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CRISPR Links to Long Noncoding RNA Function in Mice: A Practical Approach

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Abstract

Next generation sequencing has uncovered a trove of short noncoding RNAs (*e.g* microRNAs) and long noncoding RNAs (lncRNAs) that act as molecular rheostats in the control of diverse homeostatic processes. Meanwhile, the tsunamic emergence of clustered regularly interspaced short palindromic repeats (CRISPR) editing has transformed our influence over all DNA-carrying entities, heralding global CRISPRization. This is evident in biomedical research where the ease and low-cost of CRISPR editing has made it the preferred method of manipulating the mouse genome, facilitating rapid discovery of genome function in an *in vivo* context. Here, CRISPR genome editing components are updated for elucidating lncRNA function in mice. Various strategies are highlighted for understanding lncRNA function in intergenic sequence space, as host genes that harbor microRNAs or other genes, and as natural antisense, overlapping or intronic genes. Also discussed is CRISPR editing of mice carrying human lncRNAs as well as the editing of competing endogenous RNAs. The information described herein should assist labs in the rigorous design of experiments that interrogate lncRNA function in mice where complex disease processes can be modeled thus accelerating translational discovery.

Keywords

CRISPR; long noncoding RNA; mouse; genetics; genome editing

Nearly 50 years ago, Susumu Ohno (1928-2000) correctly estimated the size of our haploid genome, but incorrectly suggested there being “so much ‘junk’ DNA in our genome” [1]. Due in large measure to efforts of the ENCODE Consortium [2], we now appreciate that much of our genome, beyond protein-coding genes, is functional with tens of thousands of noncoding genes and millions of transcription factor binding sites (TFBS). Among noncoding genes, the class of long noncoding RNAs (lncRNAs), defined broadly as processed transcripts >200 nucleotides in length with no translated open reading frame, bear

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striking resemblance to protein-coding genes inasmuch as they are RNA polymerase II-dependent, multi-exonic and spliced, 5' capped, and frequently polyadenylated [3]. LncRNAs encompass numerous subclasses of noncoding RNA genes, distinguished primarily by genomic position relative to other gene loci (below). The first lncRNAs (*XIST* and *H19*) were identified in the early 1990s [4, 5], but very few would be reported over the next 20 years. Current estimates of the number of human lncRNAs vary considerably but a recent update of LNCipedia (<https://lncipedia.org/>) reveals nearly 50,000, a number that more than doubles all protein-coding genes [6]. Unlike microRNAs, with anticipated functions related to mRNA degradation and/or translational repression, lncRNAs have diverse, unpredictable functions in the cell ranging from nuclear gene transcription and architecture to cytoplasmic roles in translation, signaling, cellular trafficking, and mitochondrial homeostasis [7, 8]. Importantly, of the 50,000 human lncRNAs listed in LNCipedia, only 3% (1,555) have some functional annotation [6]. Further, much of the sequence variation associated with cardiovascular disease occurs in noncoding sequence space where lncRNAs reside [9, 10]. These facts highlight the urgent need to define the function of lncRNAs in vivo, particularly those expressed in cells of the normal and pathological vessel wall [11-14].

As detailed in the reviews of this special issue of *Vascular Pharmacology*, a growing number of vascular lncRNAs have been discovered since 2010 (Figure 1). Initial studies to elucidate vascular lncRNA function are most often done in cell culture models; however, definitive proof for the function of these and other soon-to-be discovered vascular lncRNAs will require genetic experiments in animal models where an lncRNA is deleted, mutated, or ectopically expressed [15, 16]. This practical review serves to update advances in CRISPR genome editing of the mouse and the opportunities and challenges of harnessing this game-changing technology to elucidate lncRNA function in an in vivo context.

CRISPR Components

Three components comprise the CRISPR system: (1) a programmable guide RNA that escorts (2) the Class 2, Type II-A Cas9 endonuclease for double-strand breakage and, when directed, (3) precision-mediated repair through a single-stranded or double-stranded DNA template carrying sequence substitutions or small to moderately-sized inserts such as a polyadenylation signal sequence. Two-component CRISPR editing (Cas9 and guide RNA alone) is used to disrupt protein-coding reading frames (not discussed here), to modulate expression of a gene via deactivated or 'dead' Cas9 (dCas9) conjugated to either a transactivator or transrepressor [17], or to generate small or very large deletions [18, 19]. Three-component CRISPR editing (Cas9, guide RNA, and repair template) is used to introduce precision edits such as a single base substitution or the introduction of foreign sequences such as *loxP* sites for the generation of a conditional knockout. For more historical information about the basic biology of CRISPR components and applications in mouse genome editing, the reader is referred to several previous reviews [20-23].

Updated CRISPR Editing in Mice

Innovations in Guide RNA

CRISPR-Cas9 genome editing in the mouse was first reported in 2013 [24-29] and has now all but supplanted traditional methods of modifying the mouse genome. Early CRISPR studies injected in vitro transcribed and purified guide RNA into the mouse zygote. The in-house cloning, purification, and activity testing of guide RNAs was time- and labor-intensive, often taking weeks to complete. More recent developments have enhanced the speed and efficiency of CRISPR-Cas9 genome editing in mice by improving the preparation and activity of the guide RNA. For example, it is no longer necessary to clone and purify the guide RNA. Instead, many companies provide synthetic guide RNA either as a single-guide RNA as originally reported [30] or as independent CRISPR RNA and *trans*-activating CRISPR RNA, ready for injection into the mouse zygote (e.g., <https://www.synthego.com/>). Importantly, synthetic guide RNA lacks a 5' triphosphate which has been shown to elicit innate immunity with massive induction of interferon-dependent genes in both rodent and human cells [31, 32], though this is unlikely to present a problem in the mouse zygote. Defining optimal guide RNAs is made simple with on-line programs such as "Breaking-Cas" (<http://bioinfo.gp.cnb.csic.es/tools/breakingcas>) [33] and CRISPOR (<http://crispor.tefor.net/>), which combines different measures of specificity as well as an informative summary of guide RNA off-targeting [34]. Note that the original design tool from Feng Zhang's lab (crispr.mit.edu) [35] is no longer available. Recently, additional insights have been gained in optimal guide RNA design with the finding that 3' terminal GC-rich sequences are inefficient in positioning Cas9 at targeted sites in the genome [36].

An important consideration in guide RNA design is unintended editing or off-targeting. In contrast to editing cultured cells where off-targeting events can be permanent, off-target edits in mice can be segregated from the on-target edit through breeding so long as the unintended edit is not in linkage disequilibrium with the intended edit. In this context, the off-target summary in CRISPOR [34] provides notation as to the potential for linkage disequilibrium. Although a previous report indicated pervasive off-targeting in the mouse [37], this work was subsequently retracted based on independent labs showing flaws in design and interpretation of findings [38-42]. The latter reports are in agreement with several studies showing little off-target CRISPR editing in the mouse [43-45]. The fact that a low-scoring guide RNA (with 100s of predicted off-targets) can induce many off-targets in the mouse [46] underscores the importance of carefully selecting an optimal guide RNA for mouse genome editing. A guide RNA score of 66 or higher was considered optimal to minimize off-targeting in mice [44]. Even still, investigators should evaluate whether predicted unintended edits in linkage disequilibrium with the intended edit exist and, if so, interrogate any sequence changes through PCR and Sanger sequencing of founder mice. Of course, on-target sequence fidelity must also be evaluated by Sanger sequencing since insertions and deletions, beyond the desired outcome, are possible. A recent, unbiased approach for assessing off-targeting in vivo has been reported with very high efficiency [46].

Innovations in Cas9 Endonucleases

A number of innovations have been reported for Cas9 endonucleases. For example, most mouse cores now inject purified Cas9 protein with the synthetic guide RNA as a ribonucleoprotein (RNP) complex [47]. Since the vast majority of guide RNAs successfully target Cas9 endonuclease to the desired sequence for editing, complex activity assays such as the T7E1 assay or split reporter assay [48] are no longer used in some mouse facilities. On the other hand, a simple in vitro digestion should be done to confirm proper sequence targeting of the Cas9 endonuclease (e.g., <https://www.synthego.com/>). RNPs mediate rapid (one hour) genome editing in cultured cells [49], and elegant imaging studies from the labs of Jennifer Doudna and Robert Tjian showed that the RNP surveils the genome and finds its target in minutes [50]. This is relevant because a major hurdle to overcome in any type of germline genome editing is the phenomenon of mosaicism [51, 52]. The rapid cleavage of zygotic DNA with RNPs minimizes mosaicism and enhances germline transmission of CRISPR edits in mice [47]. In contrast, the time necessary for Cas9 mRNA translation and nuclear import may exceed the time to zygotic division, thus favoring mosaicism. Importantly, germline transmission of CRISPR edits in the mouse is highly favored though on rare occasion we have seen the absence of a transmitted CRISPR-edited allele to the first filial generation; PCR analysis of founder male sperm can avoid protracted breeding efforts that fail to reveal passage of the edited allele through the germline.

Another innovation has been the genetic engineering of Cas9 variants that display reduced off-targeting [53-56]. Recently, the so-called high fidelity Cas9 was shown to be superior to previous recombinant Cas9 variants and is readily available commercially, though it has yet to be tested in mouse zygotes [57]. Smaller Cas9 endonucleases from other species of bacteria exist with longer guide RNA and PAM sequences that likely confer even greater specificity than the more popular *Streptococcus pyogenes* Cas9 variants [58]. Moreover, other classes of Cas endonuclease have distinct cutting properties and PAM recognition sequences to expand the CRISPR armamentarium for genome editing in the mouse [59-62]. Transiently acting Cas9 is an additional development that reduces mosaicism and attending toxicity associated with Cas9, a subject that has largely been overlooked in the editing community, particularly with respect to somatic editing [63, 64].

Base editing involves yet another innovation of Cas9 where the catalytic endonuclease domains are mutated and base modifying enzymes, cytosine or adenine deaminases, are fused to Cas9. The resultant recombinant dCas9 can directly edit a single base in the genome without introducing a double-strand break [65]. Base editing has enormous potential for precision editing of clinically-relevant variants in noncoding RNAs and other noncoding sequences such as regulatory elements. There has been some reporting of base editing in mouse embryos [66-68], but most mouse labs continue to use wild-type Cas9 protein for germline editing, likely because of the limited 4-5 base pair window for base conversion with so-called 'bystander editing' [65].

Innovations in Donor Templates

Innovations in donor templates for three-component CRISPR have also evolved. Previously, there was a limitation of 200 nucleotides for the synthesis of single-strand oligonucleotides

(ssODNs), the preferred donor template in three-component CRISPR. Thus, insertions of many polyadenylation signal sequences for transcriptional arrest of an lncRNA using ssODNs were not possible. Today, there are commercial sources of long ssODNs that are synthesized up to 2,000 nucleotide in length (e.g., Megamers, <https://www.idtdna.com>). These long ssODNs can also be designed to carry *loxP* sites flanking the intervening sequence to be removed upon Cre-mediated excision in a process known as efficient additions with ssDNA inserts CRISPR [69]. If longer donor templates are needed (e.g., knocking in a human lncRNA into the mouse), then targeted integration with linearized dsDNA-CRISPR (Tild-CRISPR) may be considered, where up to 6-kb of sequence has been successfully integrated in mouse embryos [70].

Innovations in Delivery of CRISPR Components to Mouse Embryos

Finally, there are a number of new methods that simplify the delivery of CRISPR components into mouse zygotes and/or enhance editing efficiency. Electroporation of mouse embryos has been widely adopted in mouse cores. This approach circumvents the technically challenging task of microinjecting zygotes while improving targeting efficiency [71-73]. CRISPR with long ssODNs inducing conditional knockout alleles utilizes electroporation in zygotes to generate floxed mice [74]. Electroporation of embryos has also been extended to the intact pregnant female in a process known as genome-editing via oviductal nucleic acids delivery [75]. This method further democratizes CRISPR editing such that most any lab can perform simple noncoding sequence deletions in mice. Three-component CRISPR editing is less efficient than two-component editing in the mouse [22]. However, a recent method of injecting mouse embryos at the two-cell stage, where the G2 phase of the cell cycle favoring three-component CRISPR editing outcomes is extended and chromatin is more accessible, has increased efficiencies of three-component CRISPR knockins more than 10-fold [76].

To summarize, several innovations in CRISPR components and delivery mechanisms have simplified and democratized this revolutionary technology in the mouse zygote. It is now possible, from conception of the experiment to genotyping founder mice, to obtain CRISPRized mice in as few as 5-6 weeks (Figure 2). Moreover, the cost of generating such mice has fallen to as little as two-thousand dollars at some academic centers. The continued refinement of CRISPR editing in the mouse coincides with a growing interest in lncRNAs in vascular biology (Figure 1). As more lncRNAs are discovered, especially in mouse tissue and cell screens, there will be a pressing need to ascertain function in the complex milieu of a living animal where disease modeling can be done and the phenotyping is more extensive over simple cell culture models.

CRISPR Editing lncRNAs in Mice

The reviews in this special issue of *Vascular Pharmacology* extend and complement earlier reviews of functional noncoding genes in vascular biology [11-14] as well as general strategies for the discovery and functional assessment of lncRNAs [15]. Initial lncRNA studies are necessarily done in cultured model systems where full transcript annotation is done by 5' and 3' RACE. These experiments provide much-needed information such as the

presence of additional lncRNA variants arising from alternative splicing, transcription initiation, or polyadenylation. Expression level profiling and the definition of transcriptional control of the lncRNA would be informative for later in vivo editing experiments. Moreover, cellular localization studies using quantitative single transcript RNA-FISH are essential in formulating hypotheses that test the function of lncRNAs. Loss and gain-of-function experiments in cultured vascular cells provide some insight into lncRNA function, but are of limited utility as they do not address lncRNA function in the complex milieu of an animal that can be subjected to an array of pathophysiological stressors. Several lncRNAs have been genetically inactivated in mice and in zebrafish, with almost all knockouts compatible with embryonic development and viability, consistent with the idea that lncRNAs fine-tune regulatory processes such as local gene expression [77-83]. Although several studies have interrogated the biology of lncRNAs under pathophysiological conditions related to the vessel wall, none have used the CRISPR editing system in the mouse zygote [84-89]. Below, we consider several strategies for germline transmitted CRISPR editing to elucidate lncRNA function in the mouse based on the genomic position of the lncRNA. We begin with the simplest scenario and work up to the more complex, highlighting strengths and weaknesses of each CRISPR editing approach.

CRISPRing Intergenic LncRNAs

We define long intergenic noncoding RNAs (lincRNAs) as those lncRNAs residing between two other gene loci where all three gene loci are independently transcribed. Accordingly, an lncRNA whose 5' end is found closely juxtaposed to a neighboring 5' locus may not be a bonafide lincRNA as it would likely be co-regulated by shared promoter elements. There are thousands of lincRNAs and many show sequence conservation and chromosomal synteny between mouse and human [90, 91]. LincRNAs are the easiest class of lncRNA to target with CRISPR in the mouse and are amenable to all methods of CRISPR editing. Consider *Neat1*, an lincRNA shown recently to promote VSMC phenotypic modulation [88]. This lincRNA is transcribed ~20-kb upstream of another lincRNA (*Malat1*), shown to have function in both EC [92] and VSMC [93], and ~6kb downstream of a protein-coding gene (*Frdm8*) (Figure 3). One could delete the entire 21-kb *Neat1* gene using two RNPs as shown (RNP1 and RNP2, Figure 3). Indeed, a similarly-sized deletion was done for the lincRNA, *Rian*, the first lncRNA to undergo CRISPR editing in the mouse [94]. Another lincRNA, *Gene model 26878 (Gm26878)*, was deleted in the mouse and found to result in neonatal lethality [95]. More recently, a 6.7-kb deletion of *Norad* revealed a premature aging phenotype in mice [82]. Genotyping such lincRNA deletions is relatively straight-forward with primers located at least 100-200 nucleotides outside the RNPs, though larger deletions or inversions have been reported requiring careful assessment of founder mice [96]. One obvious concern with such a deletion approach is the inadvertent excision of smaller transcription units or regulatory elements that control distal gene expression. Though the latter concern could be addressed by assessing chromatin marks within and around the lncRNA as well as RNA-seq experiments in a specific vascular cell type, accurate interpretation is complicated by the presence of deleted sequences across all cells of the mouse with potential cell non-autonomous effects. On the other hand, the locus of an lincRNA could be floxed with the insertion of two *loxP* sequences on the same allele to allow for vascular cell-restricted loss of the lincRNA, using appropriate Cre driver mice [97,

98]. The original method of floxing mice with CRISPR calls for Cas9, two guide RNAs, and 2 ssODNs and while the method was reported to have moderately high efficiency [27], this notion has recently been challenged (<https://www.biorxiv.org/content/early/2018/09/01/393231>). RNA-seq experiments would still need to be done to determine whether a distal-acting regulatory region was removed. If there is only one promoter driving lincRNA expression, it should be informative to either make a deletion of the promoter and a portion of first exon using two RNPs or insert *loxP* sites around the promoter and first exon for vascular cell-restricted inactivation as has been done for some protein-coding genes [99] (RNP1 and RNP3, Figure 3). However, in the case of *Neat1*, we see a potential problem with each of these approaches; loss of a shorter, more abundantly expressed transcript located at the 5' end of the gene (Figure 3). Prior to CRISPR, differentiating the effects of these two variants of *Neat1* was problematic due to their identical sequences at the 5' end. Recently, however, the function of the two *Neat1* variants was elucidated through clever positioning of RNPs and insertion of transgenes that could differentially arrest transcription of each isoform [100]. This example underscores the critical importance of fully annotating lincRNA transcripts prior to any CRISPR editing.

There is at least one more consideration with deleting an lincRNA and that relates to generating microdeletions (less than 100 nucleotides) within the lincRNA itself. Unlike protein-coding genes, where well-defined functional domains are observed, lincRNA sequences rarely offer insight into functional motifs that can be easily excised with one or two RNPs. However, biochemical studies that define contact points between an lincRNA and RNA, DNA, or protein may reveal functional sequences [101, 102] which could be deleted with RNPs in close proximity to the motif. Note, however, that such an approach could disrupt the overall structure of the lincRNA, thus imparting new macromolecular associations and potential non-physiological activities. In vitro studies that narrow the binding sequence could result in a more subtle edit of a functional motif within an lincRNA.

Assuming there is only one lincRNA transcript type, as suggested in the well-studied *Malat1* lincRNA (Figure 3), what other options beyond deleting sequences are available to the investigator? One idea would be to insert a polyadenylation signal sequence just downstream of the transcription start site to arrest transcription of the lincRNA (RNP4 in Figure 3). A similar strategy was used in cells to show that despite >90% suppression of the *Lockd* lincRNA, there was no effect on expression of the neighboring *Cdkn1b* gene suggesting that transcription of *Lockd* is dispensable for *cis*-acting transcriptional events [103]. On the other hand, deleting the 25-kb *Lockd* lincRNA resulted in 70% inhibition in *Cdkn1b* transcription supporting loss of a critical enhancer and TFBS functioning independently of *Lockd* transcription to effect *Cdkn1b* expression [103]. A contrary example exists with the *Upperhand* lincRNA, located upstream of *Hand2*, an essential cardiac transcription factor. Here, transcriptional arrest of *Upperhand* with a polyadenylation signal sequence abolished expression of *Hand2* whereas knockdown of *Upperhand* had no effect [104]. The disparate findings between *Lockd* and *Upperhand* highlight the unpredictable nature of lincRNAs, and the regulatory elements in and around the locus, in controlling local gene expression. More importantly, they demonstrate the need for combinatorial experiments in vitro that can inform the best in vivo strategy to follow for elucidating lincRNA in the mouse. The length of polyadenylation signal sequences are generally >100 nucleotides requiring a dsDNA

repair template for insertion into the genome [103-105]; however, a shorter polyadenylation signal of 49 nucleotides can easily be synthesized as an ssODN and was used to arrest lincRNA transcription in vitro [105]. Moreover, the *Charme* lincRNA was recently inactivated in mice by inserting a short polyadenylation signal followed by strong transcriptional termination sequences in an ssODN [106]. An even smaller polyadenylation signal sequence has yet to be tested in arresting lincRNA transcription [107].

Another method to study loss-of-function of an lincRNA in vivo is through CRISPR editing of an enhancer or critical TFBS. Several studies have used CRISPR-Cas9 to delete essential enhancers associated with protein-coding genes in mice [108-111], but there is virtually no such information with respect to the regulation of lincRNAs. A recent study of *lincRNA-Cox2* (aka *Ptgs2os*) used a variety of CRISPR approaches to suggest the presence of an enhancer RNA upstream of the lincRNA [112]. The use of chromatin signatures (e.g., H3K27ac) and sequence conservation can guide investigators to define potential enhancers (RNP5 and RNP6, Figure 3). Enhancers are often hundreds of bases in length and comprise multiple TFBS, the smallest regulatory unit in the genome. Thus, genotyping deletion of an enhancer with primers flanking the predicted double-strand break is straight-forward as long as a larger than expected deletion does not occur.

For finer resolution edits, it should be possible to mutate a single TFBS using three-component CRISPR (RNP6, Figure 3). Historically, this has been a rather daunting task with only one study ever reported using conventional knockout technology to modify a TFBS without leaving a genomic scar or cavity [113]. The first regulatory element edit using CRISPR in the mouse involved a three nucleotide substitution within a TFBS in the first intron of *Cnn1*; mice homozygous for this edit showed a virtual knockout of this smooth muscle-restricted gene [114]. Genotyping such subtle edits requires either an RFLP assay or PCR primers that discriminate the wildtype versus mutant allele [114]. A subsequent study, using paired TALENs surrounding a GATA1 site in the *Alas2* locus, revealed a sharp reduction of *Alas2* expression and embryonic lethality in mice [115]. The results of editing TFBS controlling *Cnn1* and *Alas2* are somewhat surprising given that gene transcription is thought to involve multiple, often redundant, regulatory elements. It will be interesting to determine if additional examples exist in other protein-coding genes as well as lincRNAs and, if so, whether conserved single nucleotide variants are present in human populations that can be modeled in mice for further functional analysis.

An advantage of targeting a single enhancer or, better, a single TFBS, is the subtle nature of the edit and the ability to override loss in target gene expression with, for example, CRISPR activation (CRISPRa) [17]. The latter studies would likely require in vitro culturing of vascular cells from CRISPRized mice since CRISPRa is a transient phenomenon that is not amenable to the aforementioned types of CRISPR editing in the mouse zygote given that no permanent editing of DNA occurs. Recently, CRISPRa was used to reverse obesity in a haploinsufficient mouse [116].

A final method of targeting an lincRNA in vivo could be to directly repress transcription using CRISPR interference (CRISPRi), which originally was reported as a single-guide RNA-mediated method of targeting the non-template strand (that is the plus or Watson

strand of DNA) with dCas9 to sterically block transcription of a target gene [117]. Subsequent work showed greater repression of target genes by joining dCas9 with the Krüppel-associated box (KRAB) domain found in an array of zinc finger transcriptional repressors [118]. Although CRISPRi has already been used to interrogate lincRNA function in cultured cells [119, 120], and shown to be superior to other methods of gene silencing (e.g., RNAi) [121], its application in the mouse for analyzing lincRNA function has yet to be reported. It should be possible to inducibly activate CRISPRi in a vascular cell-restricted manner to repress an lincRNA in vivo. Note that the guide RNA (RNP7, Figure 3), which would need to be delivered to the vessel wall via viral-mediated transfer, is targeted to the proximal promoter region within a window of -50 to +300 bp from the TSS; guide RNAs outside this interval exhibit distance-dependent loss in effective gene repression [120]. This fact, again, emphasizes the need for careful in vitro characterization of the 5' end of an lincRNA prior to studies in the mouse.

Because lincRNAs, as defined here, are well-separated from neighboring gene loci, each method of CRISPR editing may be used to elucidate loss-of-function phenotypes in the mouse. However, as is the case with protein-coding genes, variations in phenotype may be observed depending on the strategy of inactivating the lincRNA [78, 104]. Strain (or sub-strain due to genetic drift), age, gender, and the nature of a stressor are other important variables to consider in the interpretation of lincRNA loss-of-function phenotypes in mice.

CRISPRing Host LincRNAs

Some lincRNAs are a host gene for other lincRNAs or microRNAs. For example, *Carmn*, first reported as a conserved super-enhancer associated lincRNA [122], is a host gene for *miR143/145*, a known mediator of the VSMC differentiated phenotype [123-125] (Figure 4). Moreover, *Carmn* overlaps at its 3' end with *Bvht*, a convergently transcribed mouse-restricted lincRNA shown initially to be involved in cardiac muscle specification [126], but apparently dispensable for normal mouse development [78]. Several CRISPR strategies are available to elucidate the role of *Carmn* in the mouse. The key here and in other host lincRNAs is to differentiate between the host lincRNA and gene loci within it. Accordingly, and in contrast to an lincRNA (Figure 3), deletion of the entire *Carmn* locus with or without *loxP* sites would be contraindicated given that two other gene loci would be deleted or disrupted (RNP1 and RNP2, Figure 4). Note, however, that deletion of the 5' promoter region and first exon of *Carmn* (RNP1 and RNP3, Figure 4) may be informative so long as the *miR143/145* cluster is not dependent on the *Carmn* promoter for expression. Previous work has shown the presence of an upstream, conserved enhancer region harboring an SRF-binding CARG box that controls *miR143/145* expression in the context of reporter mice [124, 125]. Whether this CARG box is necessary for *miR143/145* expression in its native genomic milieu, as shown for *Cnn1* [114], can easily be addressed using three-component CRISPR editing (RNP4, Figure 4). The presence of an H3K27Ac chromatin signature in this region highlights the importance of utilizing existing ENCODE data to gain insight into potentially important regulatory regions of a host lincRNA or its internal gene loci. If the host lincRNA is transcriptionally uncoupled from the internal gene(s) within, it should be possible to target a critical upstream promoter element (RNP1, Figure 4), insert a polyadenylation signal within

the first exon (RNP5, Figure 4) or target the promoter region with CRISPRi (RNP1, Figure 4). The latter, however, is technically challenging for reasons outlined above.

If internal gene loci are co-transcribed and processed with the host lncRNA, what CRISPR strategy could be used to elucidate the function of the host lncRNA apart from the internal gene(s)? If the host lncRNA is multi-exonic, as is *Carmin*, one approach could be to make moderate deletions of a terminal exon (RNP2 and RNP6, Figure 4) or internal exons (RNP4 and RNP7, Figure 4). It would be essential not to disrupt splicing of the host lncRNA (or in the case here, the 3' overlapping *Bvht* locus) or excision of a critical regulatory element. RNA-seq experiments could address the latter. Note that in the present example, excision of internal *Carmin* exons (RNP4 and RNP7, Figure 4) would be contraindicated since a potentially important CARG box for *miR143/145* expression would be excised. The best case scenario for any loss-of-function analysis of an lncRNA would be the in vitro elucidation of a critical motif that could be point mutated, much like what has been done for other lncRNAs [101, 102]. The recent categorization of lncRNAs based on short, 8-10 nucleotide sequences called *k-mers*, which appear to predict protein binding and cellular localization of lncRNAs, offers some computational guidance to functionality and subtle, three-component CRISPR editing [127].

CRISPRing Intronic lncRNAs

lncRNAs may be found within introns of coding genes and noncoding genes in either the sense or antisense orientation. Sense intronic lncRNAs have recently been included in the class of stable intronic sequence RNAs and can arise through splicing-dependent and splicing-independent processes [128]. They generally are co-transcribed from the host gene; thus, targeting sense lncRNA transcription with RNPs that insert a polyadenylation signal, enable CRISPRi, or disrupt a proximal regulatory sequence (RNP1, Figure 5) may be ineffective approaches to mediate loss-of-function. On the other hand, each of these approaches could be utilized with the less frequent antisense intronic lncRNAs or sense intronic lncRNAs that are independently transcribed from the host gene. It is likely that elucidating the function of most sense intronic lncRNA will involve deleting most or all of the lncRNA either with or without *loxP* sites (RNP2 and RNP3, Figure 5). For example, the muscle-restricted *Syisl* sense intronic lncRNA was deleted with two RNPs resulting in poor expansion of satellite cells during skeletal muscle repair in the mouse [129]. Importantly, despite its close juxtaposition to a splice donor in the host (*Synpo2*) gene, there was little change in expression of *Synpo2* upon deletion of *Syisl*, consistent with the independent transcription of *Syisl* [129]. Since a binding site for MYOD1 was identified in the proximal promoter region of *Syisl* [129], it may prove informative and less aggressive to make a subtle mutation in the MYOD1-binding E-box to inactivate *Syisl*. There is precedent for effectively generating loss-of-function mice with subtle mutations in a single TFBS [113-115]. The nuclear intronic sense lncRNA, *Panct1*, was floxed and shown to have a critical role in the maintenance of pluripotency in mouse embryonic stem cells [130]. Interestingly, a short sequence motif was discovered in *Panct1* through chromatin isolation by RNA purification and this mutant *Panct1* failed to rescue the loss in stem cell pluripotency observed when the endogenous *Panct1* gene was deleted [130]. The latter result demonstrates a rare example of a functional lncRNA motif and its candidacy for subtle,

three-component CRISPR editing in the mouse. Thus, we can see that depending on the nature of the intronic lncRNA and insights gained through in vitro biochemical and promoter studies, most any approach to generating a loss-of-function mouse with CRISPR editing could work.

CRISPRing Natural Antisense or Overlapping lncRNAs

Thousands of annotated lncRNAs are found in close proximity to other gene loci either as natural antisense transcripts (NATs) or 5' or 3' overlapping lncRNAs that do not have exons intersecting opposingly transcribed exons. Several vascular cell NATs have been defined [13], including the very first vascular-restricted lncRNA discovered called *Tie1-AS* [131]. In contrast to the aforementioned lincRNAs and intronic lncRNAs, those of the NAT and overlapping classes are less easily targeted in the mouse with CRISPR editing. For example, the conserved NAT *Has2os* (os for opposite strand), which is involved with matrix remodeling in VSMC [132], has two exons (Ex3-4) that overlap the opposingly transcribed exon (Ex1) of the protein-coding gene, *Has2* (Figure 6A). Deleting the entire *Has2os* locus may disrupt expression of *Has2* (RNP1 and RNP2, Figure 6A). However, since exon 1 of *Has2os* is several kilobases away from exon 1 of *Has2*, a microdeletion of the promoter/exon1 of *Has2os* is attractive, especially given the absence of highly conserved sequences that would be deleted (RNP3 and RNP4, Figure 6A).

Alternatively, targeting exon 1 of *Has2os* for polyadenylation signal sequence insertion (RNP3, Figure 6A) or the promoter region with either CRISPRi or a subtle edit in a *Has2os*-specific regulatory element with three-component CRISPR (RNP4, Figure 6A), would likely confer *Has2os* restricted loss-of-function (Figure 6A). Importantly, when a first exon of a NAT lncRNA falls within a few hundred bases of the overlapping exon 1, then CRISPRi or edits to the promoter region of the lncRNA may impact the overlapping gene. However, insertion of a polyadenylation signal sequence in an lncRNA exon in close juxtaposition to an overlapping exon would unlikely impact overlapping transcription [133]. Thus, three-component CRISPR-mediated insertion of a polyadenylation signal should be widely applicable across different lncRNA classes. Of course, defining a structural or regulatory motif in a single exon of a NAT lncRNA, as has been described in other lncRNA genes [101, 102, 129], would allow for subtle editing of an exon in three-component CRISPR (RNP5, Figure 6A), so long as the edit does not influence an overlapping protein-coding exon. In this context, an intriguing synonymous SNP found in an exon of the *EGFR* locus was found to affect a NAT lncRNA, conferring heightened sensitivity to cancer therapy in humans [134]. This unexpected finding should alert vascular biologists to variations in lncRNA sequences that may have hidden, clinically-relevant functions worthy of dissection in the mouse. The lncRNASNP2 database (<http://bioinfo.life.hust.edu.cn>) contains a trove of lncRNA sequence variants that, if found in conserved sequences of an orthologous mouse lncRNA, could be modeled in the mouse with three-component CRISPR editing as reported for a vascular-associated coding SNP [135]. Of note, polymorphisms in the promoter region of the *NEAT1* lncRNA result in reduced expression and protein binding making these potentially viable substitutions to study in the mouse [136].

A more difficult scenario exists with the NAT lncRNA, *Flicr* [80]. Here, we see *Flicr* exons overlapping exons in two flanking protein-coding genes (Figure 6B). Deleting the *Flicr* locus in its entirety is a bad strategy as it would likely result in a triple knockout of the lncRNA and the two overlapping protein-coding genes (RNP1 and RNP2, Figure 6B). Targeting the first exon or promoter region of the annotated *Flicr* locus would impact the overlapping and oppositely transcribed *Ppp1r3f* coding sequence (RNP1, Figure 6B). Thus, the *Flicr* gene was targeted with either a single RNP that disrupted splicing at exon 2 (RNP3, Figure 6B) or a pair of RNPs that deleted much of exon 2 (RNP3 and RNP4, Figure 6B). Remarkably, a phenotype associated with reduced *Foxp3* expression in regulatory T cells was discovered with reduced incidence of experimentally-induced diabetes [80].

As with *Flicr*, a 5' overlapping lncRNA could be challenging to target with CRISPR editing. For example, *Vax2os* is a multi-exonic lncRNA whose complete deletion would impact the expression of the *Vax2* protein-coding gene (RNP1 and RNP2, Figure 6C). However, as with *Has2os* above, a microdeletion of the promoter/exon 1 of *Vax2os* represents a more desirable strategy since it would less likely impact *Vax2* expression (RNP2 and RNP3, Figure 6C). Alternative approaches to targeting *Vax2os* would be inserting a polyadenylation signal in exon 1 (RNP2, Figure 6C), making an internal deletion of exons (*e.g.*, RNP1 and RNP4, Figure 6C) or, as with *Flicr*, targeting specific splice sites to generate a truncated lncRNA (*e.g.*, RNP4, Figure 6C). In general, targeting regions of high sequence conservation may be ill-advised since such sequences could represent small transcription units or regulatory elements controlling distal gene expression (RNP5, Figure 6C). It should be noted that targeting the promoter or first exon of 3' overlapping lncRNAs may be less intrusive to the 3' end of the overlapping gene, whether protein-coding or noncoding though disruption of annotated microRNA binding sites in the 3' untranslated region should be avoided (not shown). As with any of the aforementioned strategies of CRISPR editing an lncRNA in mice, basic experimentation in cultured vascular cells should be done to gain some insight into how the lncRNA should be edited in vivo [15].

CRISPRing Human lncRNAs by BAC Editing in Mice

In contrast to protein-coding genes and microRNAs, lncRNAs generally lack sequence similarity across species. While there are clear experimental advantages to studying mouse-specific lncRNAs, their relevance to human biology may not be so readily apparent (*e.g.*, *Bvht*). On the other hand, the study of human-specific lncRNAs is complicated by the limited experimental tools to unravel function in vivo. Here, we highlight a human vascular lncRNA called *SENCR*, which functions to mediate or maintain EC and VSMC differentiation [137-139]. *SENCR* is a multi-exonic 5' overlapping lncRNA that coincides with the oppositely transcribed *FLII* transcription factor (Figure 7). One could perform CRISPR editing of such an lncRNA in an established human EC line (*e.g.*, EA.hy926 cells); however, these cells are aneuploid making three-component CRISPR editing of a regulatory element or the insertion of *loxP* sites or a polyadenylation signal an arduous task. Accordingly, investigators working with immortalized human cell lines should define by karyotyping the number of chromosomes harboring the lncRNA. CRISPR editing human embryonic stem cells or inducible pluripotent stem cells represents a suitable alternative, but experimentation is rather limited.

On the other hand, humanizing mice with bacterial artificial chromosomes (BACs) that carry human genes and all neighboring regulatory elements has been successful in pinpointing enhancers and regulatory elements (or variations therein) effecting gene expression in vivo [140-143]. CRISPR has been used to replace a region of the mouse genome with a BAC carrying orthologous human sequences [144]. The latter approach, however, carries the risk of deleting a critical mouse-specific regulatory element that results in embryonic lethality [145]. Consequently, we recently used *piggyBAC* transposon-mediated BAC transgenesis to integrate a BAC carrying *SENCR* in the mouse [139, 146]. *SENCR* was expressed on the surface endothelium of the mouse aorta and, more importantly, was shown to be most abundant in regions of the aorta exhibiting laminar shear stress, a finding observed in cultured human endothelial cells [139]. Of course, BACs often carry additional transcription units. In the case of an lncRNA-mRNA gene pair, such as *SENCR-FLII*, this could be advantageous in elucidating any local *cis*-acting effects on gene transcription. Moreover, it should be possible to inactivate the endogenous *Fli1* gene and study the biology of human *FLII* ± *SENCR* in the mouse. The overarching assumption in humanized mouse models is that the human gene(s) faithfully recapitulates expression and function in the mouse, which may not always be the case.

How might a human lncRNA be edited in a humanized mouse within the context of a BAC? Any of the aforementioned strategies is possible depending on the type of lncRNA. However, it is necessary to first validate expression of the human genes and delineate the boundaries of the BAC. In addition, it is imperative to define founder mice with a single copy of the BAC using digital droplet PCR or Southern blotting before commencing with BAC editing; accurate BAC editing will be very difficult with multiple copies of the BAC. Further, BAC editing is best done in hemizygous mice where only one chromosome carries the BAC-containing lncRNA. Similar to other lncRNAs in close apposition to a protein-coding or noncoding gene, BAC editing of *SENCR* would not involve the deletion of the entire *SENCR* locus as there would be simultaneous deletion of the *FLII* promoter and first exon, effectively creating a double knockout (RNP1 and RNP2, Figure 7B). Similarly, removal of terminal exons in *SENCR* could disrupt proximal promoter elements controlling *FLII* expression (RNP2 and RNP4, Figure 7B). A reasonable starting point would be to either create a microdeletion of the first exon (RNP1 and RNP3, Figure 7B) or insert a short polyadenylation signal in the first exon of *SENCR* (RNP1, Figure 7B). Note that insertion of a polyadenylation signal in the sense strand of exon 1 of *SENCR* would not be expected to impinge on transcription of *FLII* [133]. If key upstream regulatory sites controlling *SENCR* were discovered, a more subtle CRISPR edit could be performed as previously reported for a protein-coding gene [114]. The same could be said for a functional sequence motif within *SENCR* identified through computational and biochemical means. Any deletions or insertions, however small, would necessitate RNA-seq experiments to rule out unintended consequences on the general transcriptome [147]. We recommend performing RNA-seq experiments, even with regulatory SNP edits, to rule out potentially confounding changes in gene expression following CRISPR editing in the mouse.

CRISPRing Competing Endogenous (Sponge) RNAs

Regarding the competing endogenous RNA (ceRNA) concept, which posits that an lncRNA “sponges” a specific pool of microRNA to derepress target protein expression, much of the hyperbole stems from an unusual circular RNA (*CDR1as*) that has 73 binding sites for miR-7 and is resistant to miR-7-mediated deadenylation and degradation [148]. Hundreds of papers have proposed lncRNAs functioning as competing endogenous RNAs. However, since most lncRNAs are of low abundance, the vast majority of these may not be of much biological significance in vivo due to asymmetry in expression levels of the lncRNA (lower/less stable) versus the microRNA and target mRNA (higher/more stable). Moreover, there is a notable lack of rigor in formally proving a bonafide sponge RNA [149]. For example, demonstration of a ceRNA would require colocalization studies of the lncRNA and microRNA as shown originally for *CDR1as* [148] and CRISPR-mediated deletion of the putative ceRNA with subsequent measures of microRNA target proteins [150]. The penultimate experiment would be to CRISPR edit the microRNA binding element (MRE) within the lncRNA and assess microRNA targets; however, this has yet to be done for any lncRNA. There have been efforts to target MREs in protein-coding genes of *Drosophila*, zebrafish, and mice [151, 152] and at least one MRE within a risk allele for coronary artery disease exists that could undergo CRISPR editing in the mouse [153]. A website has been created (miR-CRISPR) to facilitate CRISPR targeting of putative MREs in lncRNAs [151]. If several MREs in an lncRNA are suspected to function as a sponge, the multiplex nature of CRISPR allows for targeting of each MRE simultaneously [154, 155]. This may require longer dsDNA templates [70] carrying the MRE mutations; however, if the MREs are in close proximity to one another (within 20-30 bases) then an ssODN approach might work [156]. Alternatively, multiplex base editing could be done as reported for targeting two TFBS located 9-kb apart in the mouse [68]. The advantage of the latter would be the absence of double-strand breaks that could delete intervening sequences between two MREs. On the other hand, base editing generally alters only one base and that may not be sufficient to functionally knockout an MRE. We strongly recommend against continued reporting of so-called sponge lncRNAs in the absence of more rigorous analysis.

Summary and Perspective

Mammalian genomes are pervasively transcribed with an expanding class of lncRNAs [157]. In addition, there are thousands of other long noncoding transcripts such as pseudogenes, circular RNAs, and enhancer RNAs, each of which is amenable to approaches of gene editing outlined here. Before embarking on any CRISPR editing project involving a long noncoding RNA in the mouse, its genomic landscape must be thoroughly interrogated with the latest version of the UCSC Genome Browser [158]. Full length transcripts must be defined to ensure all transcribed sequence is delineated. Moreover, cellular localization, transcriptional control in reporter assays, and knockdown experiments are necessary to gain insight into the regulation and function of a new lncRNA [15]. A recent report suggests most lncRNAs may not be targetable with CRISPR editing [159]; here, however, we propose a number of strategies that should allow most any lncRNA to be targeted in the mouse, although advantages and disadvantages exist for each based on the position of the lncRNA. These approaches capitalize on new innovations in CRISPR-Cas9 editing of the mouse,

including in vivo BAC-editing of a human lncRNA. When contemplating CRISPR editing in the mouse, ‘less is more’; therefore, the more subtle the edit (*e.g.*, microdeletions or single/multiple base substitutions), the less chance of confounding effects stemming from loss of a protein-coding gene or structural/regulatory element. It should also be mentioned that previously annotated lncRNAs have turned out to be protein-coding genes [160] and some protein-coding genes have noncoding functions [161, 162]. Three-component CRISPR editing of AUG (or CUG) codons could distinguish between a coding versus noncoding function.

In this review, we have promoted the broadened use of CRISPR to edit lncRNAs in the mouse. The time and expense in generating a mouse over a cell line has diminished considerably and there is much more flexibility in generating a mouse line, where diploid vascular cells carrying edited lncRNAs can be readily cultured, than an aneuploid cell line carrying indelible edits. We do recognize, however, there is great power in CRISPR editing human inducible pluripotent stem (iPS) cells where human disease modeling in the mouse is feasible [163]. Indeed, recent CRISPR editing of the cardiovascular risk haplotype in the *ANRIL (CDKN2B-AS1)* lncRNA revealed intriguing pathological phenotypes when edited human iPS cells were differentiated to a VSMC phenotype [164]. An atherosclerosis risk allele exists in another lncRNA that awaits formal proof of function using CRISPR in iPS cells [165]. Of course, all biomedical research using CRISPR editing has the ultimate goal of effecting meaningful measures to improve the health of human beings. The recent revelation of reckless germline genome editing of the *CCR5* gene in twin Chinese girls named “LuLu” and “Nana” [166] must galvanize scientists and the global community to proceed with this powerful technology in an ethically, socially, and scientifically responsible manner [167].

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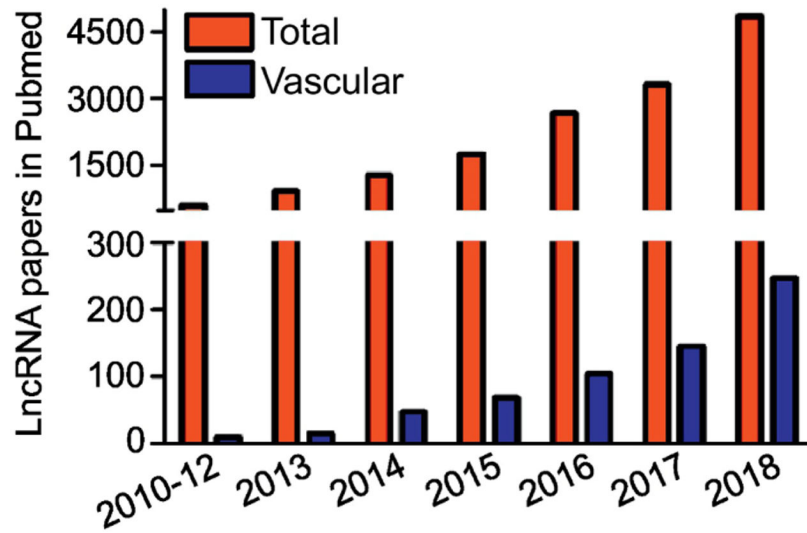


Figure 1. Pubmed lncRNA publications since 2010. Shown are the total and vascular biology-related publications on lncRNAs in Pubmed beginning in 2010. A steady rise in vascular lncRNA publications is evident beginning in 2013.

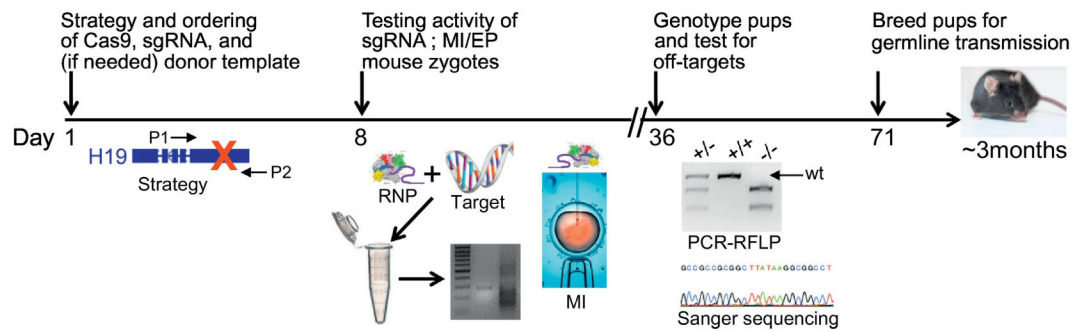


Figure 2. Timeline for generating CRISPRized mice.

The continued refinement in CRISPR components (*e.g.*, store-bought ribonucleoprotein complexes [RNP] of Cas9 and guide RNA) as well as methods of delivery (microinjection, MI versus electroporation, EP) allow for the generation of genetically-modified mice in as little as 5 weeks. Critical steps include the strategy in design with PCR primers (P1, P2) for genotyping founder mice by a PCR restriction fragment length polymorphism (RFLP)-like method and testing for off-targets by Sanger sequencing. Note that germline transmission of CRISPR edited genes typically can occur within 3 months from conception of the project.

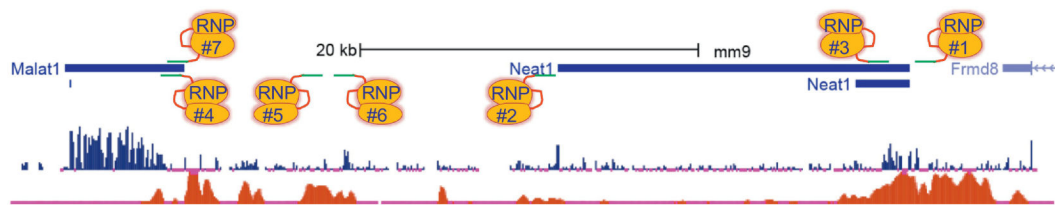


Figure 3. CRISPR editing strategies for lincRNAs.

Screenshot of UCSC Genome Browser tracks are shown with (from top to bottom) the protein-coding and lincRNA genes, vertebrate conservation track (highly conserved sequences indicated by blue peaks), and chromatin signature. The chromatin signature here and in Figures 4-5 represents H3K27Ac in 8 week old mouse hearts. In this figure and below, RNPs are depicted as orange ovals (wildtype Cas9 protein) and red (invariant scaffold RNA) and green (variable, user defined protospacer sequence) lines that constitute the guide RNA. RNPs are numbered here and below to simplify discussion of their genomic location. Genomic scale bar (in kilobases, kb) and the species and genome assembly number (mm, mus musculus and assembly 9) are indicated here and below. Please note, the strategies shown here and below are examples only and are not meant to indicate actual targeting of these specific lincRNAs, some of which (*e.g.*, *Malat1* and *Neat1*) have already been targeted in the mouse. See text for more details.

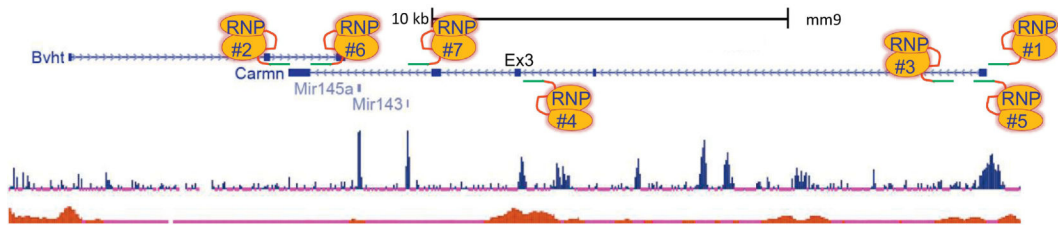


Figure 4. CRISPR editing strategies for a host lncRNA.

Screenshot of the multi-exonic *Carmn* host lncRNA. Blue squares/rectangles are exons separated by introns depicted as a line with arrows in the direction of transcription. Note the presence of highly conserved *miR143/145* cluster and an overlapping lncRNA (*Bvht*). RNP#4 overlays a conserved sequence upstream of exon 3 (Ex3) of *Carmn* that includes a consensus CArG box. Based on previous mouse studies [114], mutation of this CArG box would be expected to abolish *miR143/145* expression in VSMC of the mouse. See text for more details.



Figure 5. CRISPR editing strategies for an intronic lncRNA.

Screenshot of an lncRNA (*4930596121Rik*) within a protein-coding gene (*Dennd1b*). Note that the lncRNA is transcribed in the same (sense) direction as *Dennd1b*. See text for more details.

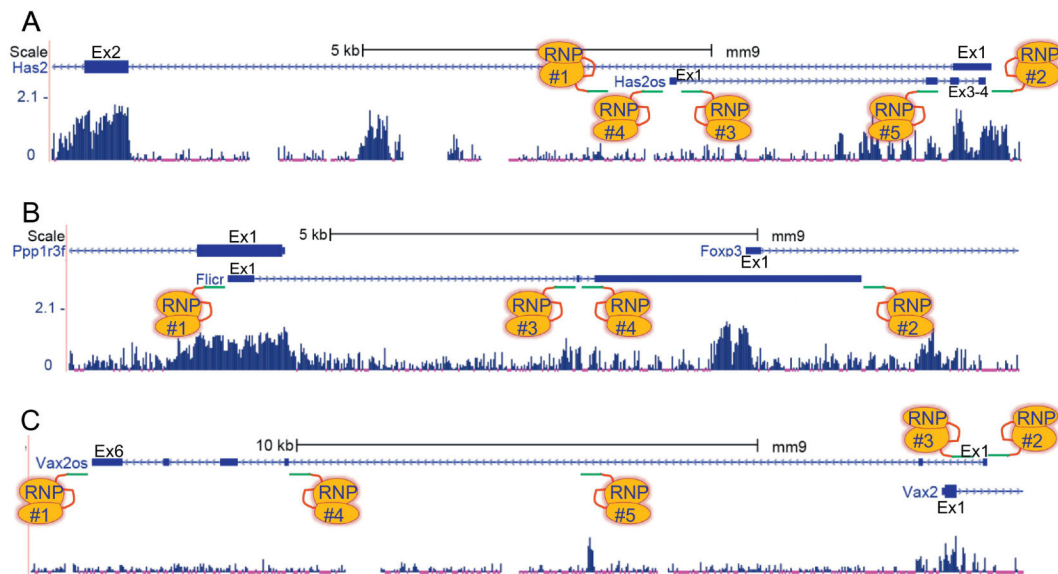


Figure 6. CRISPR editing strategies for natural antisense transcript (NAT) lncRNAs and overlapping lncRNAs.

(A) Screenshot of the NAT lncRNA, *Has2os* (os signifies opposite strand). This lncRNA is transcribed in the antisense orientation to the protein-coding gene, *Has2*. Note the much higher conservation of *Has2* versus the *Has2os* lncRNA. Exons here and below are labeled as Ex1, Ex2, etc. (B) Screenshot of the *Flicr* lncRNA that overlaps two flanking protein-coding genes. (C) Screenshot of a 5' overlapping lncRNA (*Vax2os*) whose exons do not intersect exons of the antisense transcribed protein-coding gene, *Vax2*. See text for more details.

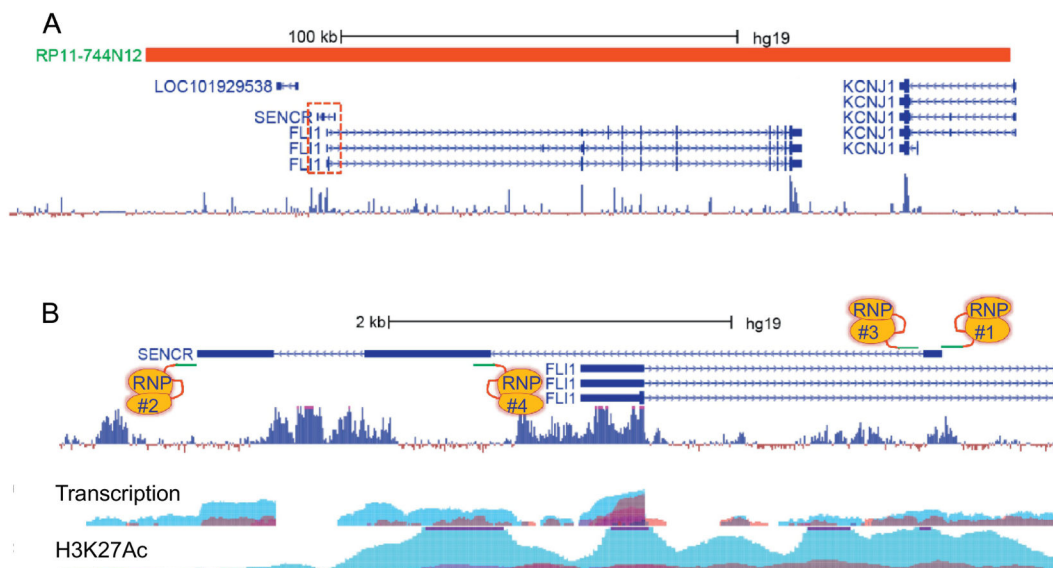


Figure 7. BAC editing strategies for a human lncRNA integrated in the mouse.

(A) Screenshot showing a BAC (RP11-744N12) harboring the human-specific *SENCR* lncRNA that overlaps the 5' end of the *FLII* transcription factor. Note that this screenshot is from human genome (hg), assembly 19. (B) Magnified region (stippled red box in panel A) showing the three exons of *SENCR* in relation to *FLII*. The tracks below *SENCR/FLII* represent ENCODE data relating to transcription and H3K27Ac. The teal colored peaks indicate transcription and H3K27Ac in HUVEC. Note the distinction in transcription of each of the exons of *SENCR* (teal associated HUVEC) versus *FLII* (multi-colored exon 1, indicating transcription in multiple cell types). Note scale bars in panel A versus panel B. See text for more details.