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Review

REVIEW

Beyond classic editing: innovative CRISPR approaches for functional studies of long non-coding RNA

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Abstract

Long non-coding RNAs (lncRNAs) makeup a considerable part of the non-coding human genome and had been well-established as crucial players in an array of biological processes. In spite of their abundance and versatile roles, their functional characteristics remain largely undiscovered mainly due to the lack of suitable genetic manipulation tools. The emerging CRISPR/Cas9 technology has been widely adapted in several studies that aim to screen and identify novel lncRNAs as well as interrogate the functional properties of specific lncRNAs. However, the complexity of lncRNAs genes and the regulatory mechanisms that govern their transcription, as well as their unique functionality pose several limitations the utilization of classic CRISPR methods in lncRNAs functional studies. Here, we overview the unique characteristics of lncRNAs transcription and function and the suitability of the CRISPR toolbox for applications in functional characterization of lncRNAs. We discuss some of the novel variations to the classic CRISPR/Cas9 system that have been tailored and applied previously to study several aspects of lncRNAs functionality. Finally, we share perspectives on the potential applications of various CRISPR systems, including RNA-targeting, in the direct editing and manipulation of lncRNAs.

Keywords: Long non-coding RNA; CRISPR/Cas9; lncRNA function; Gene editing

Introduction

Although non-coding regions make up around 97% of the human genome, little is known about these regions functionality [1, 2]. Large-scale biochemical studies such as Encyclopaedia of DNA Elements (ENCODE) project and Road-map Epigenomics, indicate that the majority of non-coding DNA is functional [3, 4]. Despite lacking a concrete universal definition, long non-coding RNAs (lncRNAs) are commonly defined as transcripts that are longer than 200 nt with no or limited protein-coding potential and are highly tissue-specific [5]. They share some characteristics with protein-coding RNAs such as the 5’ caps, the 3’ poly-A tail, as well as similar histone modifications profile and splicing mechanisms [6, 7]. LncRNAs could regulate gene expression by several mechanisms, including acting as scaffolds, decoys, guides and signals (7). In addition, a number of lncRNAs exert their effects by the mere act of their transcription [8, 9]. The human genome is rich in lncRNAs; GENCODE v26 (a manually curated database of lncRNAs) contains 15 787 annotate lncRNA genes and 2720 lncRNA transcripts [10]. However, it has been difficult to identify the genomic loci of lncRNAs as well as dissect their functionality or their interaction with other molecular pathways until recently, largely due to the challenges associated with manipulating their expression [2, 11].

Several genomic editing tools had recently emerged, such as zinc-finger nucleases (ZFNs) [12] and transcription activation-

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like element nucleases (TALENs) [13]. Although these tools have been mostly used to edit coding regions [14, 15], some attempts were made to apply them in lncRNA editing. For example, ZFNs were considerably efficient when used to deplete the lncRNA MALAT1 [16]. Alternatively, a strategy that is based on replacing genomic sequences with LacZ marker sequence was successfully used to generate mouse models knocked out for selected 18 lncRNA molecules [17], although this strategy was rather costly and applied only in animal models [18].

Clustered regularly interspaced palindromic repeats (CRISPR) systems were initially identified as means of bacterial adaptive immunity. They generally comprise arrays of DNA repeats interspersed with sequences that had been acquired from invading organisms, such as phages [19, 20]. Among these systems, the type II CRISPR/Cas9 system from *Streptococcus pyogenes* is the most widely studied and utilized in genomic editing. The system in essence consists of two major components: the Cas9 nuclease, guided by a crisprRNA (crRNA) and a tracrRNA that together form the guide RNA (gRNA) duplex [21]. Specific recognition and cleavage of the invading DNA by the gRNA–Cas9 complex is facilitated by the presence of a protospacer adjacent motif (PAM), a sequence of 2–6 nucleotides that is present exclusively in the viral DNA but not the bacterial. Ever since its discovery, the CRISPR/Cas9 system has been employed by several groups as a genomic engineering tool, due to the unprecedented ability of the gRNA–Cas9 complex to target and cleave genomic regions in a sequence-specific manner [22–26]. In genomic editing applications, a gRNA sequence that is complementary to a given target genomic sequence is designed, and used to target the Cas9 endonuclease to this specific locus, thereby causing double-stranded breaks (DSBs). Traditionally, these DSBs are repaired through the homology-directed repair (HDR) pathway [27, 28], although newly emerging variations of the CRISPR technique are non-homologous end joining (NHEJ)-dependent [29–31] (Fig. 1a).

The ‘classic’ CRISPR toolbox

CRISPR-Cas9 technology is generally faster, cheaper and more efficient than most existing gene expression manipulation methods. Most notably, the CRISPR/Cas9 system targets genomic regions, whereas the RNA interference (RNAi) machinery targets and cleaves transcripts using classic base complementarity [22, 32, 33]. This particular characteristic enables the editing of any genomic element through CRISPR, including regulatory elements such as promoters, enhancers, as well as intergenic regions and introns, whereas RNAi targets only transcripts which could be limiting. Moreover, unlike RNAi that functions through RISC (RNA-induced silencing complex) complex, CRISPR/Cas9 targets the genome directly and is not known to employ any mediator machinery [34]. CRISPR/Cas9 is also effective in producing homozygous knockouts (KO) making knockout screens more efficient than RNAi-based screens, that only transiently suppresses target gene expression levels [24, 25]. A recent study evaluated the efficiency of CRISPR interference (CRISPRi) knockdowns compared to randomly chosen short hairpin RNA (shRNA) and RNAi, and found that CRISPRi was more effective than most shRNA, and 3–5 times more effective than RNAi [35].

When used in its wild-type form, the Cas9 enzyme produces DSBs in a protein-coding region (Fig. 1a), resulting in mutations if these breaks are repaired through the NHEJ. This leads eventually to the efficient knockout of the target-coding region, an approach known as CRISPRn mutagenesis [36]. Another approach, CRISPRn HR, depends on the HDR when repairing the DSBs and is used in gene corrections, gene knock-in or overexpression, tagging as well as knock-out [37, 38]. Deletion of certain DNA stretches could also be achieved using Cas9 by inducing multiple DSBs, an approach referred to as CRISPRn excision [39, 40] (Fig. 1b and c). Finally, a version of Cas9 that lacks nuclease activity (deactivated or ‘dead’ Cas9; dCas9) while maintaining the RNA-dependent recognition of DNA could be fused with functional domains, thereby producing customized transcription factors. In bacteria, recruiting dCas9 to a promoter region is sufficient to create steric hindrance that might obstruct proper functioning of transcription machinery, hence causing reduced expression [41]. In eukaryotic cells, however, dCas9 should be combined with additional inhibitory domains, such as the KRAB (Kruppel-associated box) domain of *ZNF10,* in order to form a potent transcription inhibition complex (CRISPR interference or CRISPRi) [42]. Similarly, fusing dCas9 with activator domains such as p65, VP64 or Rta results in activating the targeted genes *in cis* (CRISPR activation or CRISPRa) [43–45] (Fig. 1d). In the context of lncRNA functional studies, the CRISPRi/a approaches have major advantages: first is the ability to detect in *cis* effects which is not possible when using plasmid-based overexpression or inhibition by RNAi, both produce in *trans* effects. A second advantage is the ability to activate endogenous promoters through CRISPRa, producing variant transcripts that are often non-coding.

Classic CRISPR editing and the complex architecture of lncRNA genomic loci

The genomic regions coding for lncRNAs are distributed over the whole genome, including intra-and intergenic regions [5]. Long intergenic non-coding RNAs (lincRNAs) are the subset of lncRNAs that are produced from non-coding regions between two coding genes. They are either produced from intergenic exclusive promoters, or from bidirectional promoters that could be shared with other coding or non-coding genes [46, 47] (Fig. 2i).

On the other hand, ‘internal’ lncRNAs lie fully within the body of other ‘host’ genes [48–50] (Fig. 2ii–iv). Internal lncRNAs could be transcribed from the coding strand, in which case they are called ‘sense’, and usually share exons with the protein-coding genes, either partially overlapping or covering the entire length of their host genes. Inversely, internal lncRNAs that arise from antisense strands of protein-coding genes are named ‘antisense’ lncRNAs. According to GENCODE [5], antisense lncRNAs could fall under one of three categories, (i) lncRNA exonoverlapping with a part of a sense gene, (ii) non-coding transcripts that span the whole sequence of sense gene, or (iii) the whole transcription unit of the lncRNA is embedded within an intron of a coding host gene, in the latter case they are termed ‘intronic’. Inversely, coding genes that are located within lncRNAs introns are called ‘overlapping’ genes [47, 50]. Both sense and antisense lncRNAs could comprise more than one exon [51, 52]. In addition to intergenic promoters, intragenic lncRNAs might be regulated by promoters that lie within gene bodies and therefore termed ‘internal’ promoters [46, 53, 54]. Expectedly, compared to lincRNAs that were extensively studied [55–58], only a small fraction of intronic lncRNAs are deeply explored due to the risk of disrupting the expression of their host genes [50]. In the mouse genome, around 87% of genes produce antisense transcripts [59], while 23% of human lncRNAs are produced from the antisense strands of coding genes [5], indicating an important functional role of antisense lncRNAs in regulating gene expression.

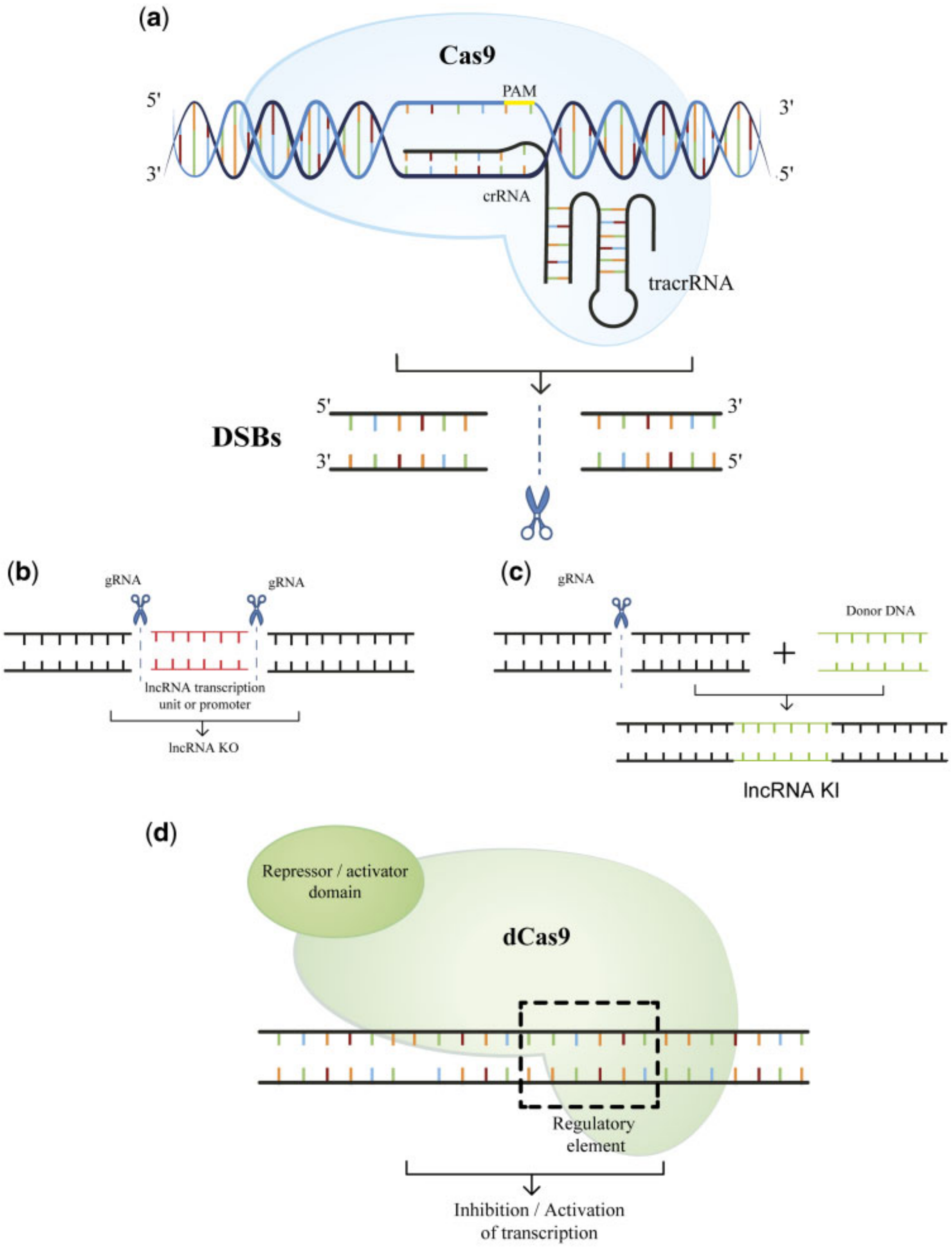

Figure 1: Classic CRISPR/Cas9 systems and editing of lncRNAs. (**a**) General scheme of the basic strategy of CRISPR-mediated double-stranded breaks. The guide RNA, which encompasses a scaffold tracrRNA bound to the Cas9 nuclease and a crRNA that recruits the gRNA–Cas9 complex to a specific genomic regions based on base complementarity. The PAM sequence (yellow) functions as a recognition and binding sequence for the Cas9 nuclease, resulting in a DSB in the genomic sequence; (**b** and **c**) are the two basic strategies often employed in lncRNA editing. (**b**) Knocking out the expression of lncRNAs by eliminating a large portion of their transcription unit and/or promoter (red), usually using multiple gRNAs. (**c**) Introducing a donor DNA sequence, usually a transcription inhibition signal, this leads eventually to knocking out lncRNA expression. (**d**) Deactivated Cas9 (dCas9) fused with either repression or activation domains. When recruited to promoter regions, dCas9 could sterically interfere with the binding of transcriptional activation factors causing transcription inhibition. In mammalian cells, however, dCas9 alone was not as effective in inhibiting transcription as in other cell types (reference). Thus, fusing dCas9 with repressor or activator domains yields a transcription repression/ activation complex that could effectively inhibit/ activate gene expression in mammalian cells. This strategy was utilized to manipulate the expression of lncRNAs both in a targeted manner and in a wide-scale screening format.

This intricate architecture of lncRNA genomic loci (outlined in Fig. 2), in addition to the enigmatic mechanisms of lncRNAs functions pose several limitations on the utilization of the CRISPR toolbox in the functional characterization of lncRNAs. For example, methods that rely on NHEJ for single base mutagenesis or knocking out expression by inducing small frame-shift mutations (e.g. CRISPRn, base editing) are generally not applicable to lncRNAs genes, because the exact sequence motifs responsible for exerting their effect remain largely uncharacterized. Besides, if the lncRNA of interest exert their effect by the act of transcription *per se*, such mutations will not affect its function. In the latter case, however, it might be useful to target regulatory elements such as promoters, although for most lncRNAs those remain to be identified. In addition, due to potential intersections of promoters, this could affect the expression of other coding or non-coding regions which might

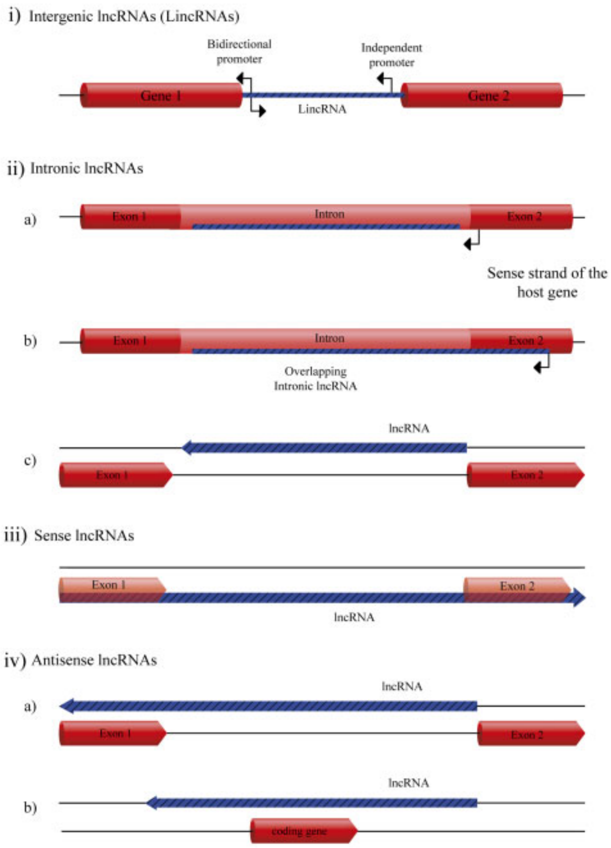

Figure 2: The complexity of lncRNA loci. (i) Intergenic lncRNAs (lincRNAs) arising from the non-coding regions between two coding genes. They could fall under the transcriptional control of independent promoters (i.e. exclusively control the lncRNA gene) or bidirectional (i.e. control both the lncRNA gene and an adjacent coding gene). (ii) Intronic lncRNAs arise from introns within coding genes. They are either completely intronic or overlapping one or more coding exons on the sense strand as in (**a**) and (**b**), respectively. Alternatively, intronic lncRNAs could arise from the antisense strands of introns as in (**c**). (iii) Sense lncRNAs. They come from the same strand as the coding exons, usually overlapping the whole length of a coding gene through one or more introns. (iv) Antisense lncRNAs could be either (a) partially overlapping their sense host gene, or (b) covering the whole length of the sense gene.

result in phenotypes falsely attributed to lncRNAs [47]. HDR-based CRISPR approaches could be used to knockout lncRNA by homology-directed insertion of a transcription termination signal or other destabilizing elements immediately downstream its transcription start site (TSS) [60, 61]. However, this strategy cannot be applied to lncRNAs whose expression is controlled by internal promoters or promoters proximal to other genes without potentially disrupting their sequence and expression. An added layer of complexity comes from the high frequency of sequence repeats within lncRNA loci [62], which could pose certain limitations on designing efficient gRNAs and shRNAs and is probably why RNAi and CRISPR are not so systematically used for the study of lncRNAs.

Functional lncRNA knockouts could be generated using CRISPRn excision, by either deleting the target lncRNAs promoters [61, 63–66] or deleting the entire lncRNA genes [61, 67]. However, excision of whole-length lncRNA would be inapplicable if the lncRNAs loci intersect with other coding or non-coding regions. Similarly, it would be impossible to delete lncRNA promoters if they are internal or bidirectional. In these cases, a good solution might be to partially delete selected lncRNA exons that do not intersect with other genes and that are distal from their promoters [17], although it remains a possibility that the undeleted part would still contain functional domain(s). Moreover, removing only a distal part of the lncRNA but not its TSS might result in generating a new transcript. Deleting large genomic regions in general could lead to inadvertently deleting uncharacterized regulatory DNA elements that influence the expression of other genes, potentially giving rise to phenotypes falsely attributed to lncRNAs [47, 60, 61, 68, 69].

Therefore, applying CRISPR methods to interrogate lncRNAs requires specific knowledge about their genomic locus, their impact on other genes and whether they exert this impact in *cis* or in *trans*. In a key genome-wide analysis by Goyal *et al.* [47], they set out to systematically assess the efficiency of targeting lncRNAs by different CRISPR methods. They found that 62% of the total lncRNAs were deemed 'non-CRISPRable', either due to having internal promoters (35%) or bidirectional promoters (20%). Furthermore, targeting 15 929 lncRNA loci by CRISPR applications was specific in only 38% of them, while almost two-thirds were susceptible to inadvertently affecting neighbouring genes. Together, these results demonstrated that the complex organization of lncRNA genomic loci could greatly limit the potential to target them experimentally in a specific manner. The study concluded, however, with three recommendations to ensure accurate attribution of resulting phenotype to the targeted lncRNA in CRISPR-mediated editing experiments: (i) careful examination of the targeted locus when designing the gRNAs to avoid potential perturbations to neighboring genes, (ii) monitoring the expression of the surrounding genes throughout the experiment alongside that of the targeted lncRNA and (iii) a validation step using RNAi or antisense oligonucleotides (ASOs) should follow and the outcome phenotype should match that of the CRISPR editing step. Alternatively, the knockout (KO) phenotype should be rescued by exogenous expression.

In addition to the limitations of Cas9 targeting, applying dCas9-based methods such as CRISPRa/i to lncRNAs could also be problematic. This was highlighted in a recent study that systematically compared the non-specific targeting and the subsequent discrepancies in gene expression profiles of RNAi, ASO-locked nucleic acids (LNA) and CRISPRi using the lncRNA MALAT1 as an illustrative example [70]. Although CRISPRi showed the fewest off-target effects, the three methods resulted in different sets of differentially expressed genes and cellular phenotypes upon depletion.

Despite these limitations, CRISPR approaches have been widely used to investigate lncRNAs functions in biological processes, under both physiological and disease conditions, either through gRNA libraries-based functional screening or through targeting individual lncRNAs for deeper understanding of their contributions to specific phenotypes. Over the past few years, more than 300 lncRNAs have been targets for studies that employ CRISPR methods according to CRISPRlnc, a database for manually curated lncRNA-targeting sgRNAs [71]. Beyond the aforementioned 'classic' CRISPR methods, several approaches have been tailored over the past years to suit the unique complexity of lncRNAs expression and function, and are utilized in lncRNA functional studies. This review highlights some of these adaptations and briefly discuss their applications in different areas of lncRNA functional studies such as tagging, manipulation of their expression and visualization, to name some examples. Finally, we share perspectives on using some of the newly

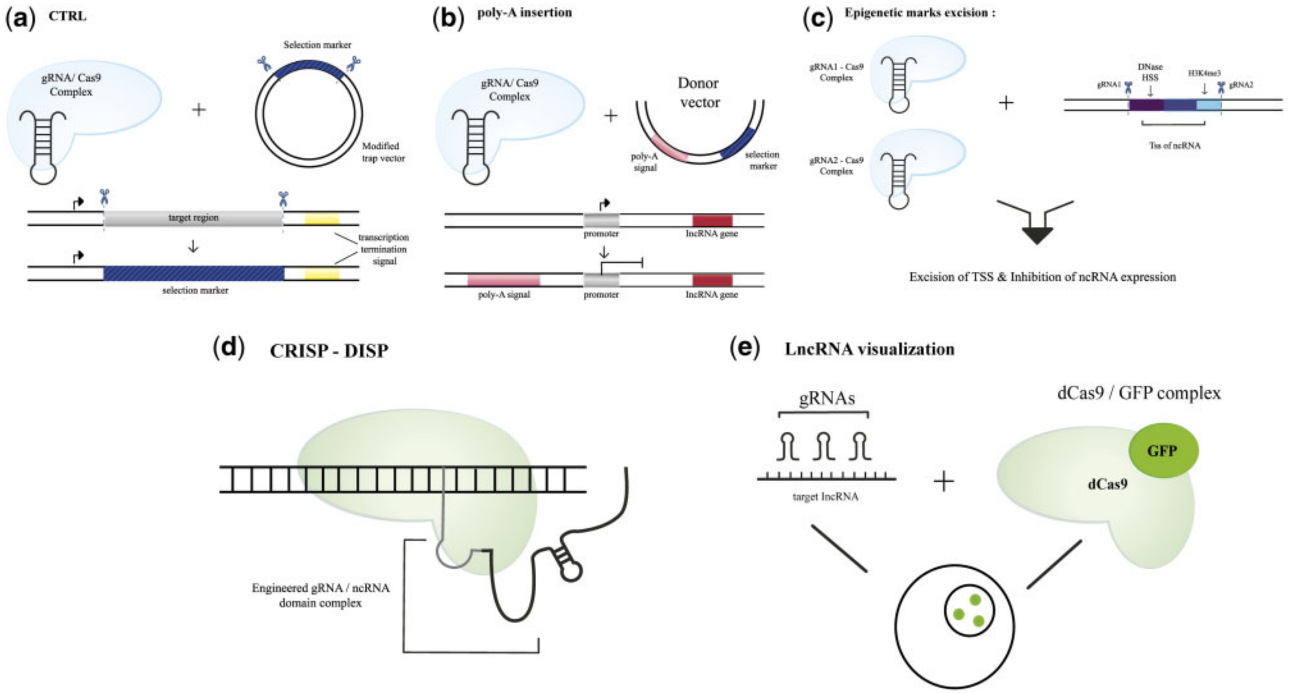

Figure 3: Different variations of CRISPR/Cas9 and their applications in lncRNA functional studies. (**a**) The CRISPR-mediated tagging and regulation of lncRNAs (CTRL) method, where deactivation of lncRNA expression and tagging with a selection marker are achieved simultaneously. (**b**) Inhibition of lncRNA expression by inserting a poly-A signal immediately upstream from its promoter. (**c**) Inhibition of lncRNA expression by Cas9-mediated excision of TSSs identified by the overlap between DNase hypersensitivity sites (HSS) and the active histone mark H3K4me3. (**d**) The CRSIPR display (CRISP-Disp) method, where the gRNA is fused to either whole ncRNAs or specific functional domains, and targeted to genomic regions to reveal the function of these ncRNAs/ domains in regulating gene expression. (**e**) Visualizing lncRNA by targeting with multiple gRNAs and a dCas9–GFP complex, followed by an imaging technique such as immunohistochemistry to enable imaging of the cell (e.g. imaging Xist).

emerging RNA-targeting CRISPR systems in the direct editing of lncRNAs.

Beyond classic editing of lncRNAs: innovative CRISPR approaches

Insertion of inhibitory and/or tagging signals

The complex arrangement of genomic regions of some lncRNAs requires alternative approaches to the direct deletion of either whole length or parts of the lncRNA gene bodies. Using CRISPR/ Cas9 system, silencing signals could be inserted in critical key regions along the lncRNA locus, thereby inhibiting its expression. Inserting a premature inhibitory signal, such as a polyadenylation (poly-A) site is an efficient and less invasive strategy to silence lncRNA expression that was applied to a number of lncRNAs in mice with considerable success [72–75]. This approach was recently utilized to silence the human lncRNA MALAT1, in addition to two coding genes [76].

The strategy is based on the biallelic integration of the poly-A signal into key sites of the target genomic regions via CRISPR/ Cas9-induced HDR (Fig. 3b). The authors employed a double marker selection to screen for clonal cells with the poly (A) signal successfully integrated. In the case of MALAT1, the poly (A) signal was inserted immediately upstream of its promoter region, resulting in efficient silencing and a sharp decrease in the MALAT1 transcript to 0.1% compared with the control cells. Interestingly, a recent study compared different CRISPR strategies of silencing lncRNAs in zebrafish concluded that the poly-A insertion was more efficient and led to complete inactivation of MALAT1 compared to deletion of either TSSs or highly-conserved sequence motifs, both resulted in attenuated expression due to usage of alternative TSSs and production of truncated transcripts, respectively [77].

Conversely, CRISPR-based targeted insertion through the NHEJ pathway in the absence of a homologous donor sequence was recently reported [30, 78], demonstrating the applicability of achieving targeted insertion with one universal vector. Gene-trap mutagenesis has been long established as an insertional mutagenesis strategy where gene inactivation and tagging of the disrupted gene with selection marker is achieved simultaneously [79]. The gene-trap method is based on hijacking the transcriptional regulatory elements of an endogenous gene to express both endogenous gene and the selection marker carried on the gene-trap vector and inserted at the insertion site (reviewed in [79] and [80]). The gene trap vectors usually contain a splice site upstream of a promoter-less reporter. Upon insertion, the *cis* regulatory elements of the trapped gene causes the expression of both the reporter and the trapped gene, resulting in the simultaneous inactivation of the targeted endogenous gene and the expression of a selection marker.

In a recent development, the CRISPR-mediated tagging and regulation of lncRNAs (CTRL) method was introduced to enable tagging and manipulating the expression of lncRNAs in mammalian cells [81]. The system in essence consists of a modified gene trap vector, which contains puromycin selection cassette and MS2 tagging sequence, and a plasmid that contains Cas9 and 2 sgRNA sequences (referred to as Cas9-2sgRNA), one is genome-targeting while the other is targeting the borders of the selection cassette thereby linearizing the trap vector (Fig. 3a). When introduced simultaneously to the mammalian cells, the selection cassette is inserted near the transcriptional termination sites of lncRNA genes inducing the expression of puromycin selection marker. At the same time, the insertion of an exogenous DNA fragment within the body of lncRNAs allows for examining the phenotype and the resulted functional disruption. For example, targeted insertion of puromycin cassette into transcription termination sites of the following six lncRNAs successfully upregulated their levels; HOX transcript antisense RNA (HOTAIR), taurine up-regulated 1 (TUG1), DICER1 antisense RNA 1 (DICER1-AS1), ZEB1 antisense RNA1 (ZEB1-AS1), PTENP1 and myocardial infarction-associated transcript (MIAT).

Interestingly, targeted insertion into the TSSs of these lncRNAs with a modified promoter trap vector that carries an exogenous poly-A signal resulted in the inhibition of only two lncRNAs (TUG1 and DICER1-AS1) and stimulation of the expression of the rest, highlighting the complexity of the regulatory circuits that govern the expression of lncRNAs. Nonetheless, the method enables successful tagging of the lncRNAs at either 5′or 3′ as well as monitoring their expression status. Taken together, the CTRL system represents a valuable tool for comprehensive analysis of lncRNA functions and could be utilized in a high-throughput format for future functional screening.

Engineering gRNAs for lncRNA relocation (CRISPR-display)

Strategies of gRNA engineering include fusing gRNAs with additional aptamer (e.g. MS2) or RNA scaffolds, for recruitment of additional effector proteins (e.g. VP64, KRAB and APOBEC) and enhanced manipulation of gene expression [82–85]. The pioneering CRISPR-Display (CRISP-Disp) method is a recent variation of the classic CRISPR system that combines both the modular nature of lncRNA [86] and the high feasibility of sgRNA re-engineering. The method employs the basics of the CRISPR technology to investigate the functional relevance of regulatory domains in a wide range of non-coding RNA molecules, including lncRNAs [87]. The method shows for the first time that, in the dCas9–gRNA complex, the gRNA sequence could be fused to large exogenous RNA domains up to 4.8 kb long, around the length of natural lncRNAs, without affecting the integrity and efficiency of the dCas9 targeting (Fig. 3d). Reporter gene activation was measured using two assays: direct activation, which involves expression of dCas9–VP64 fusion protein, and ‘bridged’ activation, with co-expression of dCas9 and a separate complex of the aptamer-binding protein PP7 coupled with VP64. The direct activation assay demonstrated the efficiency of the targeting mechanism, while the bridged activation showed that the fused RNA accessory domains retained their functional integrity and accessibility to protein binding partners. The method was then applied to lncRNAs, by fusing the RepA domain of the lncRNA Xist to a gRNA that targets a reporter gene. Xist is 17 kb in length and acts in *cis* to inactivate one of the X chromosomes by recruiting epigenetic ‘writer’ complexes that lay repressive histone marks on the Xist-coated X chromosome [88]. Consistent with the canonical Xist function, the authors reported an observable repression of the reporter gene, indicating the inhibitory function of the RepA domain and that this function is independent from the X chromosome context. As such, further experimentation could follow, such as immunoprecipitation and mass spectrometry, to identify proteins associated with functional lncRNA domains [89]. In addition, the study identified several fusion topologies that allow for engineering gRNA–ncRNA complexes while maintaining efficient targeting of dCas9. The innovative CRISP-Disp system enables repurposing and functional interrogation of either whole-length or partial domains of ncRNA, in addition to several potential applications in synthetic biology and structural characterization of ncRNAs [90]. It also allows for separately investigating the targeting and effector mechanisms of lncRNAs, and facilitates monitoring the transcription of a reported gene with concomitant imaging of another DNA locus, thus allowing for simultaneous analysis of several targets based on the available RNA motifs [91].

Epigenetic silencing of lncRNA

As discussed previously, precise knockout of lncRNAs through CRISPR-mediated frame-shift mutagenesis within genomic loci could be rather challenging. Instead, inhibiting the expression of lncRNA via targeting active histone marks is a plausible alternative. Modulating the epigenome is a well-established application of CRISPR where dCas9 is traditionally fused with an epigenetic effector protein (such as DNA methyltransferase or histone modifiers) and targeted to specific genomic loci (reviewed in [92]).

Recently, Janga *et al.* [93] developed for the first time a universal CRISPR-mediated knockout approach guided by epigenetic marks, that enables robust silencing of ncRNA loci through the excision of active histone marks (Fig. 3c). The method builds on previous work showing an overlap between the active mark histone H3 lysine 4 tri-methylation (H3K4me3) and DNase I hypersensitivity around the TSSs of most genes, and thus enables targeting loci that are poorly annotated [94]. Excision of TSS-associated active epigenetic signatures of ncRNAs genes successfully inhibited several microRNAs as well as the lncRNA MALAT1, which is constitutively transcribed in human monocytes. Although the results confirmed a role of miR-146a and miR-155 in regulating inflammatory response, they indicated no significant role of MALAT1. This epigenetically guided CRISPR-based approach represents a ncRNA knockout strategy with minimal alteration of the genomic sequence. By capitalizing on this approach, improved CRISPR libraries that alter the epigenetic code rather than the underlying sequences could be developed for systematic loss-of-function screenings of lncRNAs.

Visualization of lncRNAs

Fusion of DNA-binding proteins and fluorescent proteins (e.g. GFP) enables in principle imaging of specific genomic loci. Recently, several applications of CRISPR technology have enabled chromatin visualization via linking dCas9 to a fluorescent tag either through direct protein fusion or through an RNA scaffold. Using GFP–dCas9 fusion protein combined with an array of gRNAs tiled over a certain genomic locus enables visualization of non-repetitive genomic regions, a strategy that was used with several loci (reviewed in [95]). Similar to DNA imaging, RNA imaging is seemingly feasible using conventional methods such as fluorescent *in situ* hybridization (FISH) [96]. Nonetheless, efficient visualization of lncRNAs could be rather challenging with complementary FISH probes. The lncRNA XIST, for example, appeared as a low-resolution ‘cloud’, due to its considerably large size and complex secondary structure as well as the condensed nature of the surrounding repressed chromatin that limit the access of FISH probes [97]. Following the strategy of using dCas9–GFP fusion protein, combined with multiple sgRNAs and immunofluorescence, Wasko *et al.* were able to visualize the prototypical lncRNA Xist in female fibroblast cell line [98] (Fig. 3e). Expectedly, the images showed nuclear co-localization of both Xist and the repressive histone marks H3K27me3.

A different research direction is to exploit the specificity of gRNA targeting of genomic regions to label RNA. A recent study reported using such strategy to conduct an imaging-based pooled library screening using sgRNAs co-delivered to cells along with barcodes that are linked to reporter genes [99]. High-throughput identification of sgRNA followed using multiplexed error-robust fluorescence *in situ* hybridization (MERFISH) [100, 101] allowing for screening of RNA-binding protein. Notably, the screen revealed several modulators of the lncRNA MALAT1 localization in nuclear speckles, reflecting the potential application of this method to studies of the dynamic localization of lncRNAs to subcellular compartments as well as their interactions with other proteins.

Methods developed to visualize DNA and associated proteins *in vivo* are important to monitor the dynamic organization of chromatin within the cell nucleus. Similarly, live-cell imaging is crucial for RNA studies, albeit challenging because washing of unbound labeled probes could not be performed and hence it is necessary to increase the signal-to-background ratio. For that purpose, turn-on probes that fluoresce only upon intercalating with nucleic acids are more efficient, such as molecular beacons and forced intercalation (FIT) probes [102–104]. Alternatively, live-cell tracking and imaging of transgenic RNA is possible using MS2-MCP fluorescence system, which harnesses the specific binding between the phage MS2 coat protein (MCP) and the MS2 stem–loop RNA aptamer. The system in essence consists of GFP-tagged MCP protein in addition to multiple copies of the MCP-binding RNA aptamer, usually linked to the RNA of interest [105, 106]. Although the MS2-MCP fluorescence system was previously used in an attempt to visualize a transgenic Xist [107], it is obviously not efficient when visualizing endogenous lncRNAs. Another attempt took advantage of the sequence-specific RNA binding of the Pumilio homology domain (the PUF protein family) [108]. A Pumilio protein typically consists of eight domains, each specifically recognizes one nucleotide within an eight-nucleotide RNA sequence [109]. However, this approach is less common than MS2-MCP probably because engineering an efficient construct is rather laborious and time-consuming, limiting its potential applications in analyzing large transcripts or scaling up the techniques to transcriptome-wide studies.

Cas9 has been reportedly effective in targeting ssRNA with high specificity when combined with PAM-presenting PAM/RNA hybrid oligonucleotides (PAMmers). This RNA-targeting Ca9 (RCas9) system is a recent modification of the classic CRISPR/Cas9 system, where the PAM sequence is introduced *in vitro* as part of the PAMmer oligonucleotide [110]. A mismatch in the PAMmer allows for exclusive recognition of the target RNA and not the genomic DNA [111]. Capitalizing on this strategy, Nelles *et al.* [112] successfully used RCas9 to visualize and track RNA in live cells, by fusing dCas9 to a fluorescent protein (mCherry or GFP), a targeting sgRNA and a nuclear localization signal in addition to an exogenous PAMmer oligonucleotide. Although only mRNAs were tracked in that study, it would be interesting to explore potential applications of RCas9 in live-cell tracking and visualizing of lncRNAs. Nonetheless, proper visualization of lncRNAs might require using multiple copies of the RCas9–sgRNA complex in order to cover the whole length of molecule.

Perspectives on post-transcriptional editing of lncRNAs using CRISPR systems

Due to the aforementioned limitations of Cas9-based genomic editing, a plausible alternative might be to edit RNA molecules post-transcriptionally. Functional studies of lncRNAs often depend on loss-of-function or gain-of-function methods, whether in cell cultures or in animal models. RNA interference (RNAi) has traditionally been the method of choice to manipulate gene expression post-transcriptionally [113, 114]. Although RNAi has been efficiently utilized to deplete coding transcripts [115], it showed limited efficiency when used to manipulate ncRNA expression [116]. RNAi, which depends on short double-stranded RNA sequence to target and silence expression of target RNAs [117], had been utilized for functional studies of lncRNAs [47]. Nonetheless, there are several shortcomings for using RNAi to study lncRNAs; firstly, unlike mRNA, lncRNAs are mostly nuclear [118], and although the RNAi machinery was found to function in the nucleus [119], short interfering RNAs (siRNAs) designed to target nuclear lncRNAs showed limited efficiency [120]. Secondly, the function of some lncRNAs can be transcription-coupled, meaning that the mere act of transcription is responsible for exerting their function rather than the transcript itself [8, 121]. Lastly, some lncRNAs (e.g. MALAT1) are expressed at high levels rendering RNAi targeting and inhibition insufficient to produce a complete loss of function [122]. Alternative to RNAi, other posttranscriptional silencing approaches, such as RNA targeting and cleaving via antisense oligonucleotides (ASOs)-directed RNase H activity, can target nuclear lncRNAs with rather high success [120] and even deplete nascent transcripts [53, 123], although they could efficiently be used for only a fraction of lncRNAs. A major drawback for RNA-targeting methods such as RNAi and ASOs is that their effects remain transient. Thus, to achieve long-term silencing, functional studies of lncRNAs still required alternative approaches that act on the genomic level. Although short RNAs were successfully used to achieve stable transcriptional gene silencing (TGS) via targeting proximal regions of genes [124, 125], only a few examples of RNA-induced TGS of lncRNA were reported [126, 127].

The RCas9 system was effective in targeting and cleaving single-stranded RNA (ssRNA) *in vitro* as shown by recent reports [110]. As in the DNA targeting CRISPRa/i system, fusing dCAS9 to protein effectors could allow applications of the RCas9 system in RNA studies (reviewed in [128]). In the context of lncRNAs, fusing dCas9 with protein effectors known to interfere with a certain lncRNA could be a method of fine-tuning their level of expression. For example, fusing dCas9 with lncRNA-stabilizing signals may increase their half-life inside cells. It could be fused with epigenetic effectors to investigate the function of lncRNAs in the epigenetic modulation of gene expression. Engineering gRNA by fusing it with either whole lncRNAs or specific structural domains could represent another layer of customizing the RCas9 system to lncRNA interrogation. The aforementioned are few examples, although one could only imagine a myriad of applications for RNA-targeting CRISPR technology. Nonetheless, the off-target effects of Rcas9 are yet to be evaluated, and extensive validation is needed before it could be used in *in vivo* applications.

The newly emerging type VI CRISPR-Cas systems that recognize and, upon activation, degrade ssRNAs were recently discovered in bacteria as an adaptive immunity means [129–131]. The unique effector protein of the type VI-A CRISPR systems, Cas13 (formerly C2c2), had been recently adapted into multiple applications including nucleic acid detection [132], plant and mammalian RNA knockdown and tracking [133, 134]. In addition, a catalytically inactive form of *Prevotella sp.* Cas13b (PspCas13b) was fused with the deaminase domain (ADAR2_{DD}) of the adenosine deaminase acting on RNA (ADAR) enzymes, and efficiently used in RNA base editing, a system that was referred to as RNA Editing for Programmable A to I Replacement

(REPAIR) [135]. Although the aforementioned RNA-targeting CRISPR systems were only used to target coding transcripts, it would be interesting to explore their applicability to edit lncRNAs which, if successful, could represent an attractive tool for dissecting the functions of lncRNAs with rather high specificity, as well as enable structural probing at a single nucleotide resolution. Alternatively, the type VI effectors could be adapted for high-throughput functional screens for lncRNAs (2). On the other hand, the recent development of CRISPR/Cas9 splicing manipulation tools, such as CRISPR-SKIP [136] and TAM [137], that are based on single base editing via cytidine deaminase, could enable the functional identification of lncRNA splice variants [138]. It will probably require combining multiple tools in order to fully elucidate lncRNA function.

Conclusion

Although classic methods of CRISPR editing could be efficient when studying some lncRNAs, the intricate architecture of lncRNA genes and their transcriptional regulatory circuits require innovative approaches for their functional studies. Several innovative adaptations to the classic CRISPR system are emerging and are being widely applied in various aspects of lncRNA functional studies such as tagging and expression manipulation and visualization of lncRNAs. In addition to targeting lncRNAs on the genomic level, newly emerging CRISPR methods that directly target RNA could facilitate presumptive direct editing and/or manipulation of lncRNAs, opening the door to a myriad of applications in the study of both the functional and structural aspects of lncRNA biology.

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References

1. Ohno S. An argument for the genetic simplicity of man and other mammals. *J Hum Evol* 1972; **1**:651–62.
2. Montalbano A, Canver MC, Sanjana NE. High-throughput approaches to pinpoint function within the noncoding genome. *Molecular Cell* 2017;**68**:44–59.
3. ENCODE Project Consortium TEP, Consortium TEP. An integrated encyclopedia of DNA elements in the human genome. *Nature* 2012;**489**:57–74.
4. Roadmap Epigenomics Consortium, Kundaje A, Meuleman W, Ernst J *et al*. Integrative analysis of 111 reference human epigenomes. *Nature* 2015;**518**:317–29.
5. Derrien T, Johnson R, Bussotti G *et al*. The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression. *Genome Res* 2012;**22**: 1775–89.
6. Carninci P, Kasukawa T, Katayama S *et al*. The transcriptional landscape of the mammalian genome. *Science* 2005; **309**:1559–63.
7. Wang KC, Chang HY. Molecular mechanisms of long noncoding RNAs. *Molecular Cell* 2011;**43**:904–14.
8. Kornienko AE, Guenzl PM, Barlow DP, Pauler FM. Gene regulation by the act of long non-coding RNA transcription. *BMC Biol* 2013;**11**:59.
9. Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet* 2016;**17**:47–62.
10. Harrow J, Frankish A, Gonzalez JM *et al*. GENCODE: the reference human genome annotation for the ENCODE project. *Genome Res* 2012;**22**:1760–74.
11. Joung J, Engreitz JM, Konermann S *et al*. Genome-scale activation screen identifies a lncRNA locus regulating a gene neighbourhood. *Nature* 2017;**548**:343–6.
12. Le Provost F, Lillico S, Passet B *et al*. Zinc finger nuclease technology heralds a new era in mammalian transgenesis. *Trends Biotechnol* 2010;**28**:134–41.
13. Oost JVD. New tool for genome surgery. *Science* 2013;**339**: 768–70.
14. Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc Natl Acad Sci* 1996;**93**:1156–60.
15. Wright DA, Li T, Yang B, Spalding MH. TALEN-mediated genome editing: prospects and perspectives. *Biochem J* 2014; **462**:15–24.
16. Gutschner T. Silencing long noncoding RNAs with genome-editing tools. In: Pruett-Miller S (ed.), *Chromosomal Mutagenesis. Methods in Molecular Biology (Methods and Protocols)*. *1239*. New York: Humana Press, 2015, 241–50.
17. Sauvageau M, Goff LA, Lodato S *et al*. Multiple knockout mouse models reveal lincRNAs are required for life and brain development. *Elife* 2013;**2**:e01749.
18. Baliou S, Adamaki M, Kyriakopoulos AM *et al*. Role of the CRISPR system in controlling gene transcription and monitoring cell fate. *Mol Med Rep* 2018;**17**:1421–7.
19. Bhaya D, Davison M, Barrangou R. CRISPR-Cas systems in Bacteria and Archaea: versatile small RNAs for adaptive defense and regulation. *Annu Rev Genet* 2011;**45**:273–97.
20. Horvath P, Barrangou R. CRISPR/Cas, the immune system of Bacteria and Archaea. *Science* 2010;**327**:167–70.
21. Wang H, La Russa M, Qi LS. CRISPR/Cas9 in genome editing and beyond. *Annu Rev Biochem* 2016;**85**:227–64.
22. Jinek M, Chylinski K, Fonfara I *et al*. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012;**337**:816–21.
23. Mali P, Yang L, Esvelt KM *et al*. RNA-guided human genome engineering via Cas9. *Science* 2013;**339**:823–6.
24. Shalem O, Sanjana NE, Hartenian E *et al*. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 2014;**343**:84–7.
25. Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells using the CRISPR-Cas9 system. *Science* 2014; **343**:80–4.
26. Korkmaz G, Lopes R, Ugalde AP *et al*. Functional genetic screens for enhancer elements in the human genome using CRISPR-Cas9. *Nat Biotechnol* 2016;**34**:192–8.
27. Iyama T, Wilson DM. DNA repair mechanisms in dividing and non-dividing cells. *DNA Repair* 2013;**12**:620–36.
28. Orthwein A, Noordermeer SM, Wilson MD *et al*. A mechanism for the suppression of homologous recombination in G1 cells. *Nature* 2015;**528**:422–6.
29. Suzuki K, Izpisua Belmonte JC. In vivo genome editing via the HITI method as a tool for gene therapy. *J Hum Genet* 2018;**63**:157–64.
30. Suzuki K, Tsunekawa Y, Hernandez-Benitez R *et al*. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature* 2016;**540**:144–9.
31. Auer TO, Duroure K, De Cian A, *et al*. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res* 2014;**24**:142–53.

32. Yang L, Mali P, Kim-Kiselak C, Church G. CRISPR-Cas-mediated targeted genome editing in human cells. *Methods Mol Biol* 2014;**1114**:245–67.
33. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 2014;**157**:1262–78.
34. Doetschman T, Georgieva T. Gene editing with CRISPR/Cas9 RNA-directed nuclease. *Circ Res* 2017;**120**:876–94.
35. Gilbert LA, Horlbeck MA, Adamson B *et al*. Genome-scale CRISPR-mediated control of gene repression and activation. *Cell* 2014;**159**:647–61.
36. Cong L, Ran FA, Cox D *et al*. Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013;**339**:819–23.
37. Ran FA, Hsu PD, Wright J *et al*. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 2013;**8**:2281–308.
38. Wang H, Yang H, Shivalila CS *et al*. One-step generation of mice carrying mutations in multiple genes by CRISPR/cas-mediated genome engineering. *Cell* 2013;**153**:910–8.
39. Xiao A, Wang Z, Hu Y *et al*. Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Res* 2013;**41**:e141.
40. Essletzbichler P, Konopka T, Santoro F *et al*. Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line. *Genome Res* 2014;**24**:2059–65.
41. Qi LS, Larson MH, Gilbert LA *et al*. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 2013;**152**:1173–83.
42. Gilbert LA, Larson MH, Morsut L *et al*. XCRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013;**154**:442–51.
43. Maeder ML, Linder SJ, Cascio VM *et al*. CRISPR RNA-guided activation of endogenous human genes. *Nat Methods* 2013;**10**:977–9.
44. Perez-Pinera P, Kocak DD, Vockley CM *et al*. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat Methods* 2013;**10**:973–6.
45. Chavez A, Scheiman J, Vora S *et al*. Highly efficient Cas9-mediated transcriptional programming. *Nat Methods* 2015;**12**:326–8.
46. Sigova AA, Mullen AC, Molinie B *et al*. Divergent transcription of long noncoding RNA/mRNA gene pairs in embryonic stem cells. *Proc Natl Acad Sci USA* 2013;**110**:2876–81.
47. Goyal A, Myacheva K, Groß M *et al*. Challenges of CRISPR/Cas9 applications for long non-coding RNA genes. *Nucleic Acids Res* 2017;**45**:e12.
48. Lander ES, Linton LM, Birren B *et al*. Initial sequencing and analysis of the human genome. *Nature* 2001;**409**:860–921.
49. Venter JC, Adams MD, Myers EW *et al*. The sequence of the human genome. *Science* 2001;**291**:1304–51.
50. Ma L, Bajic VB, Zhang Z. On the classification of long non-coding RNAs. *RNA Biol* 2013;**10**:924–33.
51. Zhang X, Rice K, Wang Y *et al*. Maternally expressed gene 3 (MEG3) noncoding ribonucleic acid: isoform structure, expression, and functions. *Endocrinology* 2010;**151**:939–47.
52. Guttman M, Garber M, Levin JZ *et al*. Ab initio reconstruction of cell type-specific transcriptomes in mouse reveals the conserved multi-exonic structure of lincRNAs. *Nat Biotechnol* 2010;**28**:503–10.
53. Luo S, Lu JY, Liu L *et al*. Divergent lncRNAs regulate gene expression and lineage differentiation in pluripotent cells. *Cell Stem Cell* 2016;**18**:637–52.
54. Hu HY, He L, Khaitovich P. Deep sequencing reveals a novel class of bidirectional promoters associated with neuronal genes. *BMC Genomics* 2014;**15**:457.
55. Huarte M, Guttman M, Feldser D *et al*. A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. *Cell* 2010;**142**:409–19.
56. Guttman M, Amit I, Garber M, French C *et al*. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* 2009;**458**:223–7.
57. Prensner JR, Iyer MK, Balbin OA *et al*. Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. *Nat Biotechnol* 2011;**29**:742–9.
58. Ørom UA, Derrien T, Beringer M *et al*. Long noncoding RNAs with enhancer-like function in human cells. *Cell* 2010;**143**:46–58.
59. Katayama S, Tomaru Y, Kasukawa T *et al*. Molecular biology: antisense transcription in the mammalian transcriptome. *Science* 2005;**309**:1564–6.
60. Paralkar VR, Taborda CC, Huang P *et al*. Unlinking an lncRNA from its associated cis element. *Mol Cell* 2016;**62**:104–10.
61. Yin Y, Yan P, Lu J *et al*. Opposing roles for the lncRNA haunt and its genomic locus in regulating HOXA gene activation during embryonic stem cell differentiation. *Cell Stem Cell* 2015;**16**:504–16.
62. Kapusta A, Kronenberg Z, Lynch VJ *et al*. Transposable elements are major contributors to the origin, diversification, and regulation of vertebrate long noncoding RNAs. *PLoS Genet* 2013;**9**:e1003470.
63. Han J, Zhang J, Chen L *et al*. Efficient in vivo deletion of a large imprinted lncRNA by CRISPR/Cas9. *RNA Biol* 2014;**11**:829–35.
64. Aparicio-Prat E, Arnan C, Sala I *et al*. DECKO: single-oligo, dual-CRISPR deletion of genomic elements including long non-coding RNAs. *BMC Genomics* 2015;**16**:846–61.
65. Deng C, Li Y, Zhou L *et al*. HoxBlinc RNA recruits Set1/MLL complexes to activate Hox gene expression patterns and mesoderm lineage development. *Cell Rep* 2016;**14**:103–14.
66. Welsh IC, Kwak H, Chen FL *et al*. Chromatin architecture of the Pitx2 locus requires CTCF- and Pitx2-dependent asymmetry that mirrors embryonic gut laterality. *Cell Rep* 2015;**13**:337–49.
67. Durruthy-Durruthy J, Sebastiano V, Wossidlo M *et al*. The primate-specific noncoding RNA HPAT5 regulates pluripotency during human preimplantation development and nuclear reprogramming. *Nat Genet* 2016;**48**:44–52.
68. Groff AF, Sanchez-Gomez DB, Soruco MML *et al*. In vivo characterization of linc-p21 reveals functional cis-regulatory DNA elements. *Cell Rep* 2016;**16**:2178–86.
69. Bassett AR, Akhtar A, Barlow DP *et al*. Considerations when investigating lncRNA function in vivo. *Elife* 2014;**3**:1–14.
70. Stojic L, Lun ATL, Mangei J *et al*. Specificity of RNAi, LNA and CRISPRi as loss-of-function methods in transcriptional analysis. *Nucleic Acids Res* 2018;**46**:5950–66.
71. Chen W, Zhang G, Li J *et al*. CRISPRlnc: a manually curated database of validated sgRNAs for lncRNAs. *Nucleic Acids Res* 2019;**47**:D63–8.
72. Bond AM, Vangompel MJW, Sametsky EA *et al*. Balanced gene regulation by an embryonic brain ncRNA is critical for adult hippocampal GABA circuitry. *Nat Neurosci* 2009;**12**:1020–7.
73. Grote P, Wittler L, Hendrix D *et al*. The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. *Dev Cell* 2013;**24**:206–14.
74. Anderson KM, Anderson DM, McAnally JR *et al*. Transcription of the non-coding RNA upperhand controls

Hand2 expression and heart development. *Nature* 2016;**539**: 433–6.

75. Ballarino M, Cipriano A, Tita R *et al*. Deficiency in the nuclear long noncoding RNA Charme causes myogenic defects and heart remodeling in mice. *EMBO J* 2018;**37**: e99697.
76. Liu Y, Han X, Yuan J *et al*. Biallelic insertion of a transcriptional terminator via the CRISPR/Cas9 system efficiently silences expression of protein-coding and non-coding RNA genes. *J Biol Chem* 2017;**292**:5624–33.
77. Lavalou P, Eckert H, Damy L *et al*. Strategies for genetic inactivation of long noncoding RNAs in zebrafish. *RNA* 2019;**25**: 897. 069484.118.
78. Schmid-Burgk JL, Höning K, Ebert TS, Hornung V. CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism. *Nat Commun* 2016;**7**:12338.
79. Stanford WL, Cohn JB, Cordes SP. Gene-trap mutagenesis: past, present and beyond. *Nat Rev Genet* 2001;**2**:756–68.
80. Friedel RH, Soriano P. Gene trap mutagenesis in the mouse. *Meth Enzymol* 2010;**77**:243–69.
81. Cheng TL, Qiu Z. Long non-coding RNA tagging and expression manipulation via CRISPR/Cas9-mediated targeted insertion. *Protein Cell* 2018;**9**:820–5.
82. Dahlman JE, Abudayyeh OO, Joung J *et al*. Orthogonal gene knockout and activation with a catalytically active Cas9 nuclease. *Nat Biotechnol* 2015;**33**:1159–61.
83. Zalatan JG, Lee ME, Almeida R *et al*. Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell* 2015;**160**:339–50.
84. Hess GT, Frésard L, Han K *et al*. Directed evolution using dCas9-targeted somatic hypermutation in mammalian cells. *Nat Methods* 2016;**13**:1036–42.
85. Kweon J, Jang AH, Kim DE *et al*. Fusion guide RNAs for orthogonal gene manipulation with Cas9 and Cpf1. *Nat Commun* 2018;**9**:303.
86. Guttman M, Rinn JL. Modular regulatory principles of large non-coding RNAs. *Nature* 2012; **482**:339–46.
87. Shechner DM, Hacisuleyman E, Younger ST, Rinn JL. Multiplexable, locus-specific targeting of long RNAs with CRISPR-Display. *Nat Methods* 2015;**12**:664–70.
88. Zhao J, Sun BK, Erwin JA *et al*. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science* 2008;**322**:750–6.
89. Perez-Pinera P, Jones MF, Lal A, Lu TK. Putting non-coding RNA on display with CRISPR. *Mol Cell* 2015;**59**:146–8.
90. Lin PC, Corn JE. Co-opting CRISPR to deliver functional RNAs. *Nat Methods* 2015;**12**:613–4.
91. Plummer RJ, Guo Y, Peng Y. A CRISPR reimagining: new twists and turns of CRISPR beyond the genome-engineering revolution. *J Cell Biochem* 2018;**119**:1299–308.
92. Pulecio J, Verma N, Mejía-Ramírez E *et al*. CRISPR/Cas9-based engineering of the epigenome. *Cell Stem Cell* 2017;**21**:431–47.
93. Janga H, Aznaourova M, Boldt F *et al*. Cas9-mediated excision of proximal DNaseI/H3K4me3 signatures confers robust silencing of microRNA and long non-coding RNA genes. *PLoS One* 2018;**13**:e0193066.
94. Thurman RE, Rynes E, Humbert R *et al*. The accessible chromatin landscape of the human genome. *Nature* 2012;**489**: 75–82.
95. Wu X, Mao S, Ying Y *et al*. Progress and challenges for live-cell imaging of genomic loci using CRISPR-based platforms. *Genomics, Proteomics and Bioinformatics* 2019;**17**:119–28.
96. Larsson C, Grundberg I, Söderberg O, Nilsson M. In situ detection and genotyping of individual mRNA molecules. *Nat Methods* 2010;**7**:395–7.
97. Clemson CM, McNeil JA, Willard HF, Lawrence JB. XIST RNA paints the inactive X chromosome at interphase: evidence for a novel RNA involved in nuclear/chromosome structure. *J Cell Biol* 1996;**132**:259–75.
98. Waśko U, Zheng Z, Bhatnagar S. Visualization of Xist long noncoding RNA with a fluorescent CRISPR/Cas9 system. *Methods Mol Biol* 2019;**1870**:41–50.
99. Wang C, Lu T, Emanuel G *et al*. Imaging-based pooled CRISPR screening reveals regulators of lncRNA localization. *Proc Natl Acad Sci USA* 2019;**166**:10842–51.
100. Moffitt JR, Hao J, Wang G *et al*. High-throughput single-cell gene-expression profiling with multiplexed error-robust fluorescence in situ hybridization. *Proc Natl Acad Sci USA* 2016;**113**:11046–51.
101. Moffitt JR, Zhuang X. RNA imaging with multiplexed error-robust fluorescence in situ hybridization (MERFISH). *Methods Enzymol* 2016;**572**:1–49.
102. Sokol DL, Zhang X, Lu P, Gewirtz AM. Real time detection of DNA·RNA hybridization in living cells. *Proc Natl Acad Sci* 1998;**95**:11538–43.
103. Köhler O, Jarikote DV, Seitz O. Forced intercalation probes (FIT Probes): Thiazole orange as a fluorescent base in peptide nucleic acids for homogeneous single-nucleotide-polymorphism detection. *Chem Bio Chem* 2005;**6**:69–77.
104. Hövelmann F, Gaspar I, Ephrussi A, Seitz O. Brightness enhanced DNA FIT-probes for wash-free RNA imaging in tissue. *J Am Chem Soc* 2013;**135**:19025–32.
105. Fouts D. Functional recognition of fragmented operator sites by R17/MS2 coat protein, a translational repressor. *Nucleic Acids Res* 1997;**25**:4464–73.
106. Bertrand E, Chartrand P, Schaefer M *et al*. Localization of ASH1 mRNA particles in living yeast. *Mol Cell* 1998;**2**:437–45.
107. Ng K, Daigle N, Bancaud A *et al*. A system for imaging the regulatory noncoding Xist RNA in living mouse embryonic stem cells. *MBoC* 2011;**22**:2634–45.
108. Ha N, Lai LT, Chelliah R *et al*. Live-cell imaging and functional dissection of Xist RNA reveal mechanisms of X chromosome inactivation and reactivation. *iScience* 2018;**8**:1–14.
109. Ozawa T, Natori Y, Sato M, Umezawa Y. Imaging dynamics of endogenous mitochondrial RNA in single living cells. *Nat Methods* 2007;**4**:413–9.
110. O'Connell MR, Oakes BL, Sternberg SH *et al*. Programmable RNA recognition and cleavage by CRISPR/Cas9. *Nature* 2014; **516**:263–6.
111. Sternberg SH, Redding S, Jinek M *et al*. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* 2014; **507**:62–7.
112. Nelles DA, Fang MY, O'Connell MR *et al*. Programmable RNA tracking in live cells with CRISPR/Cas9. *Cell* 2016;**165**:488–96.
113. Tuschl T, Zamore PD, Lehmann R *et al*. Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev* 1999; **13**:3191–7.
114. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 2000;**101**:25–33.
115. Barrangou R, Birmingham A, Wiemann S *et al*. Advances in CRISPR-Cas9 genome engineering: lessons learned from RNA interference. *Nucleic Acids Res* 2015;**43**:3407–19.
116. Rna N, Fatica A, Bozzoni I. Long non-coding RNAs: new players in cell differentiation and development. *Nat Rev Genet* 2014;**15**:7–21.
117. Elbashir SM, Harborth J, Lendeckel W *et al*. Duplexes of 21 ± nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;**411**:494–8.

118. Cabili MN, Dunagin MC, McClanahan PD *et al*. Localization and abundance analysis of human lncRNAs at single-cell and single-molecule resolution. *Genome Biol* 2015;**16**:20.
119. Gagnon KT, Li L, Chu Y *et al*. RNAi factors are present and active in human cell nuclei. *Cell Rep* 2014;**6**:211–21.
120. Lennox KA, Behlke MA. Cellular localization of long noncoding RNAs affects silencing by RNAi more than by antisense oligonucleotides. *Nucleic Acids Res* 2016;**44**:863–77.
121. Engreitz JM, Haines JE, Perez EM *et al*. Local regulation of gene expression by lncRNA promoters, transcription and splicing. *Nature* 2016;**539**:452–5.
122. Gutschner T, Baas M, Diederichs S. Noncoding RNA gene silencing through genomic integration of RNA destabilizing elements using zinc finger nucleases. *Genome Res* 2011;**21**: 1944–54.
123. Vickers TA, Koo S, Bennett CF *et al*. Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. *J Biol Chem* 2003; **278**:7108–18.
124. Morris KV, Chan SW, Jacobsen SE, Looney DJ. Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* 2004;**305**:1289–92.
125. Weinberg MS, Morris KV. Transcriptional gene silencing in humans. *Nucleic Acids Res* 2016;**44**:6505–17.
126. Stojic L, Niemczyk M, Orjalo A *et al*. Transcriptional silencing of long noncoding RNA GNG12-AS1 uncouples its transcriptional and product-related functions. *Nat Commun* 2016;**7**:10406.
127. Golding MC, Magri LS, Zhang L *et al*. Depletion of Kcnq1ot1 non-coding RNA does not affect imprinting maintenance in stem cells. *Development* 2011;**138**:3667–78.
128. Nelles DA, Fang MY, Aigner S, Yeo GW. Applications of Cas9 as an RNA-programmed RNA-binding protein. *BioEssays* 2015;**37**:732–9.
129. Abudayyeh OO, Gootenberg JS, Konermann S *et al*. C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* 2016;**353**:aaf5573.
130. East-Seletsky A, O'Connell MR, Knight SC *et al*. Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. *Nature* 2016;**538**:270–3.
131. Smargon AA, Cox DBT, Pyzocha NK *et al*. Cas13b is a type VI-B CRISPR-associated RNA-guided RNase differentially regulated by accessory proteins Csx27 and Csx28. *Mol Cell* 2017; **65**:618–30.
132. Gootenberg JS, Abudayyeh OO, Lee JW *et al*. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* 2017;**356**: 438–42.
133. Abudayyeh OO, Gootenberg JS, Essletzbichler P *et al*. RNA targeting with CRISPR-Cas13. *Nature* 2017;**550**:280–4.
134. East-Seletsky A, O'Connell MR, Burstein D *et al*. RNA targeting by functionally orthogonal type VI-A CRISPR-Cas enzymes. *Mol Cell* 2017;**66**:373–83.
135. Cox DBT, Gootenberg JS, Abudayyeh OO *et al*. RNA editing with CRISPR-Cas13. *Science* 2017;**358**:1019–27.
136. Gapinske M, Luu A, Winter J *et al*. CRISPR-SKIP: programmable gene splicing with single base editors. *Genome Biol* 2018; **19**:107.
137. Yuan J, Ma Y, Huang T *et al*. Genetic modulation of RNA splicing with a CRISPR-guided cytidine deaminase. *Mol Cell* 2018;**72**:380–94.
138. Sen R, Doose G, Stadler P. Rare splice variants in long noncoding RNAs. *ncRNA* 2017;**3**:23.