silencers. (A) In a silencer reporter assay, an easily measured ectopic 'reporter' gene is expressed under the control of a minimal promoter and appropriate enhancer. A sequence to be tested for silencer activity is added and compared with a no-silencer control. Silencer activity can be detected by measurement of reporter gene expression in cultured cells (B), or visualization of the resulting expression pattern in transgenic organisms (C). (D) Silencer activity of a sequence element in its native genomic context can be assayed by targeted deletion and measurement of the resulting expression of potential target genes. Abbreviation: CRISPR, clustered regularly interspaced short palindromic repeats.

embryonic cells and identifying dozens of associated silencers by high-throughput sequencing [i.e., **silencer-FACS-Seq (sFS)**] [11]. One of these functionally identified silencers was validated by genome editing, resulting in derepression of the target gene when the silencer was deleted. Pang and Snyder also used a highly parallel reporter assay to functionally identify silencers [13]. They tested a genome-wide library of **accessible ('open') chromatin** regions in cultured human cells, and used an inducible caspase as a reporter gene to drive apoptosis in cells carrying non-silencers, thus allowing them to screen for the **repressive ability of silencer elements** (**ReSE) assay**. They identified thousands of potential silencers in K562 cells and validated two elements by showing derepression of target genes upon silencer deletion. Doni Jayavelu *et al.* performed a parallel reporter assay on thousands of accessible chromatin regions lacking

features of promoters, enhancers, and insulators for silencing activity in human K562 cells [21]. They trained a computational model using sequences from 80% of the highest- and lowest-scoring elements in their screen and used the scores from the remaining 20% to establish a threshold value that maximally distinguished elements that exhibited silencer activity. They termed those elements that exceed this threshold score 'candidate silencers', a small subset of which they validated by a reporter assay.

Finally, Ngan *et al.* used the chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) assay, which combines ChIP with proximity ligation, in mouse embryonic stem cells to identify **chromatin loops** associated with the **polycomb repressive complex 2 (PRC2)**, which applies the H3K27me3 mark to chromatin [20]. They found that genes with promoters targeted by such loops are expressed at lower levels, and also that deletion of two intergenic PRC2-bound regions caused derepression of genes connected to them by chromatin loops. Together, these studies give us an unprecedented look at silencers as a class.

A common theme highlighted in most of these five recent papers is the overlap between silencers and enhancers. It has long been known that tissue-specific silencers can be **bifunctional *cis*-regulatory elements** that also act as enhancers in different cell types. In fact, the very element for which the term 'silencer' was originally coined [6] was reported, only 2 years later, to contain sequences that can also activate transcription in reporter assays [22]; many subsequent examples have been characterized (e.g., [8,23]). Nevertheless, regulatory elements are typically classified into distinct bins, and silencers have long been treated as a separable class of regulatory elements from enhancers [24,25]. The current set of studies suggest that bifunctionality is strikingly prevalent, with the 'weak enhancer' chromatin state significantly enriched among human silencers [13], and more than half of identified silencers exhibiting evidence of enhancer activity in other studies [11,20,21]. That Huang *et al.* did not discuss bifunctionality of the silencers that they identified, may reflect the fact that their analysis method required an element to be negatively correlated with proximal gene expression across cell types [12]; they therefore may have selected for a subclass of dedicated silencer elements. Alternatively, their ability to call enhancer elements may have simply been limited by incomplete representation of cell types and conditions in reference datasets.

Some TFs are known to act as both activators and repressors in different contexts [7], and this could contribute to regulatory elements acting as both silencers and enhancers, but it is not necessary. In the simplest case, bifunctionality of a regulatory element could reflect the differential expression of activating versus repressive TFs in different tissues. Moreover, overlapping TF DNA binding sites can cause repressors expressed in one cell type to compete for binding with activators [26]; furthermore, differentially expressed cofactors can convert activators to repressors [27]. A recent report even suggests that one element can function as an enhancer of one gene and a silencer of another in the same cell type [28], possibly due to interference of intragenic enhancer transcription with RNA polymerase passage through the host gene body [29]. Taken together, these findings suggest that it is probably an oversimplification to treat enhancers as a functionally distinct class of *cis*-regulatory elements from silencers.

Chromatin Features of Silencers

A characteristic chromatin state of silencers would enable the identification of active silencers in any eukaryotic systems, from analysis of ChIP-seq data for the corresponding **chromatin marks**. A set of enhancer-associated chromatin marks featuring particular post-translational modifications of histone tails, and occupancy by the coactivators p300 or CREB-binding protein (CBP), have been identified that can be used to predict enhancers [30–32]. This knowledge has

aided the interpretation of complex disease genetics immensely. ChIP-seq for active histone marks is a critical component of the algorithm that the RegulomeDB database uses to predict the functional impact of variants [33]. In addition, population-level variation in active promoter and enhancer marks, H3K4me1/3 and H3K27ac, have proven beneficial in fine-mapping autoimmune and lipid metabolism associated variants and interpreting their mechanism [34,35]. Considering the predicted prevalence of silencers, an analogous chromatin signature would be a vital addition to aid in variant interpretation. Because the genomic distribution of dozens of histone modifications remains poorly characterized [36], it is possible there may be highly predictive silencer marks amenable to ChIP-seq that remain to be identified.

Until very recently, there were too few silencer elements identified to determine statistically rigorous enrichments of chromatin marks among silencers [18]. Silencers are also exquisitely specific to cell type and developmental stage, which limits the number of well-matched ChIP-seq resources. Because silencers are often enhancers in other contexts, bulk assays are especially prone to obscuring silencer signatures if both cell types are included in the same sample.

Broadly, silencers are expected to lie within regions of open chromatin since they are active regulatory elements. By this logic, a simple subtractive analysis attempted to identify silencers by determining which DHSs are not annotated as promoters, enhancers, or insulators [21]. While an analysis like this will no doubt capture silencers, it will have low specificity because of incomplete annotations for other genomic elements and because it assumes there are no classes of genomic elements in open chromatin that remain to be discovered.

The advent of recent high-throughput reporter assays, has made it possible to find some associations of silencers with histone modifications. The ReSE screen [13] was able to leverage ENCODE ChIP-seq catalogs because it was performed in two highly studied cell lines (K562 and HepG2). They found that their silencers were significantly enriched for monomethylation of histone 4 lysine 20 (H4K20me1), a cell cycle regulated mark that has varied effects on gene transcription. PR-SET7/SET8 is the only enzyme known to deposit H4K20me1. Notably, although the first silencer was identified in yeast, *Saccharomyces cerevisiae* does not have a PR-SET7 homolog or methylated H4K20 [37], and so if H4K20me1 is a silencer mark, then it may not be universal across eukaryotes.

Silencers are often associated with the PRC 1 and 2, although this likely represents one subclass of silencers. H3K27me3, the characteristic histone mark deposited by PRC2, has been used to prioritize chromatin regions as possible silencers. Huang *et al.* focused on H3K27me3-containing DHS fragments to identify regions negatively correlated with gene expression [12]. Similarly, Ngan *et al.* identified silencers using ChIA-PET for PRC2-mediated chromatin loops [20]. It is perhaps not surprising that the polycomb repressive complexes are involved in silencer activity given their canonical role in transcriptional repression. However, silencers appear to be a broad class of elements that operate through several different pathways (see later), so it is unlikely PRC-mediated interactions will capture all silencers. Notably, EZH2, a core subunit of PRC2, stabilizes the neuron-restrictive silencing factor/repressor element 1, silencing TF (NRSF/REST) to promote its silencing activity, but it does so without interaction with the other PRC2 subunits, suggesting a silencing role for EZH2 outside of classical polycomb repression [38].

It is possible that there are no histone marks that are found uniquely at silencers, and they are instead indicated by a combinatorial code of chromatin marks that also appear elsewhere. One scenario is that silencers are identifiable by a combinatorial code of several histone modifications on the same histone octamer. In this case, identification of silencers would be possible using

several antibodies in ChIP-Seq experiments. One possibility is that combinatorial histone modifications might be read out by factors such as L3MBTL1, which bind nucleosomes containing H4K20me1/2 and H1-4K26me1/2 (H1bK26me1/2) to recruit heterochromatin protein 1 (HP1) [39]. Alternatively, silencers may be marked by the same histone modifications as enhancers, since the same elements often function as both, although Pang and Snyder found silencers were slightly depleted for these marks. This would call into question the interpretation of known 'active enhancer' marks and suggest that they instead may also mark active bifunctional elements in both states.

Because there are, mechanistically, distinct types of silencers (see later), It is more likely that there may not be one chromatin signature that marks all, or even a majority, of silencers. Indeed, it appears likely that there is no 'final common pathway' for silencer function. Instead, all phenomena leading to transcriptional repression (including constitutive **heterochromatin**, siRNA-mediated silencing of mobile DNA elements, and the partitioning of the genome into active and inactive compartments) could contribute a silencing mechanism to one or more subsets of functionally defined silencers.

Potential Evolutionary Origins of Silencers

Silencers identified by **reporter assays** are often one readout of a bifunctional element that operates as an enhancer in other tissues or times [11], so their origins likely parallel those of enhancers to some extent. **Transposable elements (TEs)** often contain recognition sequences for TFs, and in many contexts regulatory elements are enriched for TEs. Because of this, it has been hypothesized that TEs have been widely exapted as the basis of new transcriptional programs [40]. TEs are important because they can add new functionality with a single transposition event, rather than by the accumulation of numerous smaller mutations. As such, they are able to drive large phenotypic jumps, such as the evolution of live birth in mammals [41]. Along these lines, silencers can provide a mechanism for small mutations to have large, positive effects on transcription, because a silencer can easily be lost by a mutation, but it is unlikely that an enhancer is created *de novo* from one [42]. An example of this is seen in pigmentation across *Drosophila* species. *Ebony* is regulated by an enhancer and a silencer, and mutations to the silencer have occurred several times, independently, leading to corresponding changes in pigmentation [43]. Although it is less likely that a random mutation will create a functional element rather than destroy one [42], it is possible that a silencer could be created by a mutation that creates a binding site for a repressive factor.

TEs harbor sequences with enhancer activity, but they can also contain active silencers. Mariner TEs contain an active silencer in the transposase [44]. Although some mammal-specific TEs exhibit enhancer activity, the majority of them reduced reporter gene expression in HepG2 cells [45]. One of the **TF binding site motifs** most highly enriched in ape-specific TEs was for REST, consistent with a report that REST binding sites have been propagated extensively by TEs [46], which suggests an important role for REST in recent evolution.

Potential Role of Silencers in Human Disease

The genetics underlying human diseases are driven by coding and/or noncoding variants. While coding variants exert larger effect sizes on phenotypes [47], the overwhelming majority of complex disease variants are in noncoding regions [48]. Noncoding variants are often thought of in terms of their effects on the transcriptional activity of nearby genes, specifically by creating or abolishing TF binding sites. In line with this model, enhancers are strongly enriched for genome-wide association study (GWAS) hits [21] and expression quantitative trait loci (eQTLs) [49]. As the inverse of enhancers, it stands to reason that silencers might similarly harbor large numbers of variants that affect gene expression, and in turn, complex traits. Indeed,

computationally predicted silencer elements contained similar enrichments for GWAS variants and eQTLs [12]. One suggestion of the effect silencers can have on complex disease can be seen in a strong-effect eQTL for *TAP2* which converts a repressing sequence to an activating one, and has been associated with autoimmune diseases such as Crohn's disease and sarcoidosis, as well as fecundability [26].

Transcriptional Repressors and Their Association with Silencers

In multiple studies, silencers have been found to be enriched for the DNA binding site motifs of known repressors. *Drosophila* mesodermal silencers were enriched for occurrences of the Snail binding site motif, Snail-bound silencer target genes were upregulated in *snail* mutants, and the role of Snail was confirmed by Snail motif mutations resulting in reduced silencer activity [11]. Silencers in liver-derived HepG2 cells were enriched for occurrences of the REST motif [13,45], whereas K562 silencers were enriched for the repressor KLF12; silencers in both cell types were enriched for AP2 motif occurrences, a factor known to have repression activity [13,50].

Numerous transcriptional repressors have been implicated in human disease. Increased REST expression in the brain is associated with fewer incidences of Alzheimer's disease [51] and increased lifespan in humans [52]. In a *Caenorhabditis elegans* model, the REST homologs protected against hallmarks of Alzheimer's Disease: oxidative stress and amyloid ß toxicity [51]. Furthermore, REST functions as a tumor suppressor in mammary epithelial cells [53], and overexpression or improper degradation of REST leads to an increase in chromosomal abnormalities [54]. Similarly, mutations to the repressor methyl CpG-binding protein 2 (MeCP2) have also been shown to cause the neurodegenerative disease Rett syndrome [55]. MeCP2 falls into the broader category of factors with both repressor and activator activities because it can activate targets through association with CREB1 [56].

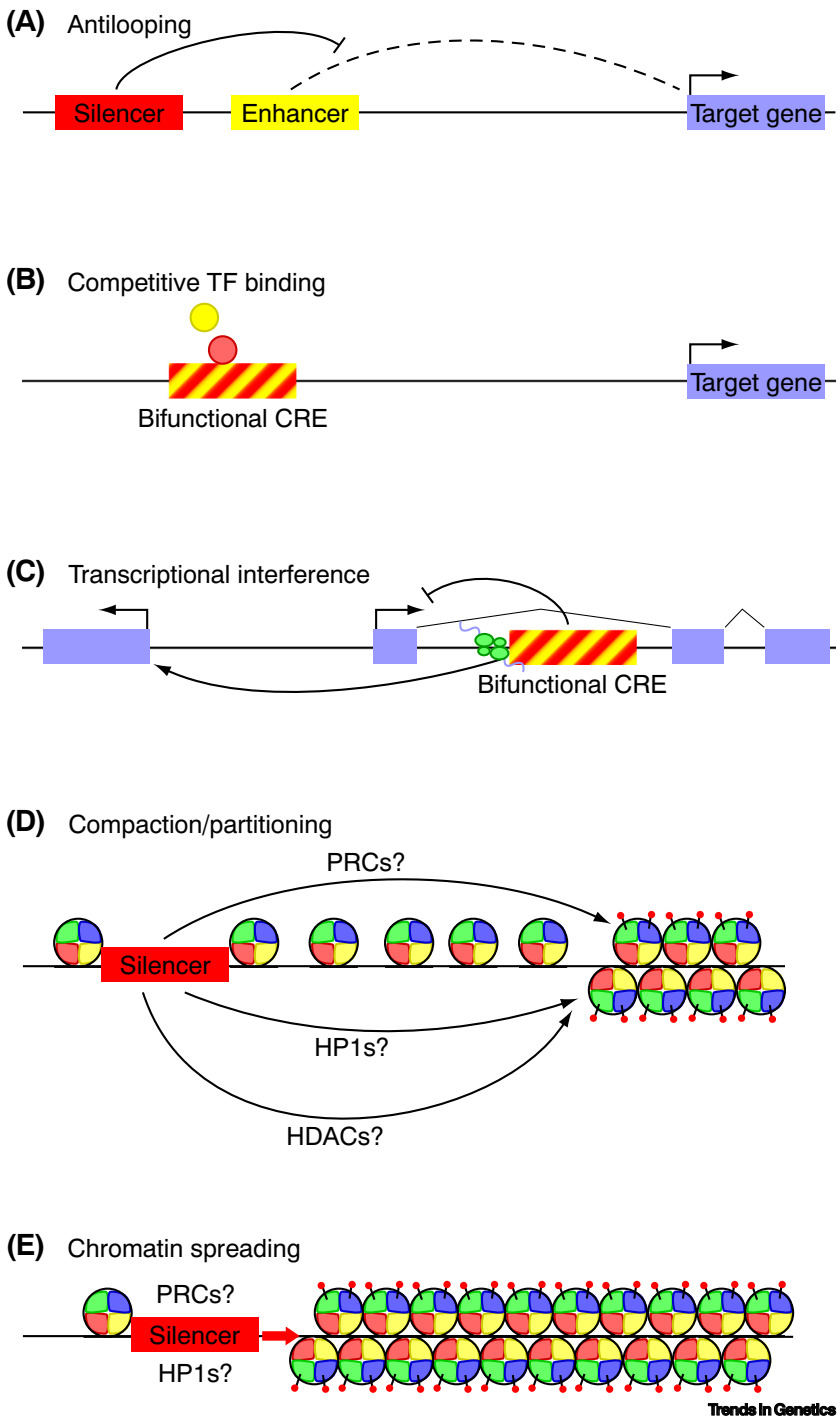
As another example, SNAI1 and SNAI2, mammalian homologs of the *Drosophila* repressors Snail and Slug, another Snail superfamily repressor, silence *CDH-1*, which encodes E-cadherin [57,58]. Loss of this tethering protein promotes the epithelial-to-mesenchymal transition and tumor metastasis [59,60]. Accordingly, overabundance of SNAI1/2 is commonly seen in myriad epithelial-derived cancers (reviewed in [61]). The ZEB family of repressors similarly regulate epithelial identity, and their misexpression can cause disease [62]. Upregulation of ZEB1 (deltaEF1) promotes the epithelial-to-mesenchymal transition [63], and, conversely, mutations to ZEB1 lead to ectopic expression of type I collagen, which manifests clinically as an overaccumulation of endothelial cells in the cornea [64]. Altogether, these results suggest that silencers, by serving as a platform mediating the effects of repressors, may play an underappreciated role in human disease.

Mechanisms of Silencer Activity

A principle goal of many studies that discover and catalog silencers, is to gain insight into the mechanisms by which these noncoding sequence elements regulate transcription (Figure 3 Key Figure). The discovery of silencers by studies focused on the genomic distribution of H3K27me3 [12] and PRC subunits that localize to H3K27me3 [20] clearly suggests the involvement of this pathway; furthermore, PRC2 previously has been implicated in silencer activity [44,65]. However, those studies which used a functional assay to define silencers did not observe a statistically significant enrichment of these chromatin marks on silencers [11,13], and only ~10% of H3K27me3-DHSs were negatively correlated with nearby gene expression [12]. This implies that PRC involvement is neither necessary nor sufficient for silencer function generally. Similarly, Pang and Snyder reported marginally significant enrichment of H3K9me3 at silencers, and the HP1 proteins that bind this mark have previously been implicated in the function of a well-characterized silencer

Key Figure

Potential Mechanisms of Silencers



(See figure legend at the bottom of the next page.)

element [66], but only a minority of identified silencers are occupied by HP1 [13]. This chromatin state (i.e., HP1 proteins bound to H3K9me3-marked nucleosomes) is most closely associated with constitutive heterochromatin, including at centromeres and telomeres [14,32]. Silencers, by contrast, mediate cell-type-specific regulation, which would be more associated with dynamically regulated 'facultative heterochromatin'.

Altogether, a parsimonious interpretation of these observations is that silencers fall into multiple functional classes, characterized by distinct associated proteins and mechanisms of action. Consistent with this view, Gisselbrecht *et al.* saw evidence for subclasses of silencers in terms of their association with target promoters [11]. The repressive TF Snail, which distinguishes these subclasses, has been suggested to function at least in part by '**antilooping**' (i.e., preventing physical contact between enhancers and promoters [67]), and Snail-unbound silencers were enriched for contacts to target promoters [11]. Notably, none of the mechanisms of silencer activity discussed here is inconsistent with a model proposed by Kolovos *et al.* [68], in which the biophysical function of silencers is to isolate their target promoters from transcriptionally active nuclear subdomains; however, this property, if true, currently would not provide an epigenetic signal that could be used to predict the locations of silencers on a genomic scale.

Concluding Remarks

Despite the first description of a silencer element nearly 35 years ago [6], silencers have remained an understudied type of *cis*-regulatory element. While computational and high-throughput genomic studies over the past 20 years significantly advanced the characterization of transcriptional enhancers and an understanding of the mechanisms by which they drive gene expression in a cell-type dependent manner (reviewed in [2,69]), the identification and analysis of transcriptional silencers has lagged tremendously. Here, we reviewed five recent studies that used different, highly parallel approaches, some using novel experimental technologies developed specifically to screen for silencers [11,13], to identify silencers in the fly [11], mouse [20], or human [12,13,21] genomes. Intriguingly, nearly all elements found to exhibit silencing activity in the Gisselbrecht *et al.* study also acted as transcriptional enhancers in a different cellular context [11]. This result is supported by recent findings in mammalian cells, in which many candidate silencer elements identified on the basis of genomic features in one cell type, showed evidence of enhancer activity in at least one other cell type [20,21]. Thus, the traditional classification of *cis*-regulatory elements as 'silencers' or 'enhancers' appears to be an over-simplification.

Broader cataloging of silencers across a wide range of cell types and cellular states in both human and model organisms, will be required to achieve a better understanding of the cell type/state specificity of silencer activity, the extent of bifunctionality of *cis*-regulatory elements, and the chromatin and sequence features of silencers (see Outstanding Questions). A new, manually curated database of published silencer elements, SilencerDB, promises to promote this aim

Outstanding Questions

Is there a silencer chromatin signature? Current studies have not identified a chromatin signature characteristic of silencers. Is there a silencer state lurking in this epigenomic dark matter?

What coregulators are necessary for silencer function? The occupancies of many well-characterized corepressors have not been found to be enriched among functionally defined silencers. Is this an artifact of heterogeneity in the profiled cells, with specific corepressors mediating the function of distinct silencer subclasses? What uncharacterized proteins are involved in silencer activity?

How do silencers behave in three dimensions? How do silencer-promoter loops interact with the structure of topologically associated domains (TADs) that structure the genome on a larger scale, and are they similar to enhancer-promoter loops?

How are functional sequences structured within silencers? How does the size of silencers compare with that of enhancers? Do silencers show similar numbers, complexity, and organizing principles of TF binding sites as that of enhancers?

How are silencers conserved evolutionarily? To what extent does evolutionary change in silencers drive evolution of regulatory networks and phenotypes? Is there a conservation signature that distinguishes silencers from other genomic elements?

How does regulatory variation affect silencer activity? How can the impact of noncoding variants in bifunctional elements on gene expression and phenotypes be predicted? What is the contribution of silencer-impacting variants to traits, including diseases?

Figure 3. (A) Silencers, particularly those bound by Snail in *Drosophila* embryonic mesoderm, can disrupt promoter–enhancer interactions in order to dampen expression. (B) Silencers may harbor transcription factor (TF) binding sites that overlap sites for activators, such that the binding of certain TFs can disrupt the induction of transcription. This is particularly relevant for bifunctional *cis*-regulatory elements (CREs) that can function as silencers and enhancers depending on context. (C) Transcription of intragenic enhancers can interfere with the passage of RNA polymerase (green) through their host genes, attenuating their expression while activating transcription of distal genes. (D) Silencers might act at a distance to deposit repressive histone marks (red circles) and/or proteins that facilitate local compaction, such as heterochromatin protein 1 (HP1) or polycomb repressive complexes (PRCs). (E) Alternatively, the silencer may nucleate the spread of heterochromatin via polycomb or HP1 that is propagated across the target gene. Abbreviations: CRE, *cis*-regulatory element; HDACs, histone deacetylases.

[70]. Comparisons of the chromatin marks enriched among silencers may allow the determination of whether there is a silencer chromatin 'signature' that could be used to accurately predict what regions of the genome act as silencers in other cell types, on the basis of ChIP-Seq data for histone post-translational modifications and regulatory cofactors.

Much also remains to be understood about the mechanisms by which silencers repress gene expression (see Outstanding Questions). What sequence-specific TFs and chromatin regulators read out and mediate the repressive effects of silencers? Different silencers likely exert their effects by different mechanisms. How are silencer-promoter interactions regulated, and do promoter-contacting silencers confer different regulatory properties from those that appear to function by antilooping?

While a wide range of DNA sequence features have been found to contribute to the activities of different transcriptional enhancers, the *cis*-regulatory logic of silencers remains unknown (see Outstanding Questions). Two different models of organization of TF binding sites within enhancers have been recognized: (i) the 'enhanceosome', in which a cooperative assembly of TFs and cofactors requires a strict organization of binding sites constrained by a network of spatial interactions among bound TFs and cofactors; and (ii) the 'billboard', in which the contributions of an assembly of individual TFs or groups of TFs, bound to flexibly arranged, clustered DNA binding sites, are integrated [71,72]. Studies of the evolutionary conservation of silencers may provide insights on sequence features of silencers that are important for their activities. Finally, more expansive cataloging and improved understanding of elements for their silencing activities, as well as their potential bifunctionality as enhancers, will allow for more accurate prediction of the effects of noncoding variants on cell-type-specific gene regulation and phenotypes.

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Declaration of Interests

There are no interests to declare.

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