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REVIEW

LncRNAs, nuclear architecture and the immune response

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ABSTRACT

Long noncoding RNAs (LncRNAs) are key regulators of gene expression and can mediate their effects in both the nucleus and cytoplasm. Some of the best-characterized lncRNAs are localized within the nucleus, where they modulate the nuclear architecture and influence gene expression. In this review, we discuss the role of lncRNAs in nuclear architecture in the context of their gene regulatory functions in innate immunity. Here, we discuss various approaches to functionally characterize nuclear-localized lncRNAs and the challenges faced in the field.

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Introduction

Long non-coding RNAs (lncRNAs) represent the largest group of RNA produced by the genome. According to GENCODE release 45, 20,424 annotated lncRNAs in the human genome outnumber protein-coding genes at 19,395 (https://www.gencodegenes.org/human/stats.html). Because of the cell-type specificity of lncRNAs, the more sequencing that is performed, the more lncRNAs that are identified, which possibly explains why the number of these genes increases with each release of GENCODE while proteins remain constant. LncRNAs can be defined by an arbitrary size cutoff of greater than 500 nucleotides, separating them from small RNAs such as tRNAs, rRNA, and miRNAs [1]. They are typically Pol II transcribed and capped, spliced, and polyadenylated like an mRNA, but they do not code for proteins. There is a whole class of lncRNAs originating from enhancer regions, referred to as eRNAs. These typically function within the nucleus at their transcription sites and are involved in chromatin architecture and looping.

LncRNAs are typically classified based on their location within the genomic space, including intergenic, intronic, antisense, and sense. LncRNA naming conventions involve naming them based on their neighboring genes if their function is unknown. Alternatively, lncRNAs are named based on their known function [1]. LncRNAs can act locally to affect neighboring genes on the same allele from which they are produced, which is referred to as acting *in cis* or the lncRNA transcript moves away from the locus to mediate effects broadly, which is referred to as *trans*. Depending on their cellular localization, lncRNAs can act as transcriptional enhancers or translational regulators for different cellular processes, such as viability [2,3], cell cycle [4,5] and differentiation [6]. LncRNAs have also been associated with the development and progression of various pathological conditions, such as cancer [7,8] and autoimmune disorders [9,10].

Over the last decade, lncRNAs have been shown to act as key regulators of gene expression, mediating effects on transcription within the nucleus and translation within the cytoplasm [1]. Their gene regulation capabilities are fundamental for cellular homeostasis and immune responses. In the cytoplasm, lncRNAs have been found to localize to the mitochondria, ribosomes, extracellular membranes, and exosomes, where they can regulate differentiation (linc-MD1), translation (BACE1-AS), and other biological functions, such as signal transduction [11–13]. In the nucleus, lncRNAs are mainly found in nucleoli, chromatin speckles, and paraspeckles, where they regulate gene expression at the epigenetic, transcriptional, and posttranscriptional levels [Figure 1 Lncrnas in the nucleus]. For example,

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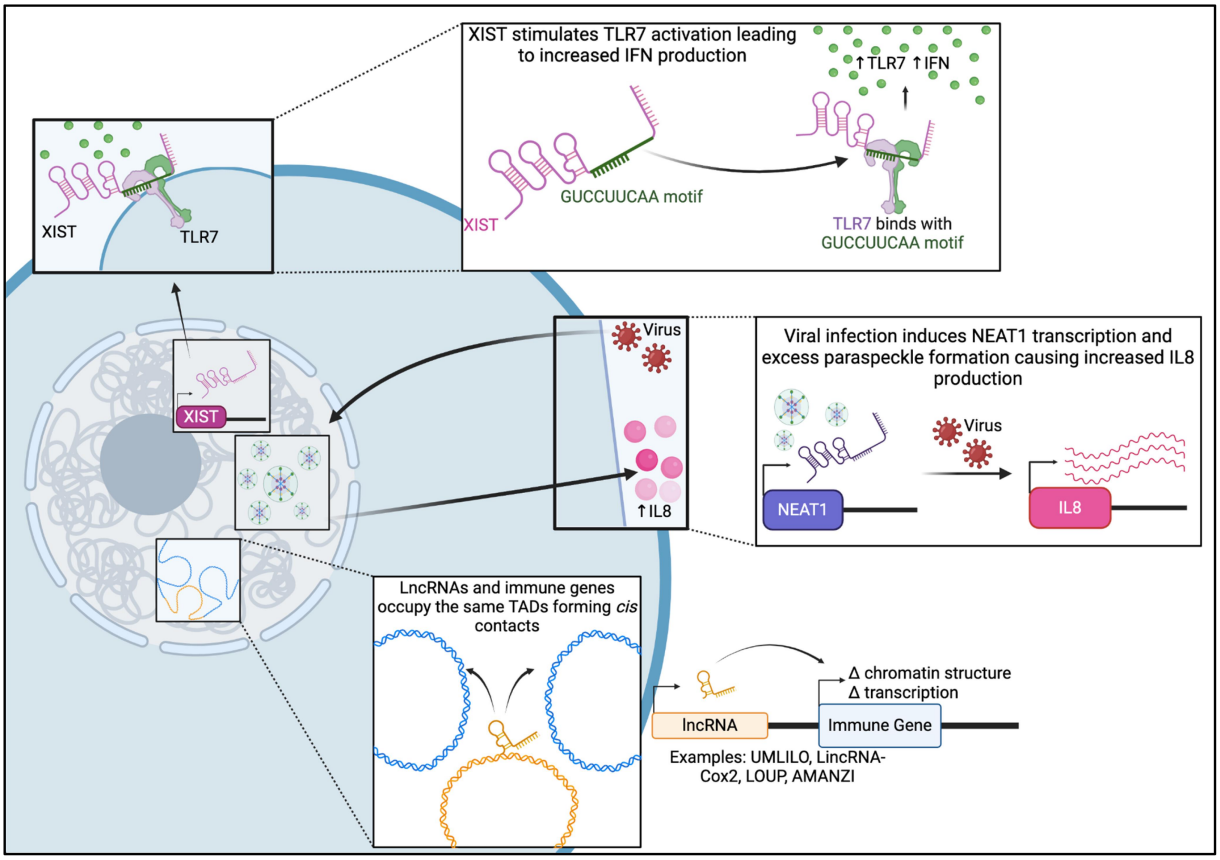

Figure 1. Mechanisms of immune gene regulation by nuclear-localized lncRNAs. **a**, female-specific X-inactive specific transcript (XIST) is a rich source of TLR7 ligands and consequent activation. XIST harbors a GUCCUUAA motif that induces TLR7. This proinflammatory role of XIST is found to be most common in women with systemic lupus erythematous (SLE), possibly explaining why women are more likely to be affected by SLE. **b**, Nuclear enriched abundant transcript 1 (NEAT1) is essential to forming paraspeckles in the nucleus and inducing antiviral cytokine production upon viral infection. NEAT1 transcription is increasingly induced upon viral infection, producing excess paraspeckle formation in the nucleus and inducing IL8 production. **c**, 3D chromatin looping allows lncRNAs to regulate immune genes *in cis*. Upstream master lncRNA of the inflammatory chemokine locus (UMLILO) directs transcription factors across chemokine promoters, enabling epigenetic priming for their transcription. *LincRNA-Cox2* is a multifunctional locus that can regulate Ptgs2 through an enhancer mechanism.

Maternally Expressed gene 3 (MEG3) transcriptionally regulates the TGFb pathway via RNA/DNA triplex formation [14]. Another example is metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), which is found in nuclear speckles and forms a pre-mRNA complex to regulate alternative splicing [15]. Mutations to the miR15/16 binding sites within MALAT1 result in the loss of cytotoxic T cell memory and function [16]. MALAT1 levels have also been shown to decrease in macrophages following viral infection and MALAT1 acts as an inhibitor of type 1 interferons (IFNs) with increased levels of the gene being identified in patients with systemic lupus erythematosus (SLE) [17].

In this review, we highlight the functional mechanisms of lncRNAs involved in regulating the nuclear architecture that have been shown to specifically impact immunity (Table 1). We will focus on emerging interests in the field of innate immune memory and recent studies highlighting that lncRNAs play an important regulatory role in this phenomenon. Finally, we touch upon some of the technical challenges we face in the lncRNA field, especially relating to determining the mechanisms of action of nuclear-localized lncRNAs and speculating on the future of this fast-moving field.

Architectural lncRNAs regulate gene expression by modulating chromatin accessibility and nuclear architecture

The nucleus has many different levels of spatial organization of DNA, RNA, and proteins. Nucleosomes are the basic units of the

Table 1. Nuclear lncRNAS impacting immunity.

LncRNA	Mode of regulation within the nucleus	Impact on the Immune system	Conservation	Reference
XIST	X inactivation in females	X inactivation is lost in activated T cells leading to higher expression of TLR7	Conserved between human and mice	[32–37]
NEAT1	Forms in the nucleus	Important in production during stress	Conserved between human and mice	[48]
LOUP	Regulates its neighbor SPI1 *in cis*	Impacts macrophage differentiation	Cis regulatory effects are conserved between human and mice	[63–64]
LincRNA-Cox2	Functions *in cis* and *in trans* to regulate immune genes	Within the nucleus it can function to regulate its important immune gene PTGS2	To date it has only been shown to function in mice	[56]
UMLILO	Functions *in cis* to regulate viral genes including *IL-8*, *CXCL1*, *CXCL2* and *CXCL3*	regulates chemokine expression important in viral defense	Only expressed in humans but if placed in the mouse synthetic locus it can function *in cis*	[51]
AMANZI	Regulates the IL1 and IL37 TAD	Involved in trained immunity in monocytes	Expressed in humans	[52]
ThymoD	Functions *in cis* to regulate expression of *Bcl11b* as transcription of the lncRNA leads to increased CTCF binding, enabling the long-range chromatin loops to form to enhance transcription	Cis regulation is critical for thymocyte differentiation. Knockout mice display loss of T cell development	Conserved between human and mice.	[18]
HASTER	Function as a transcriptional stabilizer of HNF1A	Removing the lncRNA promoter in mice causes diabetes	Conserved between human and mice	[19]
MEG3	Interacts with chromatin through RNA-DNA triplex formation	Regulates TGFb signaling through interactions with distal regulatory elements	Data shown in human cells but MEG3 does contain conserved pseudoknot structures	[14,20]
MALAT1	Interacts with SR proteins and influences splicing	Knocking out the mir15/16 binding sites in MALAT1 reduced IL2 levels and cytotoxic T cells and responses to LCMV and Listeria	MALAT1 is conserved between human and mouse	[15,16]

chromosome and are composed of DNA wrapped around an octamer of histone proteins. Histone proteins contain tails that can be modified using different histone-modifying enzymes. Depending on these modifications, chromatin can be readily accessible or inaccessible for transcription. Thus, a cell maintains a dynamic transcriptional state by frequently altering its architectural landscape. Therefore, understanding the key regulators of the nuclear architecture is of great significance.

In 1989, Nickerson et al. published a study showing the importance of RNA in the regulation of the nuclear matrix [21]. Treatment of HeLa cells with RNase A or any RNA transcriptional inhibiting drug resulted in dramatic rearrangement of chromatin and loss of nuclear architecture integrity, suggesting that RNA is key for higher-order chromatin formation [21]. Over the last couple of decades, much work has been performed to show that lncRNAs can be key players in regulating the nuclear architecture through a number of mechanisms. Over 100 modifications can occur in histones, which greatly influence gene expression [22]. One of the most intensely studied histone-modifying complexes is the polycomb repressor complex (PRC1 and PRC2) [23]. These complexes can modify histones and induce transcriptional silencing. Interestingly, they do not have DNA-binding motifs, and it has been hypothesized that RNA provides a scaffold for specific targeting although recent studies have called in question the extent to which RNA is involved. PRC2 is considered one of the most promiscuous binders of RNA, which appears critical for the silencing function of the complex, as removal of the RNA results in upregulation of the PRC2 targeted genes [24]. One study reported that PRC2 interacts with over 9000 lncRNAs in embryonic stem cells. They show some specificity in the interactions, with the dominant motifs being G-rich [25–27]. Others have reported that RNA can inhibit PRC2 through blocking its methyltransferase activity and inhibiting its interactions with nucleosomes [28,29] However, in recent months two publications have called into question the

idea that RNA acts as a bridge for PRC2 chromatin occupancy and instead suggest that the earlier conclusions are based on technical artifacts [30,31]. Healy et al., recently reported that the issue with the earlier conclusions lies in the use of RNase A which results in an overall loss of heterochromatin (H3K27me3) and ChIP signals and a gain of non-targeted chromatin [30]. They conclude RNA degradation is not sufficient to displace PRC2 from chromatin and that RNA is important for chromatin solubility during experimentation. Guo et al., have reported that many of the previously reported PRC2-RNA in addition to CTCF and YYI RNA interactions may not occur *in vivo* [31]. These studies highlight the importance of reevaluating data in light of technical advancements and further work is warranted to gain a more complete insight into the specificities of the role that RNA plays in bridging chromatin and modifying complexes.

XIST regulates chromatin architecture leading to X inactivation

In the early 1990s, XIST (X inactive specific transcript) was one of the most intensively studied conserved lncRNA genes. XIST plays a critical role in silencing one copy of the X chromosome in females with early reports indicating this was through interactions with PRC2 [32–34]. In one of the first studies, XIST was shown to be 17kb long with conserved tandem repeats at the 5' end [34]. However, no conserved open reading frames (ORFs) were observed, which led to the speculation that it might act as an architectural RNA. Furthermore, using fluorescence in situ hybridization, they showed that XIST was largely localized in the nucleus in the inactive chromosome territory. Similar studies were carried out in mice, and it was discovered that the XIST transcript was 15kb long [33] and similar to the human transcript, mouse XIST had conserved tandem repeats but no conserved ORFs.

How exactly XIST functions to regulate gene expression on the inactive X has been under intense investigation over the last few decades. At the architectural level XIST has been proposed to paint the entire chromosome impacting chromatin composition, structure, nuclear organization and inducing formation of a heterochromatic Barr Body (reviewed in [35,36]. Recently Spen Family Transcriptional Repressor (SHARP), a histone deacetylase, has been implicated as an important regulator of XIST silencing [37]. Xist recruits SHARP ampliyfing its abundance across the X chromosome. SHARP leads to recruitment of SMRT and subsequent activation of HDAC3. HDAC3 is a histone deacetylase that, once activated, removes acetyl groups from histones, leading to the exclusion of RNA Polymerase II across the X chromosome, leading to its deactivation. This interaction between XIST and SHARP has been confirmed *in vivo* using CLAP and while PRC2 complex is indeed enriched within the silent X there was no direct interaction between PRC2 and XIST observed using this technique [31]. The mechanism by which XIST inactivates the X chromosome serves as evidence that architectural lncRNAs play a spatial and temporal role in regulating gene expression by altering the nuclear architecture.

In addition to its architectural role, XIST can also affect the immune response. Toll-like receptors (TLRs) are type 1 transmembrane receptors responsible for the recognition of conserved components within microbes, leading to the activation of inflammation through the transcription factors NF-κB and IRF3 [38]. TLR7 is an endosomally localized lncRNA that recognizes single-stranded RNA and is encoded on the X-chromosome. TLR7 plays an important role in the autoimmune condition of Systemic Lupus Erythematosus (SLE) owing to the recognition of self-RNA driving pathogenic inflammation. XIST has been implicated in SLE via several mechanisms [39–42]. Studies have shown that T and B cells have altered states of X chromosome inactivation during development, and this is particularly prominent with altered localization of XIST evident in SLE patient cells. Evidence from females and males with Klinefelter syndrome (who carry an extra copy of X) indicates the biallelic expression of TLR7 in immune cells, including dendritic and adaptive immune cells [42]. If cells escape inactivation, this could lead to the increased expression of genes from X, which could play roles in the downstream inflammatory cascades. Some of these mechanisms could help explain the increased incidence of diseases such as SLE in females. This disease-associated effect of XIST is also evident in

female mouse models of lupus, including NZM2328 and MRL/lpr mice [39–41]. Another interesting implication of XIST in SLE is a recent study showing that XIST RNA can function as a ligand for TLR7 [43]. They showed that XIST levels were elevated in patients with SLE, which correlates with an associated interferon (IFN) signature. Interestingly, it appears that XIST is not an IFN-inducible gene, suggesting that it could be a driver of pathogenic IFN responses. They showed that XIST contains the GUCCUUCAA motif, which is a potent inducer of TLR7, and that knockdown of XIST reduced IFN production, indicating that it plays a direct role in receptor activation. The exact mechanism by which this incredibly large RNA can act as a ligand, or how it is processed into fragments to activate the TLR7 pathway remain unclear. There is evidence of XIST in extracellular vesicles which could again aid in its ability to activate cells, but the mechanisms by which it is exported or in what condition it is within EVs are to be determined. A recent study showed that generation of an inducible and non-silencing (repeat A mutant) XIST transgenic overexpressing male mouse resulted in the formation of autoantibodies against XIST associated proteins or ribonucleoproteins (RNPs). These autoantibodies are found in human systemic lupus patient samples also [44]. The adaptive immune repertoire of T and B cells in addition to the chromatin states of these male transgenic mice more closely resembled that of wild type females [44]. Given that the presence of these resulted in increased disease severity in the mouse models of SLE suggests that these novel autoantigens indeed contribute to the disease pathogenesis in models of SLE and in human patients suffering from SLE. These results provide an important window into the possible mechanisms underlying the sex bias observed in SLE.

NEAT1 as regulator of immune gene expression via paraspeckle formation

LncRNAs have been associated with the formation of nuclear condensates, which are important for the spatial control of gene expression within the nucleus. One of the best examples is the nuclear paraspeckle assembly transcript 1 (NEAT1) and its role in paraspeckle formation. Nuclear paraspeckles are among the most well-established condensates or membrane-less organelles (MLOs) that reside within the nucleus and play critical roles in regulating gene expression during stress responses. These nuclear structures were identified by the presence of paraspeckle protein 1 (PSPC1). Interestingly, NEAT1 encodes two gene isoforms (NEAT1_1 and NEAT1_2), with NEAT1_2 being the main driver of the paraspeckle assembly. NEAT1 binds to eight RNA binding proteins (RBPs) to form the paraspeckle, including the splicing factor proline- and glutamine-rich protein (SFPQ), the non-POU domain-containing octamer-binding protein (NONO) found in sarcoma (FUS), RNA binding protein 14 (RBM14), Brahma-related gene-1 (BRG1), DAZ-associated protein 1 (DAZAP1), and two heterogeneous nuclear ribonucleoproteins, HNRNPK and HNRNPH3 [45,46]. Paraspeckle formation is tightly regulated by active transcription and the presence of NEAT1. In the absence of NEAT1 or NEAT1 transcription or active RNA Polymerase II, stable paraspeckles fail to form [47].

Given that NEAT1 forms following a stress response, it is not surprising that it also plays an important role in controlling gene expression following immune cell activation. Imamura et al. showed that NEAT1 can be induced by viral infections (Influenza and Herpes) in addition to stimulation with the TLR3 ligand PolyI:C [48]. The activation of NEAT1 leads to increased paraspeckle formation. Mechanistically, they showed that NEAT1 influences the expression of IL8 through secretion of the splicing factor SFPQ, which typically acts to repress IL8 at its promoter. This relocation of the protein away from the promoter allowed for increased transcription of IL8 which is important for antiviral immunity. In macrophages, paraspeckles rapidly aggregate within 30 min of stimulation and then quickly disaggregate by 2 h post activation [49]. NEAT1 knockout mice cannot control the replication of *Salmonella enterica* or *Vesicular stomatitis virus*. NEAT1 knockout macrophages fail to induce a proinflammatory M1 response and are instead programmed to the M2 phenotype, which is classified as anti-inflammatory and important in wound healing. The rapid induction and removal of paraspeckles in response to

stimulation is a critical regulatory mechanism within macrophages to deal with microbial invasion.

Cis regulatory lncRNAs impacting innate immunity

As described earlier, lncRNAs can function to modulate genes *in cis* meaning they influence their neighbors, or they can work *in trans*, moving away from their site of transcription to regulate genes. Here, we focus on the important cis-regulatory lncRNAs that work within the nucleus to regulate important immune genes and processes. Recent technological advances in chromatin capture techniques, including Hi-C-seq, have shown that the genome can be compartmentalized into topologically associated domains (TADs) brought into proximity by chromosomal loops, which facilitates efficient co-regulation of these genes [50]. Many immune genes lie within specific TADs that enable rapid responses to external stimuli. In addition, emerging evidence for the importance of architectural features is implicated in a phenomenon known as immune training. This involves epigenetic changes and chromatin looping, which influence how cells such as macrophages form short-term memory following an initial stimulus, predominantly β-glucans [51]. If monocytes or macrophages are first treated with β-glucans, it induces a state of priming characterized by the trimethylation of histone H3 at lysine 4 (H3K4me3) at the promoters of primed genes. Following a secondary stimulus such as LPS, these primed genes are produced at higher levels than genes that are not primed. Here, we discuss two new lncRNAs that play important roles in these immune processes.

The regulatory function of UMLILO in the immune inflammatory response and trained immunity

Upstream Master Lnc, the chemokine LOcus (UMLILO), was one of the first lncRNAs to play important cis-regulatory roles in controlling innate immune genes during training [51]. Fanucchi et al. treated monocytes with β-glucans and identified a set of induced lncRNAs, which they named immune gene-primed lncRNAs (IPLs). They focused on the characterization of one IPL, UMLILO, which functions *in cis* directing the WD repeat-containing protein 5 (WDR5)-mixed lineage leukemia protein 1 (MLL1) complex across chemokine promoters, facilitating their H3K4me3 epigenetic priming. UMLILO is not present in the syntenic chemokine TAD in mice, but simply placing human UMLILO in this location results in priming of the neighboring chemokines, confirming that UMLILO directly works *in cis* to mediate its effects. Interestingly, UMLILO itself could be replaced with another WDR5 interacting lncRNA, HOTTIP, which could then perform the same function on CXCL genes as it is the ability of the gene to interact with WDR5, which is important in the regulation of target genes.

AMANZI functions within the nucleus to regulate IL37 and innate immune training

A recent study identified the lncRNA, AMANZI (A MAster Noncoding RNA antagoniZing Inflammation), as a critical regulator within the locus controlling IL1 and IL37 [52]. AMANZI is transcribed in the opposite orientation to IL1b and is a 1000nt polyadenylated transcript. It shares a topologically associated domain (TAD) with IL1b and IL37. Interestingly, IL1b is proinflammatory, whereas IL37 is anti-inflammatory. AMANZI plays a critical role in regulating innate immune training by inhibiting IL1b expression while promoting IL37 expression. SNPs have been identified in AMANZI and are associated with altered responses to infection. The common variant, RS16944, lies within the AMANZI. Individuals with AA at the locus are at increased risk for lethal sepsis as they have higher expression and a more stable AMANZI transcript, which promotes IL37 expression levels while inhibiting IL1b expression. In contrast, individuals with GG show enhanced inflammatory responses with higher levels of IL1b, lower levels of AMANZI, and higher levels of trained immunity in response to glucans. Mechanistically, they showed that the A-site in AMANZI is methylated (6-methyladenosine, M [6]A), which leads to an interaction with

YTH proteins, increasing the stability of the transcript. This locus plays a critical role in trained immunity. Knockdown of AMANZI using locked nucleic acids (LNAs) resulted in enhanced levels of IL1b following β-glucan induction, indicating that AMANZI is a key negative regulator of the circuit. This study provides critical insights into the mechanisms governing training and tolerance in macrophages. Importantly, SNPs lying within noncoding regions can have major impacts on a host's ability to respond to infection. In addition, it is one of a few studies showing that an RNA modification within a lncRNA plays a clear mechanistic role in its function. There is growing interest in the role of RNA modifications in biological processes. The 2023 Nobel Prize in medicine was awarded to Dr. Katalin Karikó and Dr. Drew Weissman for their discoveries of the importance of modifications in suppressing the immunogenicity of RNA [53]. However, there are approximately 160 different classes of RNA modifications and the functional importance of these and specifically their roles in long noncoding RNAs are only beginning to be investigated [54]. Emerging technologies such as direct RNA sequencing will play critical roles in the identification of all RNA modifications *in vivo* and will assist in providing insights into their cell type and context specificity. This paired with techniques such as ASOs and CRISPR will allow for rapid determination of the functional importance of such modifications to the RNA under investigation. Given the recent consensus report from the National academy on Charting a future for sequence RNA and its modifications we predict this will be a very active area of research in the coming years [55].

LincRNA-Cox2 is a key regulator of the inflammatory immune response

LincRNA-Cox2 is an example of a multifunctional locus. It is expressed in myeloid cells and can function *in cis* to regulate its incredibly important neighboring protein PTGS2 (also known as COX2), the key enzyme in the prostaglandin pathway [56–62]. *LincRNA-Cox2* also functions *in trans* to both positively and negatively regulate innate immune genes [56,59]. *LincRNA-Cox2* is capable of exerting these wide-ranging effects because it is expressed in both the cytosol and nucleus. Within the nucleus, it regulates PTGS2 through an enhancer RNA mechanism [58]. Knockout mice were generated by deletion of this locus and replacement with a LacZ cassette. One of the most downregulated genes in these mice is PTGS2. A second spliced mutant mouse was generated by removing the intron. In this mouse model, the exonic sequence of *lincRNA-Cox2* was maintained, but the transcript appeared to be unstable and was no longer inducible following inflammatory activation. Interestingly, in these mice, the PTGS2 locus remained expressed at the same level as in wild-type mice. This indicates that even a very low expression of exonic transcripts is required for PTGS2 regulation. Removal of this locus and replacement with the LacZ cassette does not work, and neither does simple overexpression of the spliced *lincRNA-Cox2* transcript, indicating that the sequence itself is important and that locus-specific expression is required for the regulation of PTGS2.

LincRNA-Cox2 also appears to function within the nucleus to form a complex with HNRNPA2B1 and HNRNPAB to negatively regulate select genes [56]. The exact mechanism by which *lincRNA-Cox2* regulates genes *in trans* remains to be elucidated. However, all the genes that are either downregulated or upregulated following the removal of *lincRNA-Cox2* can be rescued by crossing the knockout mouse with a transgenic overexpressing mouse, confirming that *lincRNA-Cox2* can function *in trans* to regulate certain immune genes. To date, all these studies have been performed in mice, and it remains unclear whether a functional homologue of *lincRNA-Cox2* exists in humans.

LOUP is a multifunctional locus that functions in the nucleus to regulate its neighboring gene SPI1

LncRNA originating from the upstream regulatory element of SPI1 [also known as PU.1] (*LOUP*) was first described in myeloid cells as a gene that functions *in cis* to enhance the expression of its transcription factor SPI1. Mechanistically, *LOUP* forms a complex with RUNX1, leading to

chromatin loop formation and enhanced expression of SPI1 [63]. *LOUP* recently emerged as a hit in two high-throughput CRISPR screens, the first to identify lncRNAs involved in monocyte-to-macrophage differentiation and the second to examine lncRNAs that regulate inflammation through the transcription factor NFkB [64]. *LOUP*, emerging as a hit on the macrophage screen, is consistent with its nuclear role in regulating SPI1, which is a well-known transcription factor involved in myeloid differentiation. *LOUP* appears to be localized in both the nucleus and the cytoplasm. Through Ribo-seq experiments, cytosolically localized *LOUP* was shown to encode short open reading frames, and functional studies indicated that it is a functional peptide that mediates the negative effects of *LOUP* on NFκB-driven inflammation in addition to regulating SPI1 protein levels. In summary, *LOUP* is a multifunctional locus that can affect macrophage development and signaling through distinct mechanisms.

Role for eRNAS in regulating immune gene expression within the nucleus

There are a couple of strategies employed to differentiate eRNAs from other lncRNA classes including using a ratio of the histone marks H3k4me1/H3k4me3. Illot et al. utilized this approach to mark eRNAs with enhancer-like signatures (H3K4me1/H3K4me3 high) and identified 76 lncRNAs that were differentially expressed following LPS stimulation in human primary macrophages [65]. Functionally, they showed that knockdown using locked nucleic acids (LNAs) of the nuclear-localized eRNA *IL1b-eRNA* that surrounded the IL1b locus resulted in a downregulation of transcription and release [65].

LOUP which we identified as an important cis regulator of SPI1 in the nucleus, lies within a super enhancer region in the murine locus [64,66]. Super enhancers (SEs) are so called because they consist of a large cluster of cell type-specific transcription factors and typically have strong histone marks associated with loci including H3K4me1 and H3K27ac modifications. These features are thought to enable cell type-specific activities at these loci and indicate their importance in cell identity and cell fate. For *LOUP* we believe this is indeed what we are observing, as *LOUP* is a strong hit in monocyte-to-macrophage differentiation. For additional information on eRNAs in inflammation, we have directed you to the following reviews [67,68].

Approaches to mechanistically understanding nuclear lncRNAs

LncRNAs are highly cell type-specific in expression, are often not conserved at the sequence level, and can be very lowly expressed, all of which can be technically challenging to gain mechanistic insights. There are currently over 20,000 annotated lncRNAs, but only a few hundred have been functionally characterized [69,70]. The development of optimal tools to characterize and rapidly functionally validate lncRNAs in biological systems is required to gain insight into these genes. *In vitro* determination of lncRNA function generally involves the use of oligo-based approaches, such as siRNAs or locked nucleic acids (LNA). Although siRNAs can be useful for studying genes in the cytoplasm, they have limited efficacy within the nucleus. To target nuclear lncRNAs, antisense oligos are typically employed, including LNAs, which are effective in targeting AMANZI in primary human cells and enhancer RNAs [52,65]. Here, we focus on attempts to functionally characterize lncRNAs *in vivo* as well as in a high-throughput manner.

Generation of genetic mouse models to determine lncRNA functions

Owing to the revolution in CRISPR technology, it is possible to quickly generate gene knockouts. For lncRNAs, a number of considerations are required in order to attempt to understand the complexity of a given locus.

Removing the locus

Cas9 and multiple guide RNAs (sgRNAs) targeting the 5' and 3' ends can be employed to remove lncRNAs. This crude method is rapid and can be a useful starting mouse model because if you do not see any phenotype in this model then perhaps the locus is not worth pursuing further. There are

several caveats to such models. Removing the entire locus could result in local destabilization of chromatin architecture and lead to off-target effects. It also does not provide insight into whether RNA is important for a particular function or whether a DNA element is important. Sauvageau et al. generated a catalog of 20 lncRNA-knockout mice by replacing the lncRNA loci with a LacZ reporter [71]. This technical approach is useful as it maintains the transcriptional activity of the lncRNA by leaving the first exon intact, and lacZ serves as a reporter read out for each gene, allowing for accurate expression mapping. This approach proved fruitful and showed the importance of several lncRNAs (*Fendrr, Peril, Mdgt, lincBrn1b and linc-Pint*) that play roles in a variety of developmental processes [71].

Transcriptional disruption

To determine whether it is indeed the RNA transcript that is important for a given phenotype, it is necessary to inhibit transcription. This can be achieved by inserting a strong stop signal in the first exon of a gene. For example, the insertion of multiple polyA stops can be effective in aborting transcription. If a phenotype is observed, then this is strong evidence that the RNA being produced mediates the effect. A recent and elegant approach to highlight the importance of genetic mouse models to unravel the complexities of the *lincRNA-p21* locus was developed by Dr. Nadya Dimitrova [72]. They heroically produced four mouse models to understand how *lincRNA-p21* controls its neighboring protein p21 *in cis*. The first mouse was generated using CRISPR-Cas to introduce a 49-nucleotide synthetic polyadenylation signal (PAS) that effectively killed transcription, and the second mouse involved the insertion of a 74-nucleotide *Twister* (TWI) self-cleaving ribozyme. The beauty of these two mice is that the first simply stops all transcription, whereas the second allows for transcription and subsequent degradation of the transcript. They found that transcription of the full-length *lincRNA-p21* is dispensable for p21, as the levels of the protein were the same in both knockout models as they were for the wild type (WT). To further understand how this locus can function *in cis* they knocked out two sections (127 nucleotides and 150 nucleotides) that involved the removal of the first exon. Both mice had severe loss of p21, suggesting that there is a regulatory element within exon 1 that is critical for the regulation of the neighboring locus *in cis*. *In vitro* they introduced dead RNAs into the first exon using Cas9 and showed that transcription of the first exon plays a role in this phenotype, as all dead RNAs tested knocked down both *lincRNA-p21* and p21. This study concluded that nascent transcription from this lncRNA locus, but not the generation or accumulation of a mature lncRNA transcript, is important for cis-regulation. This study highlights the complexities of these lncRNA loci and how a single genetic approach might not provide a complete picture of the workings of a given lncRNA.

Genetic mouse models to determine trans regulation of lncRNAs

As described thus far, determining the mechanism of action of a given lncRNA can be complex. If lncRNAs do not function *in cis* to impact a biological process, they function *in trans* away from their transcription sites. Lessons from protein biochemistry can be used to generate mouse models to understand the trans-regulation of lncRNAs. If a lncRNA functions *in trans*, it should be possible to overexpress the lncRNA from a non-native locus, cross it to a deficient animal, and rescue the phenotype to wild-type levels. There are several considerations in this approach. What locus should be selected for trans expression of the lncRNA? Examples include safe harbor loci such as the Rosa 26 locus or the H11 locus. Should the promoter of the safe harbor site be used or should a strong promoter, such as the CAG promoter, which is expressed in all cells, be utilized? Alternatively, should the native promoter of a gene be used? Should the spliced version of the lncRNA be expressed or the full transcript allow for splicing? Each of these considerations can affect the phenotype of a given animal. We utilized a version of this approach to understand whether the lincRNA, *lincRNA-Cox2*, can function *in trans* to regulate immune genes. We

placed the spliced gene into the H11 locus using the TARGATT system and used the CAG promoter to drive optimal gene expression. We crossed these mice with *lincRNA-Cox2* deficient mice and rescued most of the genes back to WT levels [59,60]. If this approach failed, it could have indicated that the native promoter is important or that splicing and possible modifications that occur at the native site are important for the function of the gene. Lewandowski et al. generated genetic mouse models to elucidate the complexities of the TUG1 locus [73]. They originally generated the TUG1 knockout as part of their 18 knockout cohort mentioned earlier and showed that it is important for male fertility. Their newer model revealed that there is a DNA element within the locus that is important for cis regulation of the neighboring gene *in cis*. They generated a mouse overexpressing TUG1, which partially rescued the knockout phenotype. They showed that the locus also contains a small open reading frame that mediates trans-effects and is highly conserved. These studies highlight the importance of using genetic mouse models to elucidate the complexities of these gene loci. One important limitation of this approach is that many lncRNAs are not conserved across species and so this approach only allows for a deep mechanistic insight into murine trans regulating lncRNAs.

Using CRISPR screens to identify functional lncRNAs

While genetic mouse models are critically important for understanding the specific molecular mechanism of a lncRNA, they are limited in several ways, including low throughput and high cost. We have only begun to scratch the surface regarding the functional importance of lncRNAs in immunity. Given the fact that there are over 20,000 genes, it can be daunting to even know where to begin in terms of understanding which of the 20,000 genes are important in which biological process. The laborious process of studying genes one at a time could mean that we never determine which are important in this lifetime. One approach that allows for the rapid screening of functional lncRNAs is high-throughput CRISPR screening. As lncRNAs do not have open reading frames, CRISPR inhibition is the method of choice for screening. Once the transcriptional start site (TSS) of the gene of interest is known, catalytically inactive Cas9 can be targeted, and the attached KRAB domain induces heterochromatin and knockdown expression of the locus. This approach is powerful for targeting any gene, whether it is a lncRNA, a protein, or a microRNA, once the TSS is known. In our experience, lncRNAs are poorly annotated in the human and mouse genomes, and given their exquisite cell type specificity, it is important to map TSS sites in the cells of interest to ensure an accurate targeting library. CRISPRi works in a tight window around the TSS; therefore, it is preferable to layer additional data such as ATAC-seq or CHIP-seq for specific transcription factors from the cells of interest to help narrow the window surrounding the TSS. Long-read data from PacBio or Oxford Nanopore in your cells can also be powerful for understanding specific isoforms of genes expressed in the cell of interest, and all these data together can enhance the specificity and reproducibility of the screen. To date, only a small number of lncRNAs have been screened. Liu et al. published one of the first CRISPRi-based screens that identified 499 lncRNAs involved in cell viability [74]. This study set a benchmark for lncRNA screening and showcased the cell-type functional specificity of lncRNAs across seven human cell lines. Liu et al., used a different approach to study functional human lncRNAs by targeting their splice sites using active Cas9 [75]. They targeted approximately 11,000 lncRNAs and identified 230 lncRNAs involved in the growth of K562 cells. Screening can be difficult for a number of reasons, including complex cloning and execution for analysis and confirmation. For this reason, screens are inherently noisy and require a robust readout. Growth screening is among the most straightforward. Screening for immune regulators can be a challenge because of the rapid response speed and inability to enrich over time (an approach that is easily utilized in growth screens). We recently performed two pooled screens, the first to identify lncRNAs functioning in macrophage differentiation and the second to identify lncRNAs that are important in NF-κB signaling. The differentiation screen was more straightforward, as we utilized a THP1 monocytic cell line that is in suspension and cells differentiate into adherent cells following treatment

with phorbol esters. The second screen involved the use of our newly generated NFkB-GFP reporter, which allowed for a sorting-based screen. Cells were stimulated with LPS and the top and bottom 20% of cells represented positive and negative regulators of the pathway [64]. We identified 38 hits from the differentiation screen and 35 hits from the NF-κB screen. We characterized one hit from both screens of the lncRNA *LOUP*, as described above. A major conclusion from our screening to date is that the hits are dominated by loci that neighbor important protein-coding genes. This suggests that lncRNAs are cis-regulators. Another impact is that CRISPRi can extend in both directions from a TSS and can impact a neighbor if it is close by. However, even with all the caveats associated with screens, these are powerful tools to rapidly identify functional lncRNAs, and when performed in a careful and thorough manner, can yield important information on candidate lncRNAs that are worth pursuing further.

Challenges and future perspectives

The field of lncRNA research remains in its infancy. Although an increasing number of lncRNA transcripts are being identified through RNA-seq, we are lagging in functional characterization. As newer technologies emerge and better approaches for teasing apart DNA, RNA, and protein functions come to the fore, the better picture we will obtain on the landscape of this large group of genes. Perhaps, they will eventually be sorted into families based on their effector functions. There are several outstanding questions in the field of lncRNA research. How many lowly expressed RNA transcripts are important in biological processes, and what are the best approaches for determining function? How critical are lncRNAs to disease pathogenesis? Most causal SNPs lie outside coding exons, and how many of them are within lncRNAs and are important in disease phenotypes? How many lncRNAs are conserved amongst species? What does conservation really mean when we discuss it in relation to non-coding loci? How important are RNA modifications within long noncoding RNA to their biological functions? These are just a small number of the important questions that we are faced with. This is a rapidly evolving field, and exciting work is needed to unravel the complete roles of these genes in various biological processes and diseases.

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C. M., C. F. A., and S. C. were involved in the conceptual design, writing, and reviewing of the manuscript.

Data availability statement

No new data were generated for this study. All referenced data are available in cited publications.

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