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Modulating the expression of long non-coding RNAs for functional studies

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Abstract

Long non-coding RNAs (lncRNAs) have emerged as important regulators of cell biology. The mechanisms by which lncRNAs function are likely numerous, and most are poorly understood. Currently, the mechanisms of functional lncRNAs include those that directly involve the lncRNA transcript, the process of their own transcription and splicing, and even underlying transcriptional regulatory elements within the genomic DNA that encodes the lncRNA. As our understanding of lncRNA biology evolves, so have the methods that are utilized to elucidate their functions. In this review, we survey a collection of different methods used to modulate lncRNA expression levels for the assessment of biological function. From RNA-targeted strategies, genetic deletions, to engineered gene regulatory systems, the advantages and caveats of each method will be discussed. Ultimately, the selection of tools will be guided by which potential lncRNA mechanisms are being investigated, and no single method alone will likely be sufficient to reveal the function of any particular lncRNA.

Keywords CRISPR; expression; lncRNA; methods

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See the Glossary for abbreviations used in this article.

Introduction

After completion of the human genome project—which revealed that only approximately 2% of our DNA code for proteins—the advent of next-generation sequencing technologies enabled the surprising discovery that a substantial proportion of our non-coding genome is transcribed into RNA. Bioinformatic studies have annotated tens of thousands of lncRNAs—transcripts > 200 nt in length that do not appear to code for proteins—and such lncRNAs can be mapped to every chromosome [1–7]. While certain lncRNAs are now known to play key roles in critical cellular processes [1–4]—such as lncRNA *XIST* in X chromosome inactivation [6–8]—the vast majority of lncRNAs have not been demonstrated to have significant biological functions. For this emerging field of research, an important next step is to identify which lncRNAs regulate important aspects of cell and molecular biology, and lncRNA loss-of-function and gain-of-function approaches are a mainstay for such discovery.

Broadly, the methods used to manipulate the levels of an RNA transcript involve either alterations at the level of the corresponding genomic DNA (e.g., gene modification or local recruitment of transcriptional regulators) or molecular strategies that directly involve the RNA transcript (e.g., RNA knockdown or transfection of RNA molecules). Importantly, not all functional lncRNA loci exert their biological effects through the transcribed RNA molecule itself. While some lncRNA loci do indeed function in *trans,* producing a lncRNA transcript that functions at locations genetically unlinked and spatially distant from their site of production (e.g., *NORAD, HOTAIR,* [5,6]) (Fig 1A), other lncRNA loci regulate gene expression in *cis* (Fig 1B), having transcriptional enhancer-like function for genes on the same chromosome (e.g., *Blustr, linc-p21* [7–9]). Both the process of lncRNA transcription as well as transcript splicing can regulate the expression of a protein coding gene neighbor [7]. While the level of lncRNA expression may predict biological function within a particular cell type [10,11], lncRNA loci can even have enhancer-like function in the absence of transcription [8]. Furthermore, for lncRNA loci known to function in *cis*, its lncRNA transcripts can have additional biological functions in *trans* [12]. Given the diversity of currently known lncRNA mechanisms (and also those still yet to be discovered), the tools used for functional studies should be carefully considered in the context of how the lncRNA may function.

Elucidating lncRNA function is also complicated by the genomic arrangement of lncRNAs. Many lncRNAs overlap with coding genes (both in the sense and in the antisense directions) [13], making it often difficult to genetically disrupt the lncRNA without affecting local coding genes. As alluded to above, some lncRNA loci map to known enhancers, which similarly complicates experimental approaches and interpretations of results. Because there are relatively few lncRNAs known to have important functions—even fewer with described molecular mechanisms—and the relative lack of evolutionary conservation [14,15], the function of lncRNA loci cannot yet be predicted from primary sequences. Furthermore, although the expression of lncRNAs is very cell type-specific

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Glossary

2′-MOE	2′-O-methoxyethylribose
Airn	antisense *Igf2r* RNA non-coding
ASO	antisense oligonucleotide
BAC	bacterial artificial chromosome
Blustr	bivalent locus upregulated by the splicing and transcription of an RNA
BRD4	bromodomain containing 4
CAGE	cap analysis gene expression
CCAT1	colon cancer-associated transcript 1
Cdkn1a	cyclin-dependent kinase inhibitor 1A
CMV	cytomegalovirus
CRISPRa	clustered regularly interspaced short palindromic repeats activation
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPRi	clustered regularly interspaced short palindromic repeats interference
dCas9	nuclease-dead Cas9
Evf2	embryonic ventral forebrain 2
FDA	United States food and drug administration
Fendrr	FOXF1 adjacent non-coding developmental regulatory RNA
H3K9me3	histone-3 lysine-9 trimethylation
hnRNP-K	heterogeneous nuclear ribonucleoprotein K
Igfr2	insulin-like growth factor receptor 2
Indel	insertions/deletions
KRAB	Krüppel-associated box
LET	low expression in tumor
LNA	locked nucleic acid
lncRNA	long non-coding RNA
MALAT1	metastasis-associated lung adenocarcinoma transcript 1
MECP2	methyl-CpG binding protein 2
MYC	myelocytomatosis
nt	nucleotides
PAM	protospacer adjacent motif
PARIS	psoralen analysis of RNA interactions and structures
Plscr4	phospholipid scramblase 4
Pnky	Pinky
polyA	polyadenylation
PVT1	plasmacytoma variant translocation 1
RNAi	RNA interference
sgRNA	single guide RNA
SHAPE	selective 2′-hydroxyl acylation and primer extension
shRNA	short hairpin RNA
siRNA	small interfering RNA
SPEN	Spen family transcriptional repressor
TALEN	transcription activator-like effector nuclease
TSS	transcription start site
Ube3a-ATS	ubiquitin protein ligase E3A antisense
VP64	tetrameric viral protein 16 transcription activator domain
XIST	X-inactive-specific transcript

[10,15,16], the specificity of expression does not always predict critical biological function [17]. For instance, despite being expressed in a diverse range of cell types, some lncRNAs can exhibit exquisitely cell type-specific function [18]. Therefore, loss- and gain-of-function experiments are paramount to understanding the function of lncRNAs.

To study lncRNA function by loss-of-function or gain-of-function methods, it is important to begin with a sound understanding of transcript properties (e.g., its primary sequence, potential isoforms, presence or lack of polyadenylation) as well as corresponding DNA loci (e.g., accurate mapping of the TSS, genomic relationship to protein coding genes and known enhancers). For the purposes of this review, we will assume that these basic aspects of lncRNA bioinformatics are known. Furthermore, we focus our discussion on the experimental tools used to modulate lncRNA expression for the purpose of demonstrating biological function. Experimental approaches for investigating the molecular mechanism of lncRNAs have been reviewed elsewhere [19–21]. Since genome-scale genetic screens have been extremely powerful for the discovery of protein coding gene networks as well as non-coding DNA elements [22–24], and similar screens of lncRNA loci have recently proven useful [18,25,26], we also discuss the suitability of different methods for large-scale screening.

Direct targeting of lncRNA transcripts

Building off its success in the knockdown of protein coding genes, RNAi has been frequently used to deplete lncRNAs [16,27–29]. Typically, after transfection of siRNAs or expression of shRNAs, RNAi triggers the degradation of target RNA molecules through direct complementarity, mediated by the RNA-induced silencing complex (Fig 2A), and for mRNAs, protein translation can be inhibited as well [30–32]. The efficiency of RNAi-mediated knockdown is variable, depending in part on the subcellular localization of the target RNA [33,34]. Although mammalian RNAi is thought to predominantly occur in the cytoplasm, RNAi factors such as Argonaute and Dicer have been found in the nuclei of cells as well [35], which may explain how RNAi achieves knockdown of nuclear enriched lncRNAs such as *MALAT1* [36] and *Pnky* [16,37]. Knockdown efficiency also relates to the degree of secondary structure of the target RNA molecule, with the extent of knockdown anticorrelated with the amount of energy required to disrupt the local secondary structure [38–40]. Therefore, consideration of the location of stem loops and helices, which can be facilitated by methods such as SHAPE and PARIS [41,42], may facilitate the use of RNAi.

Given the compact size of the RNA effectors, RNAi experiments have been successfully scaled up for high throughput and pooled genetic screens for lncRNA function [29,43]. However, RNAi-based screens have a significant risk of false positives caused by off-target effects [39–41]. The specificity of both siRNAs and microRNAs depends primarily on a 7- to 8-nt region called the "seed" sequence, which must be taken into account when designing RNAi experiments for lncRNAs, as mismatches of even one nucleotide in this region along with unintended complementarity to other genes can lead to inefficient knockdown of the lncRNA and/or extensive off-target effects, respectively [44–49]. One way to counteract potential off-target effects is to test multiple siRNA or shRNAs against the same lncRNA target and assess for concordance of phenotype. Whether or not the "pooling" of multiple siRNAs decreases off-target effects is controversial [50]. It should also be noted that ectopic expression of RNAi-resistant lncRNA transcripts may not be a suitable rescue strategy, unless the lncRNA is thought to act in *trans*, since these rescue transcripts are not likely produced from their native loci and may not be produced at normal levels.

An alternative to RNAi for the degradation of lncRNAs are ASOs (Fig 2B). ASOs are 15–20-nt single-stranded DNA oligomers

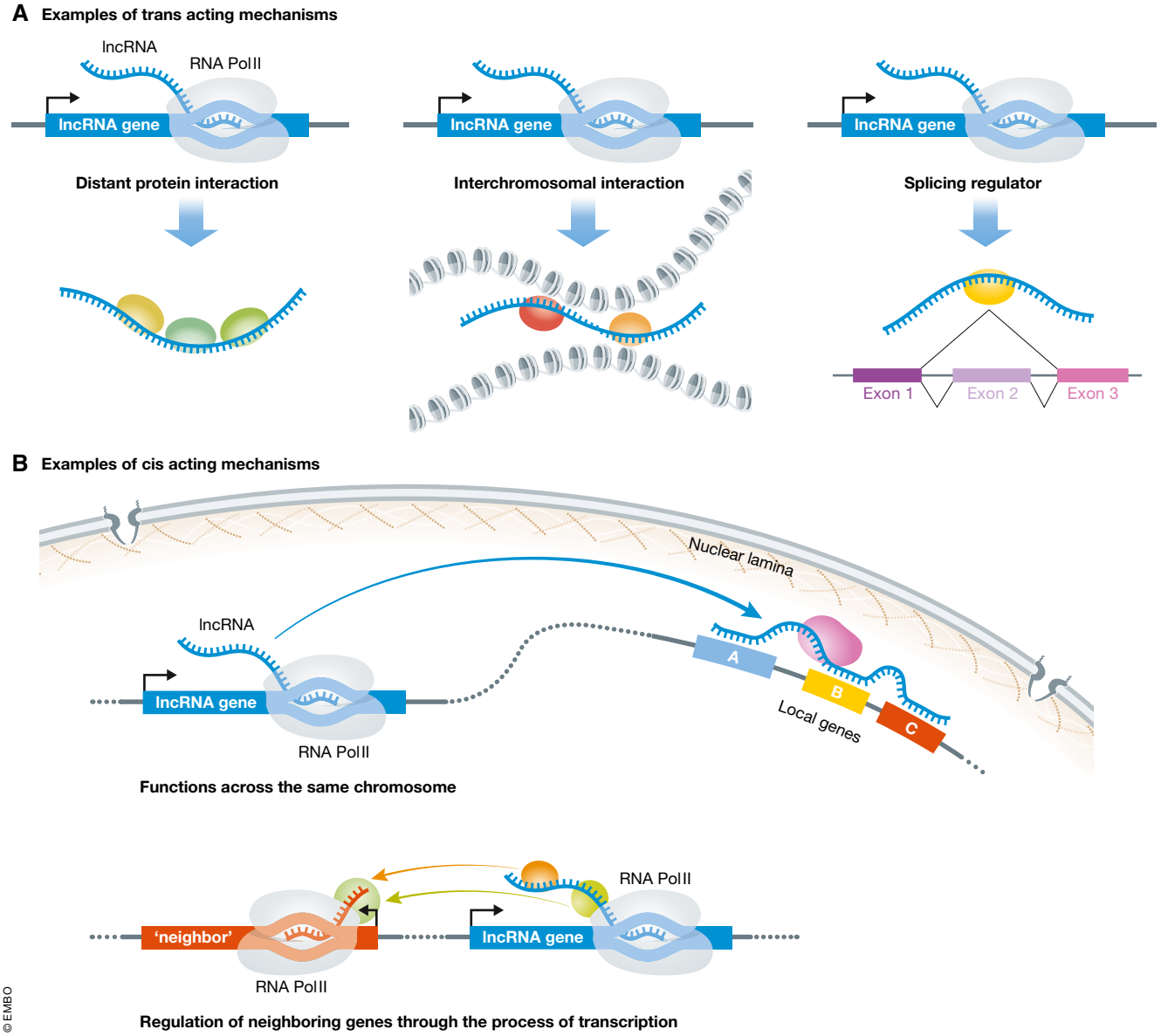

Figure 1. Mechanisms of lncRNA function.
(A) *trans*-acting mechanisms of lncRNA function include distal lncRNA–protein interactions (examples include *NORAD, HOTAIR*), interchromosomal interactions (e.g., *FIRRE*), and regulation of mRNA splicing (e.g., *Pnky, MALAT1*). (B) *cis*-acting mechanisms of lncRNA function. The lncRNA transcript functions along the same chromosome from which it is transcribed (e.g., *XIST*). lncRNAs can also function in *cis* through the process of their own transcription (e.g., *Blustr*).

that are typically chemically modified to increase the efficacy of knockdown and decrease *in vivo* toxicity. In particular, the 2′-MOE and LNA gapmer modifications have been shown to increase affinity toward target RNA transcripts and endow resistance to nucleases [51–53], allowing these modified ASOs to have half-lives between days to several weeks *in vivo* [54,55]. ASOs hybridize with target RNA transcripts through complementarity and induce RNaseH-mediated degradation of the target transcripts [56]. Thus, in contrast to RNAi-based methods, ASO-mediated knockdown is very efficient in the nucleus, making this approach suitable for studying the function of both *cis*-acting and *trans*-acting lncRNAs [9,57,58]. It remains unclear whether ASOs are suitable for identifying lncRNA genes that function through the act of transcription itself.

Through a process called gymnosis, ASOs can enter cells without the aid of transfection reagents, and there are now a number of ASOs used as pharmaceuticals to treat human disease [59,60]. While FDA-approved ASOs currently target protein coding transcripts, ASOs that target specific lncRNAs also exhibit therapeutic promise. For instance, when injected into the mouse brain ventricle, ASOs can trigger knockdown of the Angelman's syndrome associated lncRNA *Ube3a-ATS*, resulting in improvement of behavioral deficits associated with this genetic disorder [61]. In a mouse model of breast cancer, intravenous injection of *Malat1* ASOs decreases

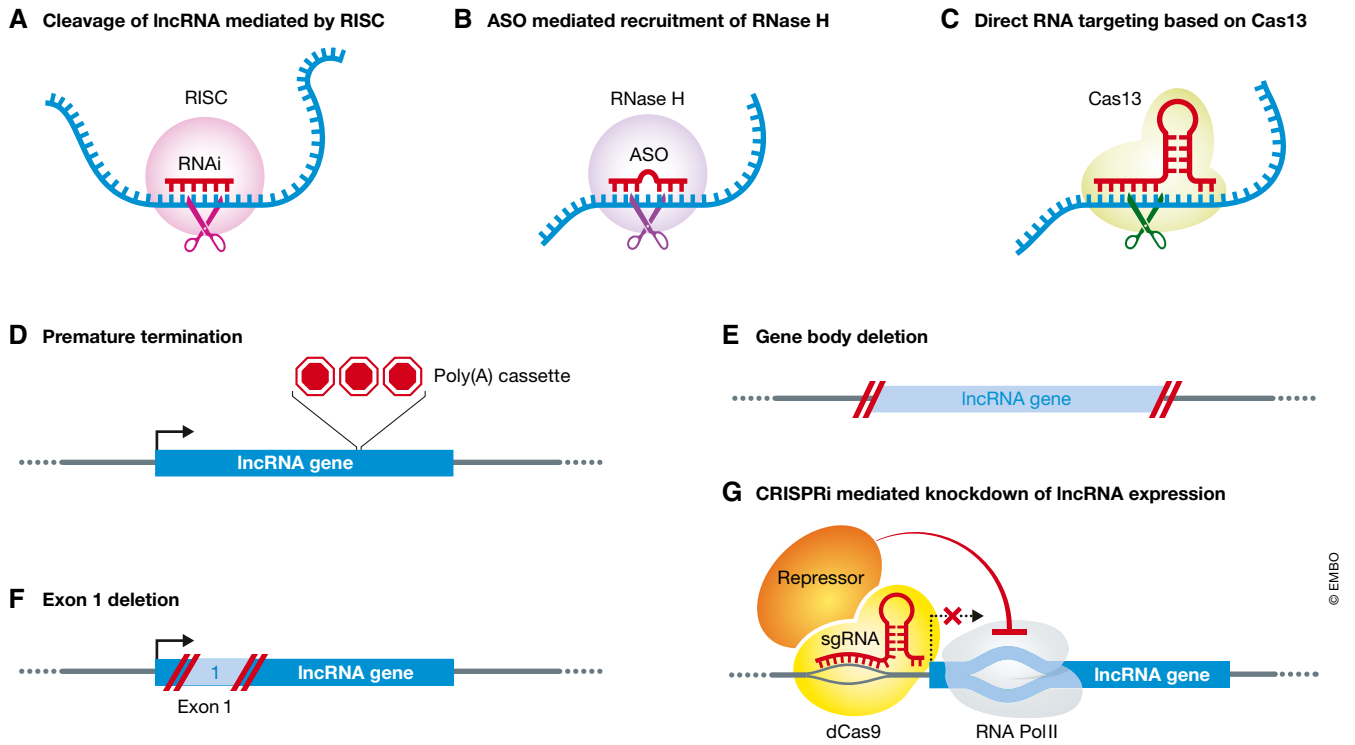

Figure 2. Methods of lncRNA loss of function.
(A) RNAi mediated by RISC cleavage of lncRNA. (B) ASO-mediated recruitment of RNAse H to target lncRNA transcript for degradation. (C) Cas13-based direct RNA cleavage of target lncRNA. (D) Insertion of polyA transcription termination signals into lncRNA gene locus. (E) Gene body deletion of lncRNA. (F) Genetic deletion of exon 1. (G) CRISPRi-mediated knockdown of lncRNA expression.

tumor metastases as compared to scrambled ASO controls [11]. However, due to the structural modifications requiring direct chemical synthesis and their relatively high cost, ASOs are currently suboptimal for high throughput or pooled screening and have not been used for genome-scale screens of non-coding RNA function.

A more recently developed method that can directly degrade lncRNAs is one that utilizes the Cas13 family of CRISPR ribonucleases [62] (Fig 3C). When provided with sgRNAs complementary to the target RNA, CRISPR-Cas13 can efficiently cleave the RNA target [52–54]. Cas13 has been used to knockdown lncRNAs in mammalian cells [52–54], and because the sgRNAs can be stably expressed from viral vectors, Cas13-based methods may be suitable for genome-scale screening for lncRNA transcript function.

Genome modification-based strategies

Genetic deletion of endogenous genes through homologous recombination is an established loss-of-function technique [63] (Fig 2E), and one that has been greatly accelerated by CRISPR/Cas9-mediated gene editing [64–66]. Two of the earliest studied mammalian lncRNAs, *XIST* and *H19*, have been deleted through replacement of the entire lncRNA locus with a drug selection cassette, revealing their roles in dosage compensation and imprinting, respectively [67,68]. More recently, 18 different lncRNAs were knocked out in mice through replacement of the lncRNA gene body with a *lacZ* reporter cassette [69]. Furthermore, conditional knockout strategies have enabled lineage-specific deletion of lncRNAs, which revealed a surprising role of *XIST* in hematopoietic malignancies [70]. Caveats of genetic deletion approaches include the potential introduction of strong promoters, which can affect nearby transcription [71]; residual *loxP* sites, which can trigger embryonic methylation of targeted genes [72]; and the unintentional removal or disruption of local DNA regulatory elements such as transcription factor binding sites, enhancers, and CpG islands [73]. Of note, these issues with genetic deletion are not unique to the study of lncRNAs (i.e., many genes that encode proteins also contain DNA regulatory elements [74,75]). Limiting the size of the genetic deletion can mitigate such concerns and provide additional insights into lncRNA mechanism. For instance, deletion of the A-repeat region of *XIST* demonstrated that these lncRNA sequences mediate X chromosome inactivation by interacting with the transcriptional repressor SPEN, in addition to Lamin B receptor, leading to the recruitment of the inactive X to the nuclear lamina [76–80]. Single exon deletions have also been performed on lncRNAs [7] (Fig 2F). However, the implementation of such size-limited genetic deletions assumes some *a priori* knowledge of how the lncRNA functions. Another point of consideration in deletion experiments *in vivo* is the genetic background in which the deletion is performed, which can contribute to the penetration of subtle lncRNA phenotypes [76–78].

Premature termination of lncRNA transcription through knock-in of polyA sites is also an effective loss-of-function strategy that may reduce the risk of disrupting known or potential DNA regulatory

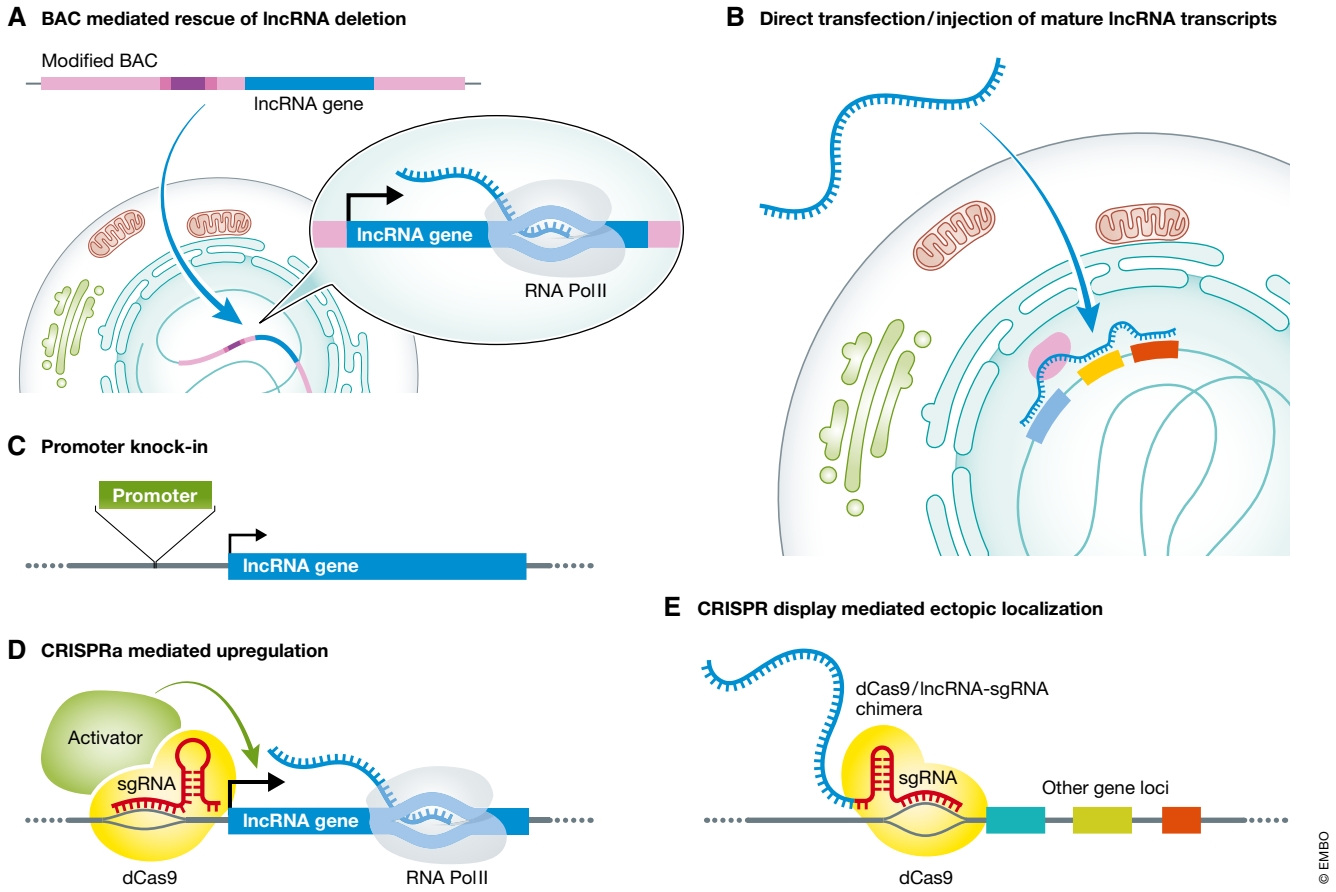

Figure 3. Methods of lncRNA gain of function.
(A) Expression of lncRNA from a BAC transgene. (B) Direct transfection or injection of mature lncRNA transcripts. (C) Genetic knock-in of strong promoter element upstream of lncRNA loci to activate lncRNA expression. (D) CRISPRa-mediated upregulation of lncRNA expression. (E) CRISPR display mediating ectopic localization of lncRNAs through a dCas9/lncrna-sgRNA chimera.

elements [79,80] (Fig 2D). For instance, insertion of a triple polyA transcription stop site into exon 1 of the neural lncRNA *Evf2* results in a truncated transcript while sparing known local *cis* regulatory regions [81]. Similarly, the mesoderm-specific lncRNA *Fendrr* was targeted by insertion of a polyA termination cassette into exon 1 of the lncRNA gene, resulting in embryonic lethality [82]. While usually effective, the insertion of polyA sites does not always result in complete lncRNA knockdown. For instance, for lncRNA *Dlx1as*, despite the presence of multiple polyA sites after exon 1, levels of *Dlx1as* remain at ~40%, perhaps due to transcriptional read-through [83].

One important advantage of polyA site insertion is that because it inhibits transcriptional elongation, mechanisms that involve the process of transcription itself can sometimes be distinguished from those that depend upon the intact, full-length lncRNA transcript. Taking advantage of the ability to make this mechanistic distinction, by using progressively downstream polyA insertions and promoter repositioning, Latos and colleagues were able to show that the lncRNA *Airn* represses its neighboring gene *Igfr2* through transcription over the *Igf2r* promoter [84]. Similarly, the lncRNA *Upperhand* was shown to regulate its divergently transcribed coding neighbor gene *Hand2* by the process of its transcription through a cardiac lineage super-enhancer. While polyA cassette insertion into exon 2 of *Upperhand* resulted in diminished *Hand2* expression, insertion of the *tdTomato* coding sequence into the same exon 2—thereby disrupting the primary sequence of *Upperhand*, did not affect *Hand2* expression [85]. By comparing polyA insertion mutants with exon 1 deletion of lncRNAs, Engreitz and colleagues were also able to demonstrate that certain lncRNAs regulate the expression of neighboring genes through processes that involve transcriptional initiation and elongation as well as RNA splicing [7]. Interestingly, the transcription of certain coding genes was also found to positively regulate the levels of coding gene neighbors. However, the relative challenge of introducing polyA cassettes through current gene targeting methods may limit its scalability in genome-scale screens.

Engineered CRISPR methods of decreasing lncRNA expression

The development of the CRISPR/Cas9 system for mammalian genome editing has greatly facilitated the interrogation of gene function, especially at large scale [22,86,87]. However, Cas9-mediated

mutagenesis through the generation of double-strand breaks and non-homologous end joining is often not suitable for the study of lncRNAs, since by definition they do not produce proteins and therefore may have biological functions that are less likely to be perturbed by small indels that produce frameshift mutations. Double Cas9 excision of DNA sequences that flank lncRNAs are more likely to inactivate lncRNA gene function and has been used to delete up to hundreds of human lncRNAs, revealing the function of previously uncharacterized lncRNA loci [26,88,89].

Engineered Cas9 variants, in particular dCas9 fused to transcriptional activators or repressors, are highly effective for modulating the expression of lncRNAs without alterations to the underlying genomic DNA sequence [90–96]. The CRISPRi system (Fig 2G), in which dCas9 is fused with the KRAB repressor domain (dCas9-KRAB), silences transcription through steric blockade of RNA polymerase elongation and local deposition of H3K9me3, which is a characteristic heterochromatin mark [90,91,97]. With a precise and relatively narrow window of activity between −50 and +300 bp relative to the TSS of the target gene, CRISPRi can site-specifically knock down lncRNA expression while minimizing disruption to the activity of *cis* regulatory regions or neighboring genes [92], making it broadly useful for lncRNAs [10,18], whose gene structures are often antisense, overlapping, or divergent to other nearby genes [13]. Empirical determination of sgRNA mismatch tolerance for CRISPRi and CRISPR/Cas9 has demonstrated exquisite sensitivity in the 12 nt most proximal to the PAM (i.e., seed region), with one mismatch in this region decreasing activity of CRISPRi by up to 100% [92]. Despite evidence of widespread binding of sgRNA/Cas9 complexes throughout the genome, it is thought that activity, whether nuclease or transcriptional modulation, requires more than transient interaction to have measurable effects on gene expression [91,98,99]. Iterations of the engineered CRISPR/Cas9 system, such as addition of the repressor domain of the transcriptional regulator MECP2 to dCas9-KRAB [96], may be useful for lncRNA knockdown. Furthermore, more accurate mapping of lncRNA TSSs from CAGE analysis [100] and optimizations to the design of sgRNA sequences [101] have augmented the CRISPRi toolbox for the study of lncRNAs, making large-scale screening more readily accessible. For instance, in a pooled screening approach to discover lncRNA function in seven different human cell lines, of over 16,000 lncRNA loci targeted, 499 exhibit cell growth-modifying phenotypes, and most of these lncRNA loci were previously unknown to have function [18]. Furthermore, with the relatively large scale of this screen that was facilitated by the robustness of CRISPRi, machine learning could be applied to the data, revealing genomic features that predict essential lncRNA function [18].

With CRISPRi, because site-specific gene repressors are recruited to the genomic DNA, it is possible that *cis* regulatory elements such as enhancers—embedded within or near the TSS of lncRNA genes—are also affected. CRISPRi is indeed capable of silencing transcriptional enhancers, enabling the identification and characterization of *cis* regulatory regions [23,97]. However, the narrow window of activity of CRISPRi complexes, their minimal off-target effects, and restriction of sgRNAs to the TSS of lncRNAs all reduce the potential of unintentional perturbation of enhancers [92,101]. Furthermore, in the study of protein coding genes, the concordance of results from CRISPRi- and Cas9 nuclease-mediated screens suggests that CRISPRi-mediated results do not generally arise from the unintentional modulation of *cis* regulatory regions [101,102]. In any case, as best practice, multiple sgRNAs targeting the same lncRNA should be tested for validation studies, and orthogonal loss-of-function experiments (e.g., ASO-mediated transcript knockdown or insertion of polyA termination sites) can also be performed to help decipher the function(s) of the lncRNA locus and its transcriptional product.

lncRNA gain-of-function strategies

The function of lncRNAs can also be discovered by their overexpression. For instance, viral vectors that produce specific lncRNAs have been used to overexpress *lncRNA-LET* and *Plscr4,* demonstrating that increased levels of these lncRNAs can affect hypoxia signaling and cardiac hypertrophy, respectively [103,104]. Of note, many "standard" expression vectors contain sequence elements that target the RNA transcript to ribosomes and enhance translational efficiency, and the inclusion of these sequences may confer lncRNAs with functions that they do not have under physiological conditions. Furthermore, ectopic expression may not target the lncRNA products to their physiological subcellular locations, such as nuclear paraspeckles, chromatin, nuclear lamina, or cytoplasm. Direct injection or transfection of *in vitro*-transcribed lncRNAs has also been performed to demonstrate lncRNA function (Fig 3B), and these methods may also be useful for the study of lncRNAs that are presumed to function in *trans* [105]. However, neither the expression of lncRNAs from vectors/plasmids nor the introduction of *in vitro*-transcribed lncRNAs preserves information encoded within and surrounding the lncRNA locus and therefore cannot be used to investigate potential *cis*-acting mechanisms.

lncRNAs can also be expressed from the local genomic context. One method employs BAC transgenes that contain the lncRNA locus, and these experiments are especially useful for establishing *trans*-acting lncRNA mechanisms *in vivo* (Fig 3A). For instance, a BAC transgene containing the intact lncRNA *Fendrr* gene can rescue certain phenotypes of *Fendrr*-null mice [82]. Knock-in strategies can also activate transcription of lncRNAs at their endogenous loci (Fig 3C). For instance, overexpression of the colorectal cancer-associated lncRNA *CCAT1* has been achieved through TALEN-mediated knock-in of a CMV promoter upstream of *CCAT1*, resulting in 15- to 30-fold increase in *CCAT1* expression and, as a consequence, increased *MYC* expression and tumorigenesis in a colorectal cancer cell line [106].

Programmable transcriptional activation using engineered CRISPR/Cas9 systems has also enabled gain-of-function studies for lncRNAs (Fig 3D). By localizing gene activation domains such as VP64 just upstream of the TSS of lncRNAs (or any other gene transcribed by RNA polymerase II) [91,93,95], lncRNA genes can be specifically overexpressed [94]. These CRISPRa approaches have been used for genome-wide screens to identify lncRNAs that play roles in drug resistance in cancer cells [107,108].

Another compelling gain-of-function strategy has been termed CRISPR display (Fig 3E). CRISPR display employs an engineered dCas9 that interacts with modified sgRNA-lncRNA chimeras, allowing site-specific delivery of lncRNA transcripts of up to several kilobases (or smaller lncRNA domains) to ectopic regions of the genome [109]. In addition to testing a subset of different *trans* lncRNA mechanisms, CRISPR display can distinguish the function of

Box 1: In need of answers

In the human genome, there are many thousands of loci that produce long non-coding RNAs (lncRNAs), and understanding their function has been challenging. The loci that produce lncRNAs can function locally in *cis*—sometimes even independently of their RNA product—but other lncRNAs can function in *trans*. Therefore, the selection of tools to modulate lncRNA expression levels, and the interpretation of experimental results, must be carefully considered in the context of how lncRNAs carry out their function. The clearest examples of lncRNA function have utilized a combination of experimental methods, and continued development of these approaches will further aid in our understanding of these enigmatic members of the non-coding genome.

the transcript from the act of transcription for potential *cis* mechanisms.

Reconciling lessons learned from perturbation of lncRNA expression levels

Given the diversity of currently known lncRNA functions and fundamental differences in the methods used to produce lncRNA gain or loss of function, it is perhaps not surprising that our understanding of lncRNA biology has evolved over time. For instance, *lincRNA-p21* was initially described as a lncRNA that regulates the p53 transcriptional response through a global *trans*-acting mechanism by interacting with hnRNP-K to localize at p53 target genes [27]. However, those phenotypes relied on RNAi-mediated knockdown of *lincRNA-p21*, which may have led to the overestimation of *trans*-acting potential of the lncRNA. Subsequent experiments using allele-specific deletion and ASO-mediated degradation of *lincRNA-p21* demonstrated that this lncRNA positively regulates its neighboring gene, *Cdkn1a* (p21), with the lncRNA transcript being required for this *cis* regulatory function [9]. Later, genetic deletion experiments demonstrated that the *lincRNA-p21* locus contains enhancers that regulate the expression of the neighboring gene *Cdkn1a*—even in tissues that do not express *lincRNA-p21*—and these findings were further validated in luciferase enhancer reporter assays [8].

Another notable example of the mechanistic dichotomies that can exist within a lncRNA locus is the cancer-related *PVT1*, which is ~55 kb away from *MYC*. *PVT1* was previously shown to be a *trans*-acting lncRNA that stabilizes the MYC protein, promoting oncogenesis [110]. Consistent with its known tumor-promoting properties, CRISPRa-mediated activation of *PVT1* conferred drug resistance to leukemia cells, as shown through pooled genetic screens [107,108]. Unexpectedly, CRISPRi-mediated repression of *PVT1* also enhanced the proliferation of leukemia and breast cancer cells [18]. Cho and colleagues subsequently showed that the promoter of *PVT1* acts as a DNA boundary element that competitively binds with distal enhancers of *MYC* [111]. When the *PVT1* promoter region is repressed by CRISPRi, these distal *MYC* enhancers interact with the *MYC* promoter, activating *MYC* transcription through a BRD4-dependent manner. While *PVT1* lncRNA transcript levels are repressed after CRISPRi, the pro-proliferation phenotype is dominated by the *cis* regulatory mechanism.

The tale of *PVT1* is not to discount the utility of site-specific gene regulatory methods for screens (both small- and genome-scale) to discover lncRNA function. Rather, these methods allow the triaging of functional lncRNA loci, which then motivates further genetic dissection using orthologous techniques discussed here and elsewhere to elucidate their molecular functions [21,73]. As illustrated by these and other well-characterized lncRNAs, the dissection of lncRNA mechanism(s) will likely involve multiple methods to modulate its expression level, and careful interpretation of results in the context of how each experimental approach functions at the molecular-genetic level.

Conclusion

The tens of thousands of long non-coding RNAs in the human genome represent a heterogenous class of genes that can function in a range of different ways. While they all share the property of not encoding proteins, they differ in how they regulate the genome and interact with the cellular machinery. It is now evident that lncRNAs can have *cis* or *trans*-acting function, and in some cases, they can have both. Furthermore, enhancers and other DNA regulatory elements embedded within lncRNA loci can function independently of the lncRNA transcript. Our continued understanding of these genes will require a toolbox of different methods to modulate their expression and test their functions. To more fully understand lncRNA function, it will also be necessary to integrate additional experimental methods that build from our growing understanding of each lncRNA mechanism, such as lncRNA–protein binding assays, RNA structure interrogation, and higher order chromatin organization mapping. Nonetheless, the methods described in this review can also be applied to the study of lncRNA mechanisms, for instance through loss-of-function screening of putative lncRNA interacting proteins. The selection of tools will be guided by the unique characteristics and underlying mechanisms of each specific lncRNA. The development and use of these tools can also facilitate the study of protein coding genes and other non-coding elements of the genome.

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Conflict of interest

The authors declare that they have no conflict of interest.

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