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Review article

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Identification of non-coding silencer elements and their regulation of gene expression

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Abstract

Cell type- and differentiation-specific gene expression is precisely controlled by genomic non-coding regulatory elements (NCREs), which include promoters, enhancers, silencers and insulators. It is estimated that more than 90% of disease-associated sequence variants lie within the non-coding part of the genome, potentially affecting the activity of NCREs. Consequently, the functional annotation of NCREs is a major driver of genome research. Compared with our knowledge of other regulatory elements, our knowledge of silencers, which are NCREs that repress the transcription of genes, is largely lacking. Multiple recent studies have reported large-scale identification of transcription silencer elements, indicating their importance in homeostasis and disease. In this Review, we discuss the biology of silencers, including methods for their discovery, epigenomic and other characteristics, and modes of function of silencers. We also discuss important silencer-relevant considerations in assessing data from genome-wide association studies and shed light on potential future silencer-based therapeutic applications.

Sections

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Introduction

The human genome includes an estimated 20,000 protein-coding genes, mutations in which cause numerous diseases[1,2]. However, genes represent only less than 2% of our genome, and most of the genome does not encode proteins[3]. The non-coding part of the genome contains introns and various intergenic regions, which were long claimed to be functionless and considered 'junk' DNA. Now we know that much of the non-coding part of the genome harbours distinct non-coding regulatory elements (NCREs) that control gene transcription, including promoters, enhancers, insulators and silencers. Among these NCREs, silencers are the least characterized elements. Thanks to recent technological advances, the characterization of silencers and the understanding of their functions in gene regulation and disease have gained more interest and entered the spotlight of genetic research.

Following the publication of the first full draft of the human genome in 2001 (refs.[4,5]) and the subsequent emergence of genome-wide association studies (GWAS), more than 90% of disease-associated sequence variants were found to lie in the non-coding part of the genome[6,7]. This distribution demonstrated the importance of annotating the functional roles of non-coding DNA elements. Recent advances in experimental and computational methods based on next-generation sequencing, developed by consortia such as the ENCODE Consortium and by individual laboratories[3,8], deepened our understanding of the functions of non-coding elements, of which most are actively involved in gene regulation[8].

Four main types of non-coding DNA elements that regulate gene transcription — promoters, insulators, enhancers and silencers — have been identified and characterized[9]. Promoters are usually defined as short DNA sequences (100–1,000 bp) close to a transcription start site of the genes they control; promoters contain transcription factor-binding sites, which recruit transcription factors and RNA polymerase for transcription initiation[10]. Transcription initiation is further fine-tuned by proximal and more distally located NCREs: insulators, enhancers and silencers, brought together into topologically associating domains by the chromatin looping factors CTCF and cohesin (Fig. 1a). NCREs can act as far as several megabases away from their target genes[11,12]. Enhancers, which are the most extensively studied type of NCRE, modulate the expression of tissue-specific and cell type-specific genes. Active enhancers and promoters are found within open chromatin, and are mainly bound by transcription-activating factors[13]. Enhancers are enriched in specific histone modifications, such as monomethylated histone H3 Lys4 (H3K4me1) and acetylated histone H3 Lys27 (H3K27ac)[14]. This unique epigenetic make-up together with other characteristics (for example, chromatin accessibility and evolutionary conservation) has led to the identification of numerous potential enhancers, both experimentally and computationally, which are catalogued in databases such as EnhancerAtlas and the VISTA Enhancer Browser[15,16].

Mammalian genomes are organized into topologically isolated loops, which insulate genes from the influence of NCREs outside their loops[17–19] (Fig. 1a). Insulators were initially defined by their ability to protect genes from genomic position effects[20–22]. They are often found between enhancers and promoters, blocking the enhancer from interacting with the promoter. Insulators also block gene silencing, by acting as a barrier between transcriptionally permissive and transcriptionally repressive chromatin domains, preventing the chromatin characteristics of the repressive domains from spreading to the permissive domains and rendering them inaccessible to the transcription machinery[23].

Of the four NCRE types, silencers remain understudied and poorly characterized. Broadly defined, silencers could be genomic regions with tight packaging of nucleosomes, such as heterochromatin[24]. In this Review, however, we adopt a more stringent definition of silencers as non-coding genomic sequences that are recognized by transcription factors (analogously to enhancers) and repress the transcription of target genes. Silencers may act in position-independent and orientation-independent manners and have important roles in development and disease[25]. Similarly to enhancers, silencers harbour binding sites for transcription factors to recruit co-repressive factors or mediate silencer–promoter interactions, resulting in tissue-specific gene repression[26,27], the mechanisms of which mostly remain to be elucidated.

In this Review, we discuss the potential mechanisms of non-coding silencers in regulating gene repression and compare methods to identify and validate silencers. We summarize the current knowledge of silencer epigenetic signatures, the potential biological functions of clusters of silencers and the possibilities of functional switching between silencers and enhancers. Furthermore, we bring attention to the roles of silencers in diseases and their potential as therapeutic targets. Finally, we discuss open questions and future directions for fundamental and translational research.

The discovery of silencer elements

A subclass of transcription factors that negatively regulate gene transcription is referred to as 'repressors'[28]. Not only repressors themselves but also the genomic sequences that they recognize and bind — silencer elements — are crucial for repressing gene expression[29]. The *Saccharomyces cerevisiae* mating-type locus *HMRE* element was one of the first identified position-independent silencers that directs an active repression mechanism[30]. The *HMRE* silencer is a *cis*-acting element responsible for turning off the expression of genes in the *HML* and *HMR* loci, which determine the mating type in budding yeast. *HMRE* is position independent, functions in either orientation and can act 2,600 bp away from its target promoter[30]. In the fruitfly *Drosophila melanogaster*, the silencer *VRE* mediates ventral gene repression in response to the dorsal morphogen gradient, thereby initiating the differentiation of the embryonic mesoderm and neuroectoderm[31]. One of the first well-studied silencers in mammals was identified in the first intron of the *CD4* gene and was found to have a key function in the development of T lymphocytes. The silencer contains two protein-binding sites, which, upon binding by their respective repressors, repress *CD4* transcription in the $CD8^+$ T cell lineage[32–34]. Further studies on the intronic *CD4* silencer have highlighted its importance during tissue differentiation[35].

Since the definition of silencers as position-independent elements that direct an active repression mechanism, many silencers have been individually identified and characterized. The neuron-restrictive silencer element (also known as repressor element 1)[36] is a 21-bp DNA sequence motif found in or near approximately 2,000 neuronal genes in the human genome[36–39]. Neuron-restrictive silencer elements are bound by repressor element 1-silencing transcription factor (REST; also known as neuron-restrictive silencer factor (NRSF)), which recruits repressive cofactors and chromatin modifiers to repress the expression of neuronal genes in non-neuronal and undifferentiated tissues[40,41]. For instance, the gene *SCG10* (also known as *STMN2*) encodes the growth-associated protein stathmin 2, which is expressed in the early development of neuronal derivatives[42]. Upstream of the *SCG10* gene lies a neuron-restrictive silencer element that represses the activity of the enhancer found in the promoter-proximal region of *SCG10*, suggesting

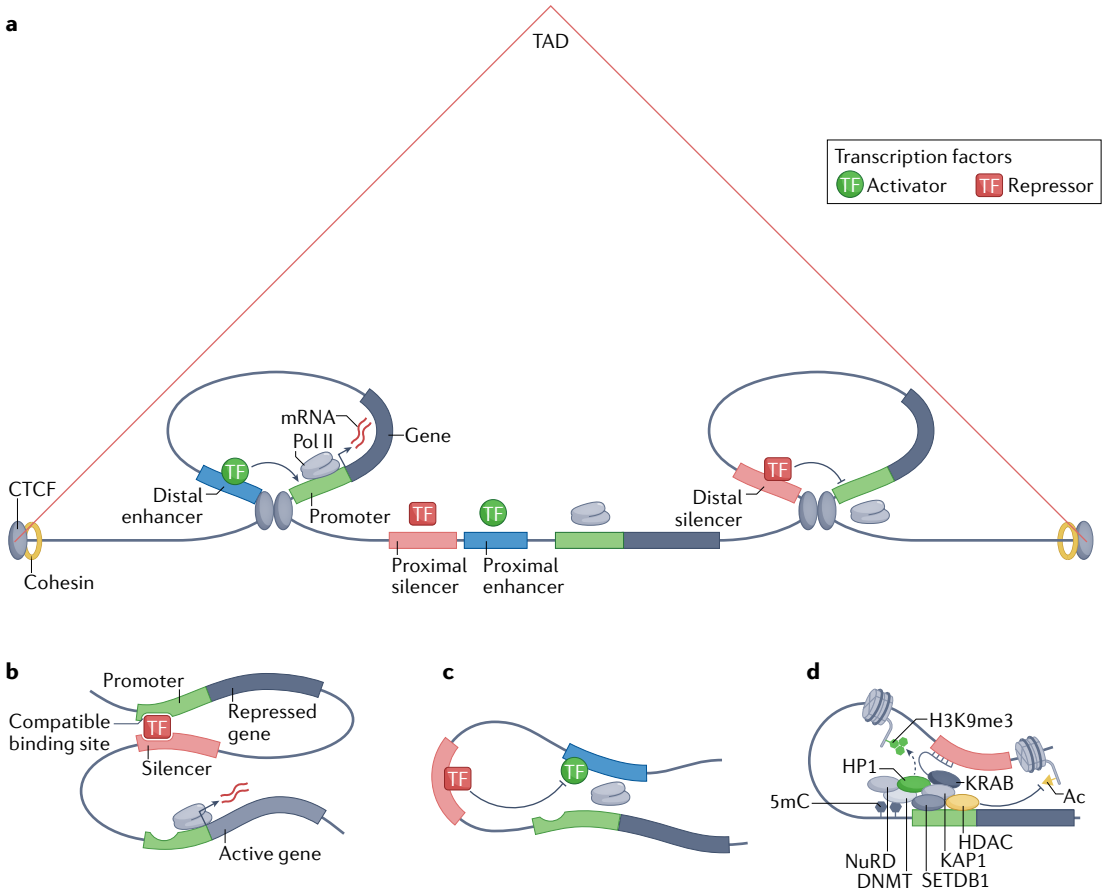

Fig. 1 | Silencer elements and their proposed mechanisms in gene regulation. **a**, Various non-coding regulatory elements, including silencers, enhancers, promoters and insulators (not shown), work together to control gene expression within topologically associating domains (TADs). TADs are demarcated by CCCTC-binding factor (CTCF) and cohesin complexes, which can also help to form smaller chromatin domains where distal enhancers or silencers are brought together with their target-gene promoters, thereby mediating transcription activation or repression, respectively. **b**, Silencer elements are bound by repressive transcription factors (TFs), which mediate specific short-range or long-range silencer–promoter interactions that lead to repression of target genes without affecting the expression of nearby genes with incompatible binding sites. **c**, Silencer elements can prevent enhancers from interacting with their target promoter, thereby repressing gene expression. **d**, Repressive Krüppel-associated box (KRAB) zinc-finger TFs bind silencers specifically through their zinc-finger domains. The KRAB domain associates with the cofactor KRAB-associated protein 1 (KAP1), leading to recruitment of various cofactors that methylate DNA and deacetylate histones, thereby repressing target genes. Ac, acetyl group; DNMT, DNA (cytosine 5)-methyltransferase; H3K9me3, trimethylated histone H3 Lys9; HDAC, histone deacetylase; HP1, heterochromatin protein 1; 5mC, 5-methylcytosine; NuRD, nucleosome remodelling and deacetylase complex; Pol II, RNA polymerase II; SETDB1, SET domain bifurcated histone-lysine methyltransferase 1.

that the expression of *SCG10* in neuronal cells depends predominantly on reversing the silencing of this enhancer[36,43]. Many silencers, like the silencer associated with *CD4*, affect their target genes directly and in a promoter-specific manner, through binding by cell type-specific repressive transcription factors such as RUNX1 and RUNX3 in *CD4* gene repression during T cell development[33–35] (Fig. 1b). However, there are also silencers, like the neuron-restrictive silencer element near *SCG10*, that indirectly repress gene expression by blocking enhancer activity[25,43–45] (Fig. 1c).

Silencers are recognized and bound by repressor transcription factors to exert their *cis*-regulatory repressive functions. Many of these repressor transcription factors, such as the REST complex, belong to the zinc-finger transcription factor family, which is the largest family of transcription factors in mammals and has widespread functions in development and homeostasis[46]. Their carboxy-terminal zinc-finger domains bind specific genomic DNA sequences, and the amino-terminal domain interacts with and recruits transcription cofactors. Approximately half of the human zinc-finger transcription factors have an amino-terminal Krüppel-associated box (KRAB) domain[47–49]. The KRAB domain recruits the cofactor KAP1, leading to the recruitment of histone deacetylases (for example, NuRD), methyltransferases (histone methyltransferases such as SETDB1, and DNA (cytosine 5)-methyltransferases) and adaptor proteins such as heterochromatin protein 1, the combined activity of which leads to reduced chromatin accessibility and subsequently to transcriptional gene silencing[48] (Fig. 1d). In the past few decades, various zinc-finger transcription factors, including CTCF, YY1 and ZBP89, have been shown to bind silencers and regulate their repressive activity[50–52].

Although these studies demonstrated the importance of silencers in development, research on the systematic identification and characterization of silencers has considerably lagged behind the extensive interrogation of enhancers. Enhancer-specific histone modifications were found to reflect cell type-specific gene expression[53–55], raising the possibility that such modifications may exist in silencers as well. Until recently, most of our understanding of silencer characteristics relied either on knowledge of silencers identified individually in small-scale experiments or on computational prediction, for example, by correlating cross-tissue epigenetic profiles with gene expression[56]. Experimental methods that facilitate a systematic and genome-wide identification of silencer elements are needed to gain more insights into the biology of silencers. In the next section, we discuss several such methods.

Methods for studying silencers

Unbiased genome-wide screening for potential silencers in mammalian genomes is currently not feasible, owing to the large size of genomes and limited screening capacities. One solution to this limitation is to focus the study on selected regions, which requires preselection of screened regions on the basis of previously described patterns. However, the incurred bias may lead researchers to overlook novel signatures of silencer elements. Recent technological advances in the systematic identification of silencers yielded multiple reports of large-scale identification and characterization of silencers throughout the genome based on distinct criteria, including computational prediction and definition of candidate elements based on chromatin organization and modification[57,58] (Fig. 2a).

Although silencer-specific chromatin signatures are still lacking, non-coding silencer elements likely mimic enhancer elements in certain aspects. Analogously to enhancers, silencers are bound by cell type-specific transcription factors which render their chromatin more accessible. On the basis of putative epigenetic characteristics and chromatin accessibility profiles, a subtractive analysis was performed to identify uncharacterized NCREs as accessible genomic regions (measured by DNase I hypersensitive site sequencing) devoid of histone modifications that characterize promoters (trimethylated histone H3 Lys4; H3K4me3), enhancers (H3K4me1) or insulators (CTCF-binding sites) (Fig. 2a,b). By means of a massively parallel reporter assay (MPRA) based on self-transcribing active regulatory region sequencing (STARR-seq)[59] (Fig. 2b), ~7,500 regions were assessed for silencer potential in K562 human chronic myelogenous leukaemia cells, of which ~3,000 showed potential silencer activity[60]. Similarly, chromatin accessibility profiles of multiple developmental stages were integrated with the corresponding gene expression profiles in the sponge *Amphimedon queenslandica*, and the accessible chromatin regions correlating with low gene expression were considered putative development stage-specific silencers[61].

Chromatin accessibility at genomic silencer regions was also exploited in other studies as a rationale for silencer screening. Accessible genomic regions from K562 cells, obtained by formaldehyde-assisted isolation of regulatory elements, were tested for silencer activity using the repressive ability of silencer elements (ReSE) screen. In ReSE screens, the silencer potential of a candidate region is assessed by its ability to repress cell death by silencing the transcription of inducible caspase 9 (Fig. 2b). In the study, ~2,600 silencers were identified in K562 cells, and ~1,660 silencers were identified in HepG2 human hepatoma cells[62]. Surprisingly, only ~2% of these potential silencer regions were shared between the two cell types, indicating that silencers have strong cell type-specific activity. With use of a different approach, genomic regions enriched by assay for transposase-accessible chromatin using sequencing (ATAC-seq) in a B lymphocyte cell line were simultaneously measured for enhancer and silencer activity and transcription factor occupancy using modified ATAC–STARR-seq episomal reporters (Fig. 2b). The analysis showed that silencers and enhancers represent distinct functional groups that are enriched in unique sets of transcription factor motifs and combinations of histone modifications[63].

There are also potential silencer-enriched genomic regions marked by H3K27me3, which is generally associated with transcriptional repression. When H3K27me3-marked regions that overlapped with the accessible chromatin as detected by DNase I hypersensitive site sequencing were annotated by bioinformatics analysis, it was found that these sites are negatively correlated with nearby gene expression in multiple cell types[56] (Fig. 2a). These H3K27me3-marked regions are thus associated with silencing, albeit not unambiguously marking silencer elements. The term 'H3K27 trimethylation-rich regions' (MRRs) was coined to denote genomic regions with a high density of H3K27me3 peaks on chromatin immunoprecipitation followed by sequencing (ChIP–seq) that preferentially contact each other and target genes, as shown by Hi-C sequencing. Compared with 'typical' H3K27me3 domains (that is, not significantly enriched in the chromatin contacts described above), MRRs could be strong indicators of silencer activity[64]. As a proof of principle, identified MRRs were intersected with previously reported silencers identified by ReSE screens[62], which revealed that these regions more predominantly overlap with each other than do silencers identified by ReSE screens and 'typical' H3K27me3 regions[56]. Although no large-scale screening for silencer activity focusing on MRRs has been performed, CRISPR–Cas9-mediated genomic deletion of two MRRs, which interact with the genes *FGF18* and *IGF2*, respectively, led to increased expression of these genes. These data indicate that MRRs are bona fide silencers with important roles in gene regulation[64].

Whereas previous high-throughput studies were mostly performed in cell lines, a parallel reporter assay was recently used to screen a library of ~600 candidate silencers in *D. melanogaster* embryos[45]. Candidate regions were selected on the basis of the presence of DNase I hypersensitive sites, H3K27me3 enrichment and binding of the transcriptional co-repressors Groucho and CtBP, which are canonically associated with long-range and short-range repression, respectively[26]. These regions were subsequently tested for their potential to repress the expression of a GFP reporter that was driven by a ubiquitous enhancer (Fig. 2a,b). Interestingly, ~75% of the identified silencers in that study were previously reported to act as enhancers in different studies, indicating that many regulatory elements have dual roles as both enhancers and silencers, depending on cell type and differentiation stage[45] (see below). However, the experimental set-up of that study did not discriminate between a direct repressive effect of silencers on the reporter promoter and a more indirect effect through repression of the enhancer activity.

In addition to the use of high-throughput screening and computational methods, assays focusing on specific markers could also be used to identify potential silencers (Fig. 2a). Trimethylation of histone H3 Lys27 is catalysed by Polycomb repressive complex 2 (PRC2), which is a key inducer of transcriptional gene silencing[65–67]. PRC2 was shown to act as a transcription repressor by forming long-range chromatin interactions, leading to epigenetic modification and the silencing of PRC2-bound gene promoters[68–70]. As such, PRC2-bound genomic

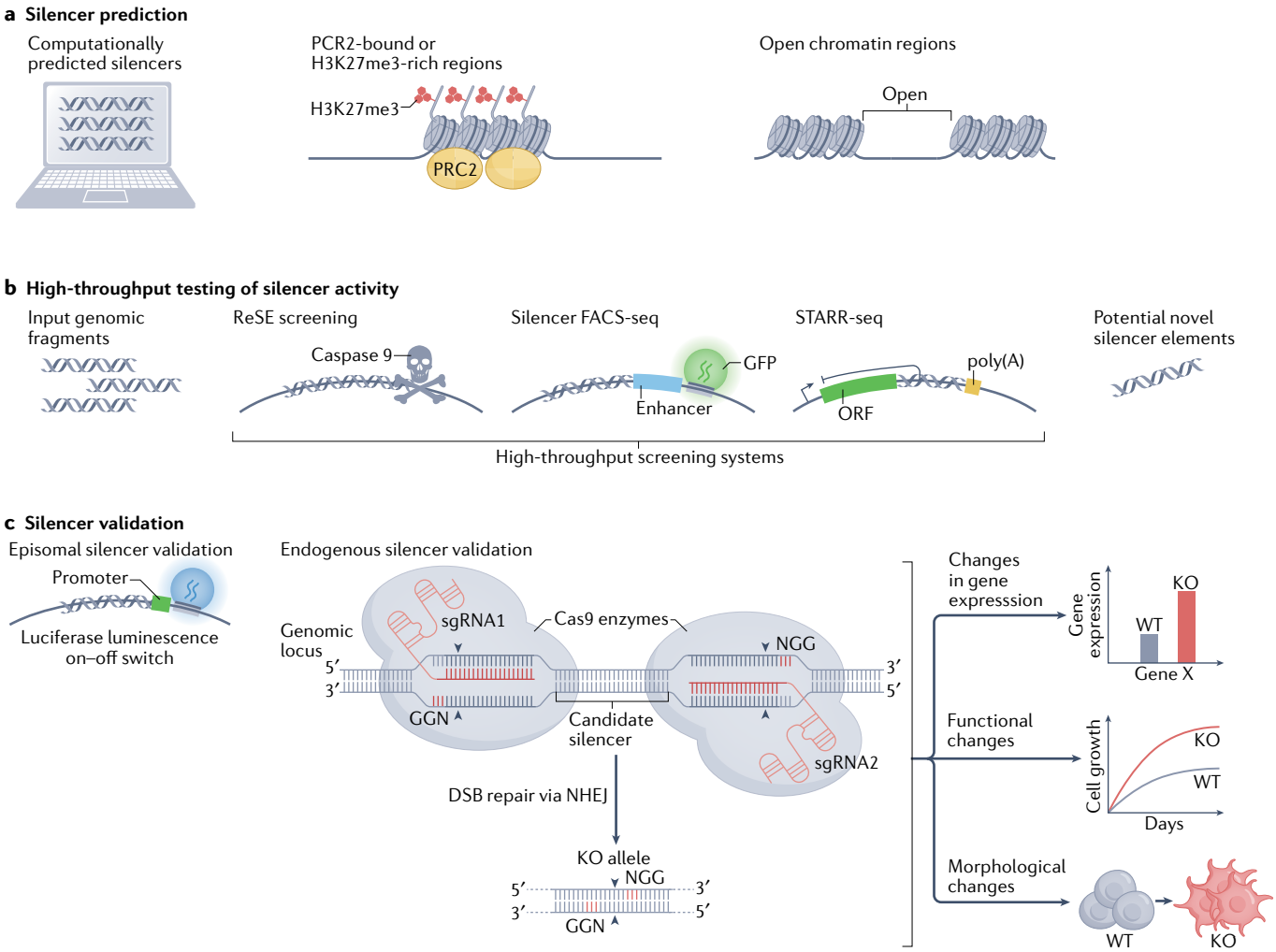

Fig. 2 | Large-scale silencer identification methods. a, Silencer prediction. Owing to the large genome size and limited library and screening capacities, silencer screening libraries are often constructed on the basis of known or predicted silencer characteristics. Recent silencer screens were performed using libraries of sequences chosen on the basis of different combinations of computationally predicted silencer regions, Polycomb repressive complex 2 (PRC2)-bound DNA fragments, trimethylated histone H3 Lys27 (H3K27me3)-rich regions and accessible-chromatin regions. **b**, High-throughput screening of silencer activity. Once candidate silencer regions are obtained, various high-throughput screening systems are used to test genomic fragments for silencer activity. The repressive ability of silencer elements (ReSE) system screens genomic sequences for potential silencers that repress the transcription of an inducible gene encoding the cell death protein caspase 9. Caspase 9 induces apoptosis, and thus cells containing non-silencer elements will undergo apoptosis, selecting for cells containing potential silencer elements that repress the transcription of the caspase 9 gene. The silencer FACS-seq (fluorescence-activated cell sorting followed by high-throughput sequencing) screening system, rather than assessing the direct effect of candidate silencers on a reporter promoter, tests candidate regions for their potential to repress the activity of a ubiquitously active enhancer coupled to a promoter (not shown) driving GFP expression. Self-transcribing active regulatory region sequencing (STARR-seq) measures the potential of candidate regions to suppress their own transcription through a defined promoter. Following transfection of the plasmid library, reporter mRNA and library DNA are isolated and amplified and the RNA to DNA ratio reveals the potential repressive activity of the candidate regions. **c**, Silencer validation. Once potential silencer elements are identified, they need to be individually validated. Episomal (plasmid)-based luciferase-reporter assays can validate the regulatory activity of potential silencer elements on a defined promoter by measuring reduced luciferase signal compared with a control vector. At the endogenous loci, potential silencer elements can be validated using CRISPR–Cas9 to generate silencer knockout (KO) cell lines using two single-guide RNAs (sgRNAs) targeting both ends of the silencers. Subsequently, silencer knockout cells can be evaluated on the basis of changes in gene expression, functionality or morphology compared with wild type (WT) cells. DSB, DNA double-strand break; NHEJ, non-homologous end joining; ORF, open reading frame.

regions have the potential to function as transcriptional gene silencers. To test this hypothesis, chromatin interaction analysis with paired-end tag sequencing was used to uncover PRC2-dependent long-range chromatin interactions in mouse embryonic stem cells (Fig. 2a). Genes interacting with PRC2-bound genomic regions are generally expressed at low levels, and ablation of contacts between identified silencers and their target-gene promoters by CRISPR–Cas9 resulted in target-gene activation and developmental defects, demonstrating the role

of PRC2-bound regions as silencers in gene regulation[71]. Whether the silencing effects are directly mediated by PRC2 or whether PRC2 is an effector of CTCF–cohesin-mediated chromatin looping is still an open question.

Recent advances in genome editing by CRISPR–Cas systems, including deactivated Cas9 (dCas9)-mediated gene activation or repression, revolutionized how the genome can be studied[72,73]. In addition to targeting coding sequences, CRISPR systems can systematically target the non-coding part of the genome in a genome-wide fashion. Using CRISPR–Cas9, small insertions and deletions can be introduced in transcription factor-binding motifs, thereby abolishing the function of NCREs and potentially resulting in deregulated gene expression or cellular phenotypes that can be used for large-scale screening[74–76]. Conversely, dCas9-mediated activation or repression systems have been used to target potential NCREs, such as insulators and enhancers, to counteract their original functions[77–80]. Although no similar screening using CRISPR–Cas9 has been documented yet to study silencers, such efforts should provide new insights into silencer biology in the future.

Once potential silencers are identified, multiple assays can be used to validate their activity (Fig. 2c). For example, luciferase activity assays in which a silencer is positioned upstream of a specific promoter (driving luciferase expression) are usually used to validate the silencer function[60,62]. However, this method tests silencer activity only on a specific promoter, overlooking the promoter specificity of regulatory elements occurring throughout the genome, and thus may be biased[81,82]. Furthermore, these are episomal (plasmid)-based assays, testing the activity of a candidate silencer outside its chromatin context. To study individual silencers within their endogenous loci, the CRISPR–Cas9 system has been used[60,62,64,83] (Fig. 2c). A pair of Cas9 endonucleases targeting both sides of the individual candidate silencer element could be designed to remove the region of interest from its endogenous locus. Genes within the same topologically associating domain that are upregulated following silencer removal are potentially controlled by the candidate silencer element[62].

Epigenomic and other characteristics

As mentioned earlier, specific epigenetic modifications are associated with distinct NCREs, and some silencers are enriched in H3K27me3 (refs.[56,64,84,85]). Trimethylation of histone H3 Lys27 is deposited by PRC2 (refs.[67,71,86]), in which Enhancer of Zeste homologue 1 (EZH1) or EZH2 is a catalytic subunit[87,88]. Independently of PRC2, EZH2 can promote the stability of the REST complex and thus its gene silencing activity[89]. Recent studies reported the enrichment of EZH2- and REST-binding motifs in identified silencers[56,60,64], indicating their involvement in silencer-mediated gene regulation. Indeed, perturbation of EZH2 expression or function led to the disruption of MRR-mediated chromatin interactions and upregulation of target genes of the identified silencers[64]. Notably, the ReSE screen also revealed enrichment of EZH2- and REST-binding motifs in the identified silencers in HepG2 cells, but not in K562 cells. Moreover, in K562 cells, a positive association was found between silencers and the binding motif for NCoR, a co-repressor of REST[90], suggesting the existence of cell type-dependent differences in silencer mechanisms of function[62].

Interestingly, data obtained from the ReSE screen also showed a positive association of silencers with methylation of histone H4 Lys20 (H4K20), a histone modification often associated with cell cycle dynamics and thought to regulate various processes related to DNA damage and chromosome condensation[62,91–93]. Although previous studies showed an association of H4K20me1 with both transcription activation and repression, the precise role of H4K20me1 in regulating gene expression and, more specifically, a possible role in silencer activity is largely unclear[91]. Discoveries of novel silencer-associated factors will greatly improve future efforts to predict and characterize new silencer elements.

Combinations of various features have been demonstrated to predict cell type-specific enhancers with relatively high confidence[94–96]. For example, in a study of genomic regions regulated by the transcription factor cone–rod homeobox protein (CRX) in mouse retinas, enhancers were found to contain binding motifs for a more variable set of transcription factors than was the case for silencers[27]. Therefore, silencer activity may depend more extensively on the interaction of chromatin (nucleosome) remodellers and transcription factors to exert their cell type-specific activity than on the effect of single transcription factors or histone modifications. However, it would not be surprising if other differences emerge once we have a comprehensive understanding of silencers. On the basis of our current understanding, it remains a challenge to pinpoint common epigenetic markers or binding motifs, if any, that could predict silencers in the genome. The studies discussed above have generated valuable insights into the mechanisms of silencer activity. However, more-thorough identification and characterization efforts in different cell types will be required to fully comprehend the role of silencers in development and disease.

Of silencer clusters and 'super-silencers'

Most research efforts are aimed at the identification of individual silencer elements. However, in their endogenous environment, silencers may function in dynamic regulatory landscapes. Three-dimensional chromatin folding mediated by CTCF and cohesin (among other factors) can bring multiple NCREs and target genes into proximity[97]. The expression of these genes within such landscapes is often dictated by the interplay between silencers and enhancers rather than by direct, one-on-one NRCE–target gene interactions[97–99].

Recently, the term 'super-enhancer' was coined for clusters of genomic elements exhibiting higher levels of enhancer-associated histone modifications and transcription factor binding, particularly of the transcription coactivator MED1, compared with individual enhancers. Super-enhancers have been shown to robustly regulate target-gene expression, specifically of genes that determine cell identity[100–103]. Currently, whether super-enhancers have greater functionality than individual enhancers remains a topic of debate. Nevertheless, similar super-clusters were proposed for silencer regions. As mentioned earlier, MRRs[64] are more likely to reside in loci that confer tissue specificity, analogous to the definition of super-enhancers[56]. Compared with other H3K27me3-rich regions, MRRs exhibited higher overlap with identified silencer elements in K562 cells[62,64], and more preferentially interacted with each other[64]. Deletion of two of the identified MRRs resulted in disruption of silencer–promoter interactions, upregulation of the target gene and altered cell phenotype, indicating that the identified MRRs may represent tissue-specific silencers[64]. Furthermore, the deletion of two silencer components within one of these MRRs, which is close to the gene *FGF18*, led to synergistic upregulation of *FGF18* expression and cell identity changes compared with the deletion of each component alone, indicating that both components function cooperatively as part of a 'super-silencer'[64,104]. Although such clusters of high-density silencer marks are helpful in identifying functional silencer elements, whether these MRRs or 'super-silencers' provide additional functionality compared with typical silencers is still open for debate.

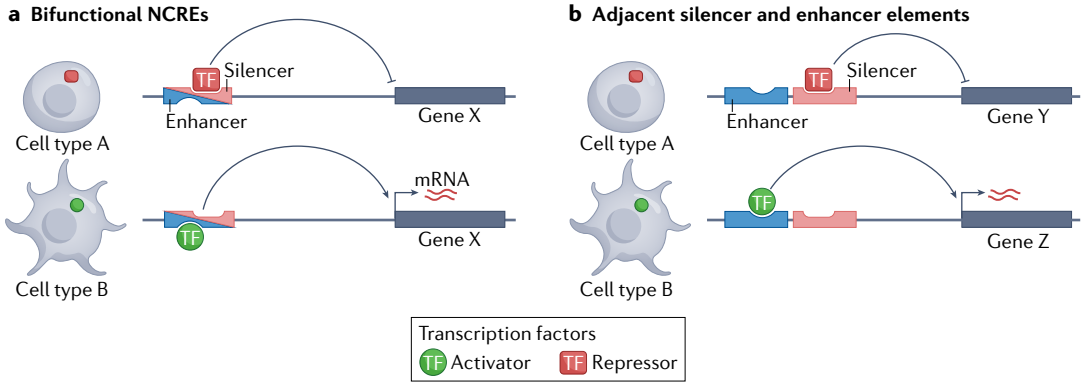

Fig. 3 | Silencer elements with dual functionality. a, A bifunctional non-coding regulatory element (NCRE) acts as a gene silencer in cell type A through binding by repressor transcription factors (TFs), whereas the same element acts as an enhancer in cell type B through binding by activator TFs. **b**, Rather than representing a single bifunctional regulatory element, a proposed NCRE could also consist of adjacent enhancer and silencer elements, and therefore function as two separate, tissue-specific NCREs with the silencer element repressing gene Y in cell type A and the enhancer element activating gene Z in cell type B.

NCRE redundancy, particularly of enhancers, has recently been demonstrated to occur often in vivo[105–107]. Thus, although a genomic region may regulate gene expression in reporter assays, deletion of this region from the genome may not affect target-gene expression owing to redundancy with other enhancers, including 'shadow' enhancers[107]. To our knowledge, such redundancy has not been described for silencers yet. Nevertheless, given the similarities between the modes of action of silencers and enhancers, redundancy might exist for silencers as well, and it should be considered when one is assessing silencer identification or activity.

Dual-function regulatory silencers

Although silencers and enhancers were assumed to be separate classes of NCREs, increasing evidence suggests that silencers can act as enhancers and vice versa, depending on the cellular context[45,56,60,71,108]. For instance, a recent study based on machine learning estimated that more than 6% of the tissue-specific silencers predicted computationally may have dual functions in human T cell development[109]. Although the extent of such dual functionality of NCREs differs between different studies[45,109,110], the phenomenon was further supported by complementary findings that some silencers are marked by histone modifications typical of active enhancers, such as H3K4me1 and H3K27ac[56,60,71], or are bound by transcription factors that bind also to active enhancers, for example, CRX[111–113]. These findings raise the question of whether silencers and enhancers are truly separate entities or whether such a dual role is a trait of NCREs that is more common than previously acknowledged. It is possible that some NCREs possess both silencer and enhancer activities because they contain binding motifs that can be recognized by both repressor and activator transcription factors, depending on the cellular context[25,45,114,115] (Fig. 3a). Consequently, the regulatory function of such NCREs in a specific cell type would be dictated by the expression levels of the corresponding transcription factors.

Alternatively, rather than representing true bifunctional elements, such dual-function NCREs might consist of separate adjacent silencer and enhancer units that function in distinct cellular processes or in distinct cell types[116–118] (Fig. 3b). The resolution of current large-scale experiments for predicting and identifying regulatory elements does not yet allow the precise definition and demarcation of NCRE boundaries at single-nucleotide resolution. Future efforts to test potential silencers identified in large-scale studies for both silencer potential and enhancer potential at single-nucleotide resolution, by disruption of endogenous loci using CRISPR–Cas9 or by reporter assays in multiple cell types, will further refine and demarcate NCRE boundaries and will contribute to a comprehensive understanding of endogenous regulatory function of NCREs in different cell types.

Silencers as therapeutic targets

Over the past decade, advances in GWAS with increased statistical power have led to the identification of many disease-associated single-nucleotide polymorphisms (SNPs), most of which are located in the non-coding part of the genome[119,120]. Although the functional annotation of SNPs and their contribution to diseases are complicated, so far most SNPs in the non-coding regions have been linked to enhancer regions[6,121], potentially perturbing enhancer function and leading to altered gene expression[120]. The functional annotation of non-coding SNPs relies on identifying the disease-causing variants among the disease-associated SNPs, and benefits from the extensive annotation of potential NCREs[122]. Although most of the disease-associated SNPs reported to date affect enhancer function, recent studies indicate that silencer elements are similarly enriched in disease-associated variants[56,60], suggesting that silencers have a role in human diseases that is similar to the role of enhancers, and underlining the importance of silencer identification and characterization in the human genome.

GWAS of oestrogen receptor (ER)-positive breast cancer identified three independent functional variants in the *FGFR2* risk locus[123,124]. Functional reporter assays revealed that these variants are located in silencer elements that control *FGFR2* expression. These risk alleles augment silencer activity, resulting in decreased *FGFR2* expression[124]. In addition, SNPs associated with ER-positive and ER-negative breast cancers were identified in the human ERα gene (*ESR1*)[125,126]. Fine-mapping and functional analysis revealed that five of these SNPs are located within NCREs that regulate the expression of *ESR1*, one of which is an orientation-dependent silencer, the mutation of which potentially increases the expression of *ESR1* and another gene[127] (Fig. 4a).

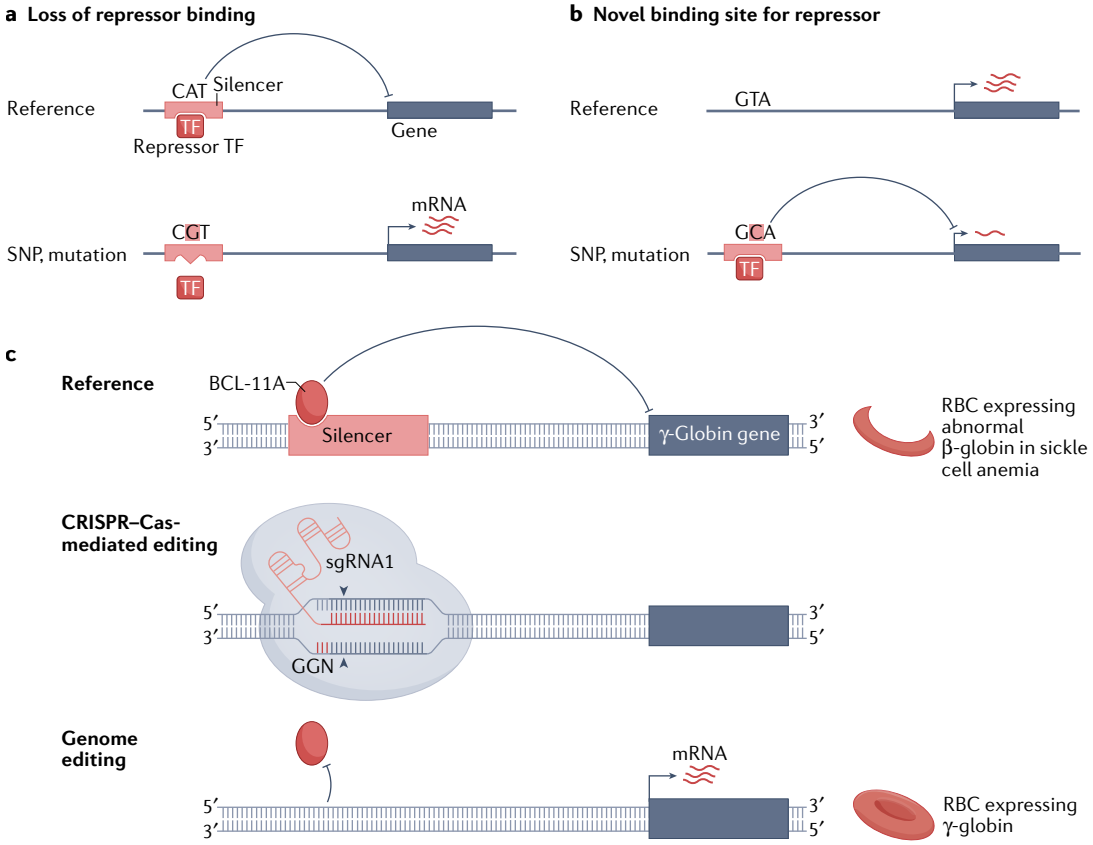

Fig. 4 | Effects of disrupted silencer function on gene expression and disease. **a**, Mutations or single-nucleotide polymorphisms (SNPs) within a silencer element can disrupt silencer function. A repressor transcription factor (TF) binds the wild type (reference) allele of the binding motif within the silencer element, leading to repression of target-gene transcription (upper panel). In the SNP-containing allele, the binding motif is disrupted and the repressor TF can no longer bind, leading to the disruption of gene repression (lower panel). **b**, A mutation or SNP can create a new binding motif for repressor TFs, thereby generating a silencer element that leads to decreased expression of nearby genes. **c**, The expression of γ-globin in erythrocytes of adults is repressed by the silencer-binding repressor BCL-11A. Re-expression of γ-globin in red blood cells (RBCs) of adults with abnormal β-globin was shown to reduce the severity of sickle cell disease and β-thalassaemia. Thus, CRISPR–Cas9-mediated disruption of the BCL-11A-binding site in this silencer element is a promising therapeutic target to specifically upregulate γ-globin and reduce disease severity. sgRNA, single-guide RNA.

Similar observations were also made in other diseases[128–132]. When ~100 cardiac QT interval-associated variants within the *SCN5A* locus were assessed for their regulatory potential, functions of multiple enhancers and silencers were found to be perturbed by different variants, suggesting a possible mechanism through which SNPs contribute to heart rhythm anomalies[131]. This concept is also illustrated by SNP rs2071473, which is associated with fecundability (the probability of being pregnant within a single menstrual cycle) in humans, and is an expression quantitative trait locus for *TAP2* (ref.[130]). This SNP is located in a progesterone-responsive NCRE that, depending on which pyrimidine nucleotide (C or T) is present at this position, functions either as a silencer or as an enhancer[130]. Another study using whole-genome sequencing for six individuals from two families led to the identification of multiple coding and non-coding mutations associated with tetralogy of Fallot, a congenital cardiac anomaly characterized by a combination of four related cardiac defects[129]. One non-coding mutation was found in a silencer regulating *NOTCH1*, which encodes a transmembrane receptor important for cardiomyocyte differentiation[128,129]. The mutation augmented silencer activity and decreased the expression of *NOTCH1*.

Therefore, tissue-specific annotation and assessment of regulatory activity are key to elucidate whether SNPs lead to silencer and enhancer disruption or strengthening, or NCRE function switching. Disease-associated variants are often mapped to predicted regulatory elements on the basis of the characteristics described above (chromatin accessibility, transcription factor binding and histone modifications). Consequently, interrogation of a potential effect is often focused on the disruption of NCRE function[121,133–135]. However, recent evidence demonstrated cases in which a SNP created a new binding motif for a repressive transcription factor, thereby creating a new silencer that represses nearby genes[136] (Fig. 4b). Unsurprisingly, the reference allele in this case is devoid of any predictive NCRE characteristics and therefore would likely be overlooked when one is assigning function to the associated SNPs. Comprehensive bioinformatics analysis of binding motifs in the sequences encompassing the associated SNPs could reveal new transcription factor-binding motifs, and could provide candidates for experimental validation that may have been previously overlooked.

The interaction between silencer regions and target-gene promoters plays an important role in gene regulation, as deletion of such

Glossary

Assay for transposase-accessible chromatin using sequencing
(ATAC-seq). A high-throughput technique to identify regions of accessible chromatin genome-wide.

Cardiac QT interval
An electrocardiographic measure of myocardial repolarization.

Chromatin interaction analysis with paired-end tag sequencing
(ChIA-PET). A high-throughput technique to identify genome-wide chromatin interactions mediated by specific factors.

Chromatin looping factors CTCF and cohesion
Architectural proteins that mediate longe-range genomic interactions by contributing to three-dimensional genome organization.

Expression quantitative trait locus
A genomic locus or single-nucleotide polymorphism that is associated with differential gene expression, thereby linking variations in gene expression levels to different genotypes.

Formaldehyde-assisted isolation of regulatory elements
(FAIRE). A high-throughput technique to discover nucleosome-depleted genomic regions, which are indicative of regulatory activity.

Genome-wide association studies
(GWAS). Studies used to identify genomic variants that are statistically associated with a particular trait or disease risk.

Genomic position effects
The influence of the endogenous chromosomal environment on the activity of a gene or a regulatory element.

Hi-C
A chromosome conformation capture technique that maps the three-dimensional organization of the genome.

Insulators
Non-coding regulatory elements with enhancer blocking or barrier function.

Massively parallel reporter assay
(MPRA). A high-throughput technique to simultaneously measure the transcriptional activity of thousands of candidate non-coding regulatory elements.

Self-transcribing active regulatory region sequencing
(STARR-seq). A type of massively parallel reporter assay in which candidate non-coding regulatory elements are cloned downstream of a reporter gene so that their enhancer or silencer activity is reflected in the abundance of the non-coding regulatory element sequence within the pool of plasmid-derived RNA.

'Shadow' enhancers
Two or more enhancers with seemingly redundant functions that regulate the same target gene or genes.

Topologically associating domain
Chromatin domain of typically ~100 kb to ~1 Mb, characterized by high intradomain contacts that contribute to NCRE-promoter interactions.

interacting regions led to the upregulation of target genes[58,64]. It is also conceivable that disruption of the mechanisms underlying this three-dimensional chromatin conformation by disease-associated SNPs may affect such physical interactions and lead to disrupted gene expression and disease[137–139]. MPRAs that simultaneously screen disease-associated SNPs for their effect on potential regulatory activity have been performed[140–143]. Adaption of similar assays to systematically screen genomic sequences for silencer activity could elucidate the functional effect of a large selection of variants on silencer function.

Dysregulation of transcription factors can lead to abolished or ectopic expression of their target genes and, consequently, to disease. In sickle cell disease and in β-thalassaemia, upregulation in erythrocytes of fetal haemoglobin (HbF) reduced the disease severity[144–146], and thus re-expressing HbF in adult red blood cells is a potential therapeutic strategy. HbF is a tetramer of two α-globin polypeptides and two fetal γ-globin polypeptides. After birth, the adult β-globin genes replace the fetal γ-globin genes as the predominantly transcribed genes within the human β-globin cluster[147]. This 'fetal switch' is mediated by the zinc-finger-containing transcription factor BCL-11A, which represses γ-globin and thereby HbF expression in erythrocytes of adults[147] (Fig. 4c). GWAS identified a few SNPs in an erythroid-specific enhancer that cause downregulation of *BCL11A* expression, thus indirectly increasing HbF expression[148,149]. These data indicate that the *BCL11A* enhancer could serve as a therapeutic target. Indeed, CRISPR–Cas9-mediated disruption of the *BCL11A* enhancer decreased *BCL11A* expression and increased HbF expression[150–155], and this approach is currently being tested in clinical trials[155,156]. It is worth noting that complete removal of the *BCL11A* gene resulted in failed erythrocyte differentiation[157], demonstrating that targeting NCREs could be a nuanced therapeutic strategy. As a transcription factor, BCL-11A may regulate other genes in addition to the β-globin cluster, so its ablation might result in unforeseen side effects. As an alternative to gene ablation, silencers containing the BCL-11A-binding sites that specifically regulate γ-globin expression could serve as potential therapeutic targets that might more specifically affect HbF expression only in erythrocytes[153,155,156] (Fig. 4c).

Biases and missing pieces

Despite the recent identification of silencer elements, general silencer characteristics such as transcription factor binding or specific histone modifications remain obscure. In this section, we discuss the strengths and caveats of recently published silencer identification methods. Similar to efforts to identify and characterize enhancers, the identification of silencer elements is hampered by the cell type-specific activity of the set of sequences to be tested. Ideally, the entire genome would be screened for regulatory potential in a systematic and unbiased fashion. Limitations of the capacity of current screening systems hamper such genome-wide screening and necessitate selection criteria for the screened library.

To facilitate studies of silencers and their role in transcription regulation during development and disease, archiving efforts such

as the SilencerDB database were made[158]. Its curators mined around 2,300 publications to generate a comprehensive database of both predicted and validated silencers in different cell types and species. Currently, this database contains approximately five million silencers predicted on the basis of extrapolating ChIP–seq- or MPRA-derived data collected from multiple studies by machine learning, and ~33,000 in vitro validated silencers from both high-throughput experiments and low-throughput experiments[158]. Of note, among these validated silencers, only 353 have been individually validated in reporter assays. The other ~32,700 silencers were demonstrated to have only potential silencer capacity in large-scale MPRA screens. Although such curation is highly valuable for silencer characterization, the predictive value of additional epigenomic features for silencer identification would greatly benefit from more unbiased, large-scale screens. Computational prediction based on epigenetic modifications also has its limitations. Even for enhancers, which are extensively characterized, a large number of predicted elements did not show regulatory activity in validation assays, suggesting that chromatin state alone does not always reflect regulatory activity[159–161].

Furthermore, despite the fact that most 'validated' silencers were shown to have silencer potential by MPRAs and ReSE screens, which are functional assessments, a major limitation of these methods is that they generally test regions out of their genomic context. These high-throughput methods assess the potential of NCREs to regulate a general promoter reporter or a strong enhancer–promoter reporter, rather than their endogenous target promoters, and in cell types in which the tested NCREs might not necessarily be active. Incompatibility issues between tested fragments and the enhancer–promoter may also produce false negative results. Other potential complexities that may affect silencer activity, such as positional effects, activity strengthening or attenuating effects through locus rearrangements, or three-dimensional chromatin organization effects, could not be assayed either. Furthermore, the tested DNA fragments are often size-selected on the basis of cell type-specific prediction (for example, ATAC-seq or ChIP–seq), and may also include regions located within heterochromatin devoid of any transcriptional activity, which introduces additional biases[59,162,163]. These limitations potentially muddle the attribution of endogenous silencer activity[164,165]. Therefore, experimental validation of individual silencers identified through high-throughput assays, either by reporter assays or by in vivo perturbation of endogenous sequence (Fig. 2c), or ideally both, is key to ensuring silencer annotation[60,62,64,83,166].

Concluding remarks

Recent advances in studying silencers led to the identification of many potential silencer elements. More extensive studies will be essential to locate silencers throughout the genome and unravel cell type-specific functions. Cataloguing both predicted and experimentally validated silencers in different cell types will, in return, facilitate the identification of repressive transcription factors or silencer-specific histone modifications, which could then be leveraged to screen genomic sequences more accurately for silencer potential and to identify even more potential silencer elements using computational methods. A joint effort of various research groups is needed to unravel the exact genomic location of silencers and their function in gene regulation. Combined with clinical data, this additional layer of gene regulation will increase our understanding of the onset and progression of diseases and could present invaluable contributions to therapeutics.

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Author contributions

B.P., J.H.v.W. and F.L.H. researched data for the article, wrote the article and reviewed and/or edited the manuscript before submission. M.P.S. contributed substantially to discussion of the content.

Competing interests

M.P.S. is a founder and member of the science advisory boards of Personalis, SensOmics, Qbio, January, Mirvie and Filtricine, and a member of the science advisory boards of

Genapsys and Epinomics. B.P. and M.P.S. hold a patent on the ReSE screening system and method (WO-2021155369-A1).

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