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Review

The how and why of lncRNA function: An innate immune perspective☆

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ABSTRACT

Next-generation sequencing has provided a more complete picture of the composition of the human transcriptome indicating that much of the "blueprint" is a vastness of poorly understood non-protein-coding transcripts. This includes a newly identified class of genes called long noncoding RNAs (lncRNAs). The lack of sequence conservation for lncRNAs across species meant that their biological importance was initially met with some skepticism. LncRNAs mediate their functions through interactions with proteins, RNA, DNA, or a combination of these. Their functions can often be dictated by their localization, sequence, and/or secondary structure. Here we provide a review of the approaches typically adopted to study the complexity of these genes with an emphasis on recent discoveries within the innate immune field. Finally, we discuss the challenges, as well as the emergence of new technologies that will continue to move this field forward and provide greater insight into the biological importance of this class of genes. This article is part of a Special Issue entitled: ncRNA in control of gene expression edited by Kotb Abdelmohsen.

1. Introduction

One of the most profound discoveries from the sequencing of the human genome is that over 85% of the genome is transcribed, yet < 2% encodes protein-coding genes [1]. Large consortiums such as ENCODE and FANTOM have embarked on attempting to characterize all functional coding and noncoding elements in the genome and have compiled important regulatory data for these elements [2–4]. Long noncoding RNAs (lncRNAs) represent the largest group of non-coding RNAs produced from the genome. LncRNAs are defined as transcripts > 200 nucleotides in length, lacking protein-coding potential. In the most recent GENCODE V30 release, there are 16,193 annotated lncRNAs in the human genome [4]. Additionally, there are over 14,000 pseudogenes, that could fall under the description of long noncoding RNAs which is simply based on them being 200 nucleotides or greater in length. Less than ~3% of annotated lncRNAs have ascribed functions. Hence this class of RNAs is greatly in need of further investigation [4]. From those that have been characterized, it is clear that lncRNAs can function through a variety of mechanisms to regulate gene expression both at the transcriptional and post-transcriptional levels [5].

As we will discuss through this review lncRNAs can mediate their functions through interactions with proteins, RNA, DNA, or a combination of these. Furthermore, the function of lncRNAs can often be dictated by their localization, sequence and/or secondary structure. There are many categories and sub-categories of lncRNAs, but some of the major classifications include: antisense [6], bi-directional [7], enhancer-associated [8], intergenic lncRNAs (lincRNAs) [9], pseudogenes [10], while a full review of all classifications can be obtained from the recent review by Jarroux et al. [11]. LncRNA function cannot be determined simply based on the lncRNA classification. However, the classification can sometimes provide insight into its mechanism of action, such as antisense lncRNAs impacting their neighboring genes. However, this same classification can also lead to erroneous assumptions about how the lncRNA regulates gene expression. Recently, some lncRNAs have been demonstrated to actually encode small peptides indicating that these genes are misclassified as noncoding, although it is possible that they could also have functions as a noncoding RNA in addition to their peptide coding capacity [12–17]. It is therefore, important to have a logical methodology to study the biological importance of these genes.

The innate immune system functions as a rapid initial response against specific pathogens, while also promoting the activation and development of the adaptive immune system [18]. Macrophages and dendritic cells are important innate immune cells that initiate the immune response through recognition of specific pathogen-associated molecular patterns (PAMPs) through their germline-encoded pattern

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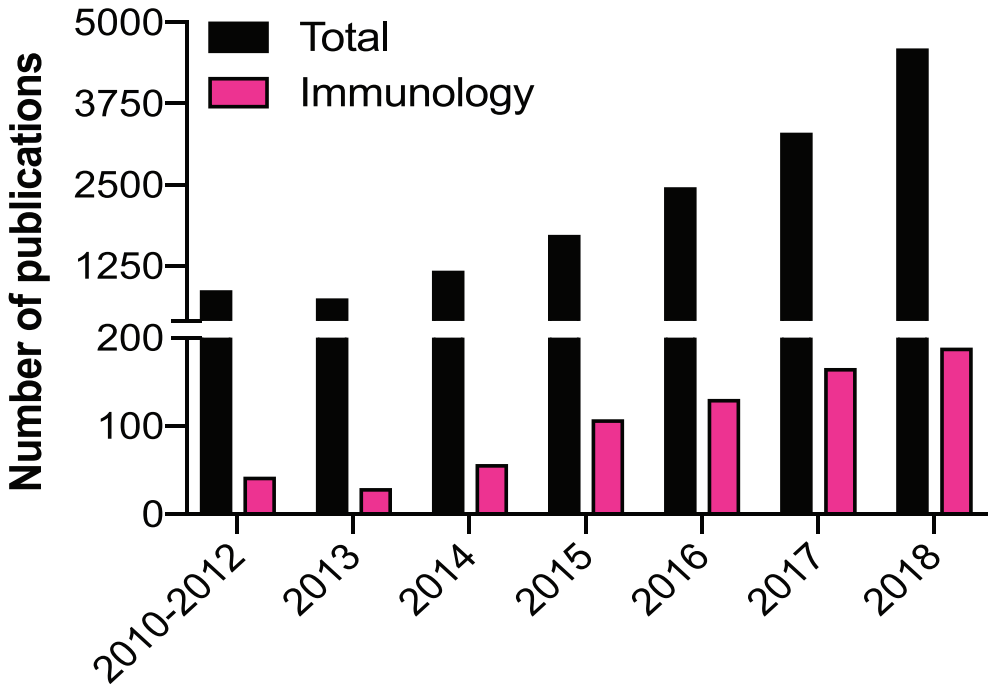


Fig. 1. LncRNA publications.
Graph representing the total number (*black bar*) or immunology related (*pink bar*) publications in the lncRNA field since 2010 to 2018.

recognition receptors (PRRs) [18]. These receptors couple pathogen-sensing to activation of downstream signaling cascades resulting in up-regulation of numerous inflammatory pathways [19]. While a robust immune response is crucial for eliminating pathogens, prolonged activation can be detrimental to the host [20]. Not surprisingly, many aspects of the inflammatory response are tightly regulated at both transcriptional and post-transcriptional levels allowing for a transient antimicrobial response while subsequently promoting a return to homeostasis [21]. Perturbations in this regulation can have significant consequences that can manifest in diseases, such as arthritis [22], multiple sclerosis [23] and cancer [20,24]. While the role of coding genes in immune cell function has been well characterized, the role of lncRNAs in these processes is just beginning to emerge [25] (Fig. 1). Here, we use the biological model system of macrophage activation as a framework to demonstrate how we approach the study of lncRNA biology. We provide a step-by-step guide to consider when studying lncRNAs. Furthermore, we discuss the challenges, as well as the emergence of new technologies that are helping evolve the ways we study these genes.

2. Getting started

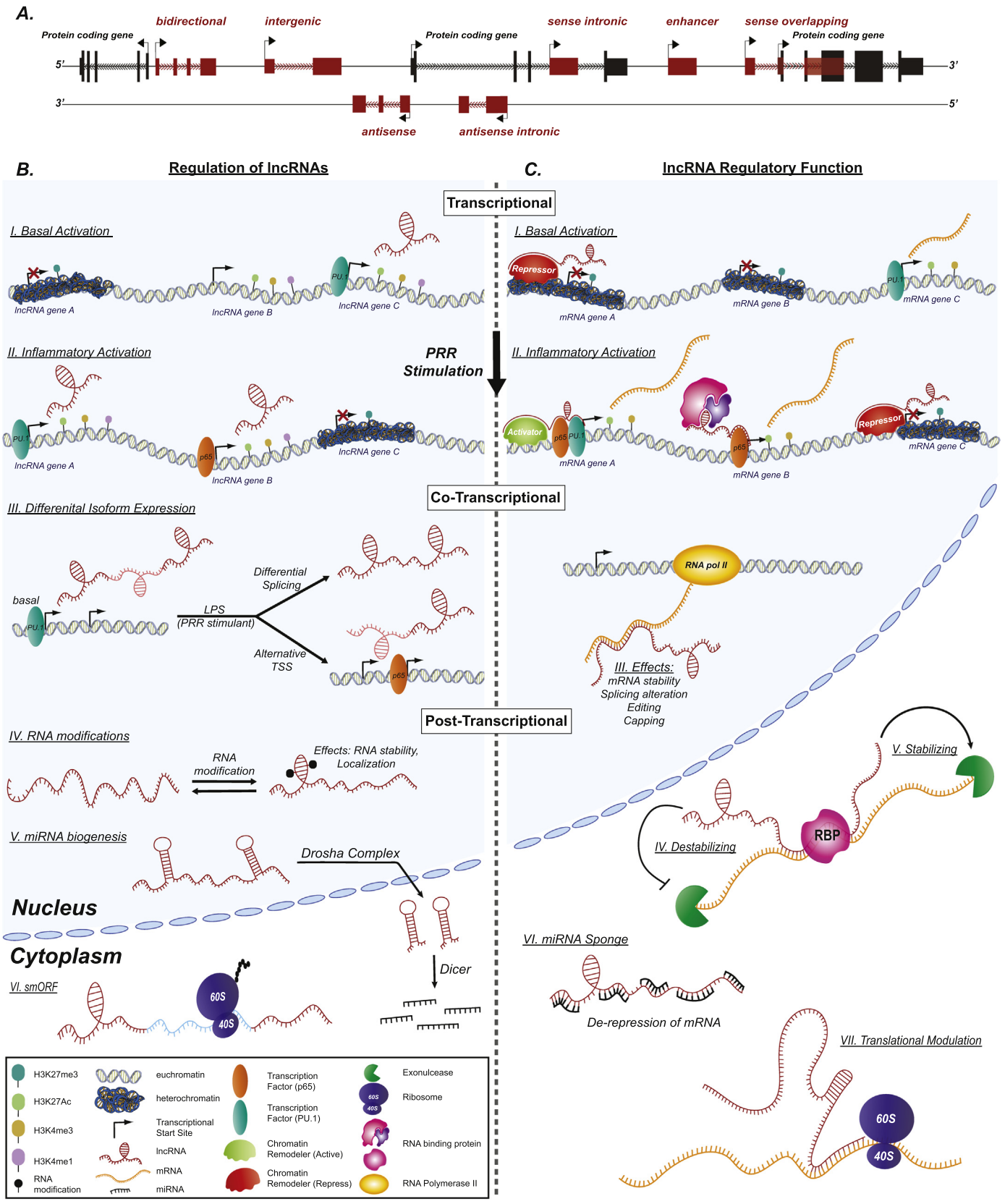
2.1. The biological question

The lncRNA field is in its infancy yet from what we do know we find that lncRNAs play critical roles in a wide variety of biological processes and diseases from cell differentiation, tissue organ development, flowering in plants, to cancer metastasis to name just a few [26–30]. We believe that lncRNAs play regulatory roles in many biological processes and diseases. Therefore, no matter what your research area is, there is a rich source of information to be obtained from the study of lncRNAs in your field of interest.

The bulk of lncRNA studies to date have focused on the cancer field [31–33]. Meanwhile, studies of lncRNAs in the context of innate immunity have lagged, making up only ~4% of all lncRNA papers to date (Fig. 1). The innate immune system provides one of the first lines of defense against infection through the induction of inflammation [34,35]. The inflammatory response of murine macrophages offers a powerful system for applying genomic approaches for studying novel lncRNAs within the framework of a pathway that has been studied for decades. Macrophages are important mediators of inflammation and initiate this response through recognition of specific pathogen-associated molecular patterns (PAMPs) through their germline-encoded pattern recognition receptors (PRRs). These receptors couple pathogen-sensing to activation of downstream signaling cascades resulting in activation of numerous transcription factors, including NF-kappaB (NF-κB) and interferon regulatory factors (IRFs) that can act in combination to both positively and negatively regulate the expression of thousands of genes [36,37]. There are 10 different TLR genes in the human genome and 13 TLR genes in mice [38–40], each binding a different PAMP [41]. Using this extensively studied biological system, we identified the first example of a TLR-stimulated lncRNA, lincRNA-Cox2, which was capable of positively and negatively regulating distinct types of innate immune genes [42–46]. Knockdown of lincRNA-Cox2 resulted in impaired production of proinflammatory genes (*i.e.*, IL-6), while IFN-related genes were hyperactivated in the absence of lincRNA-Cox2 [42–46]. Numerous other studies have made use of the TLR-signaling biological system, uncovering and characterizing dozens of novel lncRNAs that act in a wide range of mechanisms to either positively or negatively regulate this pathway as reviewed in Carpenter et al. and Hadjicharalambous et al. [47,48].

2.2. LncRNA candidate selection

As mentioned lncRNAs are categorized into five main classes of long noncoding RNAs based on their genomic location: antisense, bidirectional, intronic, enhancer-associated, and intergenic. Intergenic and enhancer lncRNAs contain their own promoters and are distinct from protein-coding genes. Bidirectional lncRNAs share a promoter and are transcribed from the opposite strand of a protein-coding gene, while intronic lncRNAs are transcribed within an intronic region of a protein-coding gene (Fig. 2A) [49,50]. The specific class of lncRNAs can often provide significant insight into how it may regulate gene expression. For example, lncRNAs antisense to a coding gene have been



(caption on next page)

Fig. 2. Classification and regulation of LncRNAs.
(A) Positional classifications of lncRNAs based on their genomic location in respect to nearby protein coding genes: bidirectional, intergenic, antisense, antisense intronic, sense intronic, enhancer, sense-overlapping. (B) The lower left panel of the figure represents how lncRNA activity can be regulated transcriptionally. Transcriptional activation is depicted basally 'Part I', as well as during inflammatory activation 'Part II' following stimulation of a pattern recognition receptor (PRR). Three lncRNA examples genes are shown A, B and C. Part III depicts how a lncRNA can undergo differential isoform expression which is regulated co-transcriptionally. During active transcription a lncRNA can either undergo differential splicing or utilize a new transcriptional start site post inflammatory stimulation, such as lipopolysaccharides (LPS). Post-transcriptional regulation of a lncRNA is broken up into three parts: IV, V and VI. After transcription is completed there is several processes that a lncRNA can undergo. Part IV depicts RNA modifications, which can change the structure of a lncRNA molecule. These modifications can be added or removed depending on the inflammatory state of a cell. Part V depicts the process of miRNA biogenesis, where a lncRNA can be processed to a mature miRNA. Part VI shows that a lncRNA can also be translated if it has a small open reading frame (smORF). (C) The lower right panel of the figure illustrates the regulatory function of a lncRNA in the nucleus and the cytoplasm. During active transcription (basally 'Part I' or inflammatory 'Part II'), a lncRNA can function to repress genes (mRNA gene A and C) or activate genes (mRNA gene A and B). A lncRNA can either be a scaffold for transcription factors to enhance activation, or a scaffold for chromatin remodeler proteins to open or close chromatin. A lncRNA can also regulate a mRNA transcript co-transcriptionally 'Part III' by affecting either the stability, change the splicing activity, editing of modifications, or even the capping of the mature mRNA. Finally, post-transcriptionally a lncRNA can function to regulate the mRNA in several ways. A lncRNA can affect the stability of a mRNA transcript: 'Part IV and Part V.' Alternatively, a lncRNA can function as a miRNA sponge which indirectly de-represses the expression of a mRNA that would be targeted by the miRNAs. Lastly, a lncRNA can modulate the translation of a mRNA by binding to ribosomes or mRNA transcripts during translation.

demonstrated to be involved in transcriptional interference, negatively affecting the expression of their coding gene [51]. While all categories of lncRNAs will no doubt be important in various biological processes, the most targetable lncRNAs are intergenic lncRNAs. A benefit to studying an intergenic lncRNA (lincRNA) is the immense variety of molecular techniques that would not apply to other types of lncRNAs, such as antisense, bidirectional and intronic lncRNAs, which often overlap coding genes and their targeting could lead to possible unwanted interference of that coding gene.

2.2.1. Cis regulators

Numerous studies have established that lncRNAs can regulate the expression of their neighboring coding genes (*cis* regulation) [51]. A recent study by Engreitz et al. demonstrated that a significant portion of lncRNAs had *cis* effects on their neighboring genes [52]. Interestingly, in most cases, the *cis* effect did not require the production of the lncRNA transcripts themselves, but instead required the processes associated with their production, such as transcription and splicing [52]. There are many examples of lncRNAs that regulate their neighboring coding genes, for example: lnc-MARCKS, lnc-TNFAIP3, AS-IL1α, lnc-IL7R, and IL-1β-eRNA [52–56]. Recently we used genetic mouse models to show that lincRNA-Cox2 can function as an enhancer RNA *in cis* to regulate its neighboring gene Ptgs2 [46]. For these reasons, one aspect to consider when selecting a candidate, no matter the class, is to investigate the effect of the transcriptional expression on neighboring coding genes. This candidate selection approach is sometimes referred to as "guilt by association" [57,58]. This bioinformatic approach drives an initial hypothesis that the lncRNA could be involved in the similar biological pathway as their neighboring protein-coding gene due to their co-expression.

2.2.2. Trans regulators

A large number of studies to date have also shown that lncRNAs can regulate gene expression on different chromosomes (*trans* regulation) [59] (Fig. 2C). The majority of lncRNAs studied in immunity were initially identified following RNA-sequencing to examine their expression profiles in specific cell lines or tissues during inflammatory activation. For example, lincRNA-Cox2 was initially identified as an up-regulated lncRNA in murine dendritic cells following TLR4 stimulation [60], as well as murine bone marrow derived macrophages (BMDMs) following TLR2-depedent stimulation [42]. Studies have also highlighted the functions of lncRNAs that are highly downregulated post inflammatory activation, such as lincRNA-EPS [61] and lnc13 [62]. Both lincRNA-Cox2 and lincRNA-EPS were discovered in BMDMs post TLR inflammatory activation and were chosen for further characterization based on their extreme expression profile. LincRNA-Cox2 is rapidly upregulated and regulates a large number of interferon stimulated genes (ISGs) and NF-κB regulated genes [42]. Meanwhile, lincRNA-EPS is rapidly down-regulated during inflammation and acts as an inflammatory brake on all ISGs during periods of homeostasis [61]. These are just two examples of lncRNAs that have provided critical insights into the roles of lncRNAs in immunity. For more information on specific lncRNAs that are involved in innate immunity we direct you to the following recent reviews on this topic [42,47,63]. In addition to these bulk RNA sequencing studies, a small number of single cell RNA sequencing studies have been performed in both human and mouse that can be utilized to examine differential expression of lncRNAs in basal *versus* treatment conditions or between cell types [64–68]. Numerous RNA-seq (both bulk and single cell) datasets are available for a variety of primary cells, cell lines or tissues of interest either basally or under a multitude of inflammatory or cellular differentiation treatments. These datasets outlined in Table 1 [42,46,74–77, 57, 61, 64, 69–73] and Table 2 [53,56, 82–90, 65–68, 78–81] provide a rich source of lncRNAs for further investigation.

EVLncRNAs [91], NONCODE [92], or LNCipedia [93] are databases that categorize published information on all annotated lncRNAs. These databases can be utilized to determine if a lncRNA is experimentally validated within one or more studies. Additionally, these databases can provide information on whether a lncRNA possesses multiple isoforms, secondary structure, cross-species conservation and/or disease-association *via* presence of single nucleotide polymorphisms (SNPs).

2.3. LncRNAs expression and specificity

Multiple studies have demonstrated that lncRNA expression is more cell type specific compared to protein-coding genes [94–96]. Such specific expression patterns can often provide important clues into the specific biology that the gene could be involved in [46] (Tables 1 and 2). A variety of consortiums exist for both human and mouse and can be utilized to determine cell type specificity of a lncRNA candidate further. For instance, GTEx [97] and XENA [98] are two websites that include RNA sequencing on healthy primary human tissue samples, in addition to samples from patients with diagnosed cancers. This will further assist the initial understanding of the expression of the lncRNA in specific tissues as well as obtaining information on whether a lncRNA is involved in cancer. In contrast, if a researcher is studying a mouse candidate lncRNA, the Mouse Cell Atlas (MCA) [99], as well as Tabula Muris [100] are excellent tools to assess specificity in cellular expression, as well as differential splicing isoforms amongst differentiated cell types. Additional websites for mouse and human expression datasets can be found at the ENCODE project [101], the European Bioinformatics Institute Expression and the FANTOM projects [102]. These sites are filled with raw and analyzed data sets from either single cell or bulk RNA sequencing from primary cells, tissues or immortalized cells ready to use to determine the statistical significance of expression for any annotated lncRNA candidate.

Table 1
Murine sequencing datasets.

	Type	Treatment	Duration (h)	Sequencing	PMID
Spleen	Tissue	TLR4	0, 6	RNA	30404006
Lung	Tissue	TLR4	0, 6	RNA	30404006
		SARS-CoV	0, 24	RNA	27462873
		PR8	0, 24, 48, 120	RNA	20978541
Monocyte	FACs sorted	–	–	RNA	24586061
				ATAC	
				ChIP - H3K27Ac	
				ChIP - H3K4me1	
				ChIP - H3K4me2	
				ChIP - H3K4me3	
Dendritic cells	CD103+	PR8	0, 24, 48, 120	RNA	20978541
	Bone marrow derived	None	–	RNA	19182780
		TLR1/2	0, 6		
		TLR4	0, 6		
		TLR9	0, 6		
Macrophage	Alveolar macrophage depletion	NS1-GFP	0, 12	RNA	30886410
	Bone marrow (monocyte)	–	–	RNA	25480296
	Lung tissue resident			ATAC	
	Spleen tissue resident			ChIP-H3K4me1	
	Kupffer cells			ChIP-H3K4me3	
	Peritoneal macrophage			ChIP-H3K27Ac	
	Colonic macrophage				
	Microglia				
	FACs Sorted	–	–	RNA	25103404
				ATAC	
				ChIP - H3K27Ac	
				ChIP - H3K4me1	
				ChIP - H3K4me2	
				ChIP - H3K4me3	
	Bone marrow derived	TLR4	0, 0.25, 0.5, 1, 2	RNA	22817891
				Chromatin, nucleus, cytoplasm	
		Mtb	0, 4, 12, 24, 48	RNA	29712924
		IFNγ Mtb	0, 4, 12, 24, 48		
		IL4/IL13 Mtb	0, 4, 12, 24, 48		
		TLR1/2	0, 5	RNA	23907535
		TLR4	0, 2, 6	RNA	27315481
		TLR4	0, 2, 6	ATAC	
		TLR4	0, 1	ATAC	26924576
		TLR1/2	0, 0.25, 0.5, 1, 2	RNA	
		TLR4	0, 0.25, 0.5, 1, 2	RNA	
				ChIP - SRF	
				ChIP - IRF3	
				ChIP - p65	
		TLR4	0, 1	ChIP - H3K27Ac	29779944
				ChIP- HeK4me2	27462873
				ChIP - PU.1	
				ChIP - Nrf2	
				ChIP - p65	
				ChIP - Smad3	
				ChIP - Fos	
				RNA	

2.4. Determining disease association

Genome-wide association studies (GWAS) have revolutionized the study of complex diseases by allowing quantitative disease-association of thousands of genetic loci [103]. These studies include evaluation of single-nucleotide polymorphisms (SNPs) or deletions and determination of their association with a disease phenotype. Diseases studied range from Inflammatory Bowel Disease (IDB) to schizophrenia [104,105]. Until recently, most GWAS studies focused on protein-coding genes, even though 90% of disease-associated SNPs lie in non-coding regions of the genome [106]. There are several databases that summarize the plethora of published human sequencing studies, including UK Biobank [107] and GWAS Catalog - EMBL-EBI [108]. Other databases specifically focus on SNPs within lncRNAs, such as lncRNASNP2 [109] and Lnc2Catlas [110].

To date, a couple of studies have clearly shown how SNPs from GWAS studies can be used to identify clinically relevant lncRNAs. Castellanos-Rubio et al. identified a SNP rs917997 associated with Celiac Disease and showed it was located within a novel lncRNA, Lnc13 [62]. Lnc13 regulates inflammatory genes and mediates its function *via* an interaction with an hnRNP protein [62]. Furthermore, they showed that the SNP disrupted the RNA-protein interaction, thus making the lncRNA dysfunctional [62]. Another fascinating study began by mining GWAS for atherosclerosis disease-associated SNPs, which led to the discovery of a novel lncRNA, LIN00305, that had 5 SNPs that were associated with atherosclerosis all located within an intronic region [111]. The group went on to characterize LINC00305 in human primary and immortalized monocytes as a promoter of inflammation through activating the aryl-hydrocarbon receptor repressor (AHRR) – NF-κB pathway by directly binding to lipocalin-1 interacting membrane receptor (LIMR), acting as a scaffold to promote the interaction between LIMR and AHRR [111]. Both of these studies started by investigating clinically relevant SNPs that led to the discovery of disease-associated lncRNAs, providing the groundwork for future studies on potential biomarkers or the development of novel therapeutic targets for a variety of inflammatory diseases.

Table 2
Human sequencing datasets.

	Type	Treatment	Duration (h)	Sequencing	Reference
Spleen	Tissue	–	–	Bulk RNA ChIP - H3K36me3 ChIP - H3K4me1 ChIP - H3K4me3	*26030523*
Lung	Tissue	–	–	Bulk RNA ChIP - H3K36me3 ChIP - H3K4me1 ChIP - H3K4me3	*26030523*
Liver	Tissue	–	–	Single cell RNA	*30348985*
B cell	Lymphoblastoid cell line	TNF	6	ChIP - NFkB	*30361341* **GSE31477**
PBMCs	Healthy IRF7 −/− UNC93 −/−	Influenza	0, 8, 16	Bulk RNA	*25814066*
	Blood	Healthy SLE	Longitudinal study	Bulk RNA	*22675550, 30962246*
	Macrophages	Salmonella/Listeria	0, 2, 24	Bulk RNA	*27690314*
	Monocytes	TLR4	0, 1, 4	Bulk RNA	*25855049*
	(Blood) CD14+	–	–	ChIP - H3K4me3	*25258085*
		–	–	ChIP - H3K27me3	
		–	–	Bulk RNA	
	(Blood) CD14+ CD16+	–	–		
	Monocytes (PBMCs)	–	0, 4	ChIP-H3K4me3	*22675550, 28900427*
		–	0, 4	ChIP-H3K27Ac	
		Uninfected	–	Bulk RNA	
		Influenza, S. aureus, S. pneumoniae, E. coli	–	Bulk RNA	
		LPS	0, 4, 24	Bulk RNA	
	Monocyte bead purification	LPS	0, 4, 24, 144	Bulk RNA ChIP-H3K4me1 ChIP-H3K27Ac	*27863248*
		β-Glucan	0, 4, 24, 144	Bulk RNA ChIP-H3K4me1 ChIP-H3K27Ac	
Dendritic cells	(Blood) CD141+ (Blood) CD1c+	–	–	Bulk RNA	*28475900*
	Plasmacytoid	CpG-C	0, 1, 2	Single cell RNA	*30127440*
	Monocyte derived	Differentiation	–	RNA bulk	*24744378*
		Differentiation	–	ChIP - H3K27Ac	
		Differentiation	–	ChIP - STAT1	
		Differentiation	–	ChIP - IRF1	
		A. fumigatus	0, 6	Bulk RNA	*28280489*
		C. albicans	0, 6	Bulk RNA	
		TLR4 (LPS)	0, 6	Bulk RNA	
Macrophages	THP-1 (PMA)	Mtb	0, 4, 18, 48	Bulk RNA	*22675550*
		TLR1/2	0, 8	Bulk RNA	*24371310*
		TLR1/2	0, 8	Bulk RNA	*30918008*
		TLR2/6	0, 8		
		TLR4	0, 8		
	Monocyte derived	–	–	Bulk RNA	*29475453*
		Mtb	0, 24	Single cell RNA	*28192419*
		H1N1	0, 1, 3, 6	Bulk RNA	*29475453*
		N5N1	0, 1, 3, 6	Bulk RNA	

Additionally, as previously mentioned, XENA or Lnc2Catlas are databases that combine RNA sequencing of tissue samples from healthy and cancer patients, these tools can also be adapted to assess age, gender, tissue or even disease association of splice variants [98,110]. These disease-associated SNPs or splice variants will help guide further studies to determine therapeutic or biomarker potential. In summary, assessing disease association by a variety of databases can provide insight into the function of a lncRNA [112].

2.5. Conservation of lncRNAs

Unlike lncRNAs, coding genes are highly conserved across distal and related species. The requirement of coding genes to encode functional peptides likely constrains the variation within the open reading frame (ORF) sequence [113]. LncRNAs by definition are not translated and often have poor sequence conservation across related species. Many lncRNAs display species-specific expression. Thus, inferring function based on sequence similarity is a challenge (reviewed in [114]). A more useful conservation metric is to assess whether the lncRNAs have conservation of synteny (location relative to flanking coding genes), along with expression conservation [115]. The databases LNCipedia and NONCODE have user-friendly interfaces, allowing assessment of both conservation of synteny and sequence for lncRNAs. Additionally, expression conservation can be useful to assess whether the specific lncRNA has the same biological role in similar and divergent species [116]. Conservation of lncRNA expression can also be indicative of conservation of regulatory regions, such as transcription factor binding sites within promoters [117]. However, conservation of expression does not necessarily mean the RNA product is important for lncRNA function. Enhancer RNAs (eRNAs), for example, are thought to predominantly function by creating a localized, active transcriptional state, which can activate neighboring genes [118–120]. It is unclear to what extent the specific RNA sequence of eRNAs is important for their function [118]. Nevertheless, a couple of studies have provided

examples of eRNAs for which the transcript sequence was necessary for their function [121,122].

To further investigate conservation of a candidate, BLAST (basic local alignment search tool) on NCBI (national center for biotechnology information) can be utilized to explore conservation across species [123] or lncRNAdb v2.0 [124], by inputting the entire sequence of a lncRNA of interest. If a lncRNA has a known structure, inputting the shorter structured RNA sequence can enhance conservation results. One can also, view the conservation track of the UCSC genome browser [125] to assess if this specific sequence within the lncRNA transcript is conserved across species. In summary, conservation of a lncRNA is complicated to assess using current bioinformatic methods. However, understanding if there is a functional motif (therefore a shorter starting input sequence) within a lncRNA can allow for an increased assessment of functional conservation across species.

2.6. Transcriptional regulation of lncRNAs

Activation of inflammatory pathways result in both up and down regulation of specific lncRNAs, which in turn can have either positive or negative regulatory effects on the pathway, such as activation of sequestered transcription factors [45,126] or enhanced or repressed expression of specific inflammatory cytokines [61,84,127]. Genes that are immediate regulators of immunity are poised for transcriptional activation, which can be assessed by defining the openness of promoter regions of a lncRNA pre- and post- inflammatory stimulation. Common methods for assessing chromatin accessibility of promoters include DNase-hyper-sensitivity (DNAseHS) [128] and the Assay for Transposase-Accessible Chromatin (ATAC) [129]. DNAse HS-seq and ATAC-seq datasets are available from a variety of tissues on ENCODE for both mouse and humans (Tables 1 and 2). If the promoter is open (accessible), this is indicative of either a poised or actively transcribed gene. Accessibility of promoter regions in the hematopoietic cell lineage was assessed by Lara-Astiaso et al. through performing ATAC-seq for all cells in the hematopoietic lineage [76]. This dataset provides insight into a gene's promoter accessibility, as well as cell type specificity. For instance, if the promoter is open in all cell types, it shows that it is ubiquitously accessible and possibly expressed, while if a promoter is only accessible in myeloid cells or terminally differentiated macrophages this provides insight into the cell type that could be most biologically relevant for a particular lncRNA. Another interesting data set from Tong et al. have provided ATAC sequencing from bone marrow derived macrophages (BMDMs) pre- and post- inflammatory time course stimulation [72]. This dataset assesses both poised genes and genes that undergo promoter remodeling during inflammatory activation. If a promoter of a lncRNA is inaccessible or accessible during inflammatory stimulation, this could provide insight to its regulation and biological significance during an immune response.

Once the accessibility of a promoter region is determined, defining post-translational modifications of histones on promoter regions will assess promoter activity in specific cell types or inflammatory states [130–132]. A histone modification is a covalent post-translational modification (PTM) to histone proteins which includes methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation (reviewed in [133]). Posttranslational histone modifications do not affect DNA nucleotide sequence but can modify chromatin availability to the transcriptional machinery [134]. Identifying the types of epigenetic histone marks will add additional layers of understanding if the candidate is active, poised, silenced or an enhancer. Many publicly available datasets provide this information outlined in Tables 1 and 2. While the examples we outlined here are immune focused there is a vast array of additional primary and immortalized cell line for every histone mark available through the easily accessible ENCODE database [101].

Finally, the transcriptional regulation of a lncRNA can be defined by analyzing the transcription factor (TF) motif's that lie within the predicted promoter region [135–137]. RNAReg2.0 [138] or HOMER [139,140] are useful tools to predict TF binding sites by motif analysis. TF motifs can be indicative of biological pathway regulation indicating when a gene is expressed. These findings of predicted TF can then be put into a gene ontology tool (PANTHER or DAVID) to assess how a candidate lncRNA is transcriptionally regulated. For instance, the presence of pioneering transcription factors could provide information on the cell type specificity of a lncRNA. On the other hand, if the promoter motifs are enriched for p65, Interferon Response Factors (IRFs) or Activating Transcription Factors (ATFs) this would be indicative of inflammatory specific expression. This finding can be further supported by Chromatin immunoprecipitation (ChIP) sequencing data sets from a multitude of labs, as well as data from the ENCODE project (outlined in Tables 1 and 2).

3. Functional characterization of lncRNAs

3.1. Assessing whether lncRNAs are translated

The categorization of lncRNAs as “noncoding” is initially determined bioinformatically using the arbitrary cut-off of < 100 codons [141]. This leaves the possibility that some lncRNAs may be mRNAs. Therefore, one of the first steps in characterizing a candidate lncRNA is to confirm that it is noncoding. For example, in Drosophila, a gene annotated as a lncRNA FBgn0087003, was shown to encode multiple small ~ ten amino acid peptides critical for development [142]. The discovery of these germline-encoded biologically active peptides has opened the door into new and exciting levels of regulation. However, this discovery also shows that we should be cautious when characterizing a lncRNA to confirm that indeed they are noncoding.

Several bioinformatic tools exist for predicting small ORFs (smORF) but have noted that the predictive ability for smaller ORF size is in general very poor [143]. PhyloCSF uses codon substitution frequency, together with conservation across multiple species, to provide a score metric that can be used to determine the presence of a conserved ORF [113]. Other approaches have sought to identify novel small peptides using mass spectrometry. However, a major challenge is determining whether the peptides identified correspond to novel smORFs or represent degraded intermediates of larger proteins [144].

The development of ribosomal foot-printing coupled to next-generation sequencing (ribo-seq) has provided a powerful quantitative method for assessing global translation [145,146]. While ribosome profiling allows for ribosome nuclease-protected RNA fragments to be mapped to transcripts to enable quantitative measurement of the translation efficiency [146]. In addition, to mapping ORFs, ribo-seq can be performed with a drug that stalls ribosomes at the start codon to globally map translation start sites—this revealed significant numbers of non-canonical translation initiation from CTG codons [146]. Ribo-seq has also found that many lncRNAs appear to be translated, raising the possibility that some of these transcripts could be producing small peptides [145]. Guttman et al. developed the ribosome release score metric as part of the ribosome profiling analysis pipeline to more accurately predict translational efficiency. Their findings show that ribosome occupancy of lncRNAs and 5′UTRs does not always equate to translation [147]. Furthermore, Guttman et al. concluded that most noncoding transcripts are not translated into peptides. Several additional studies have examined lncRNAs binding to ribosomes and have concluded that ribosome binding may not be functional and may serve as a quality control process to degrade transcripts with low coding potential *via* the nonsense-mediated decay (NMD) pathway [148,149]. A recent study by Jackson et al. used ribo-tagging in LPS-stimulated mouse macrophages to identify ribosome footprints within hundreds of annotated lncRNAs, raising the possibility that they may be producing functional peptides [150]. They characterized an 83aa peptide located within a previously annotated lncRNA Aw112010 and showed it was produced by non-canonical “CTG” translation initiation [150]. They demonstrated that this small peptide had a critical role in mucosal

Gene knock-down	Target	Reversible loss-of function?	Type of phenotype	Time to phenotype	Limitation(s)?
A. RNAi (RISC pathway)	Transcript	Yes	hypomorphic	Short	Minimal knockdown of nuclear lncs
B. GAPMERs/ASOs (RNase-H pathway)	Transcript	Yes	hypomorphic	Short	None
C. CRISPRi (KRAB)	gDNA (TSS)	Yes	hypomorphic	Medium	> 1000 bp from nearest coding gene TSS, can only target lincRNAs
D. CRISPR-splice-sites	gDNA	No	hypomorphic	Medium	Splice site cannot overlap coding gene
E. CRISPR-chomp	gDNA	No	Null (complete)	Medium/Long	Deleted region cannot overlap coding gene
F. poly(A) terminator	gDNA	Yes (loxP)	hypomorphic	Medium/Long	Region cannot overlap coding gene

Gene Activation	Target	Reversible gain-of function?	Type of phenotype	Time to phenotype	Limitation(s)?
G. CRISPRa	gDNA (TSS)	Yes	over-expression	Medium	> 1000 bp from nearest coding gene TSS, can only target lincRNAs
H. Ecotopic	cDNA	Yes (tet-on)	over-expression	Medium	cDNA < 5-6kb size (lenti constucts)

Fig. 3. LncRNA expression manipulation.
(A) RNA interference acts post-translationally, degrading transcripts in the cytoplasm. (B) Gapmers, are composed of RNA and core stretch of ~10 nt of DNA sequence that is critical for engaging the RNase H pathway. (C) CRISPRi uses a catalytic inactive version of Cas9 fused to a KRAB domain. The CRISPRi system can target transcription start sites (TSS) to induce heterochromatin-based transcriptional silencing. CRISPR/Cas9 can be used to target (D) splice sites or (E) to delete specific regions of lncRNA loci using two flanking gRNAs. (F) Polyadenylation signals (3–5) can be inserted just downstream of the TSS, which result in premature termination of transcription. These poly(A) signals can be flanked by loxP sites to allow their removal. (G) The CRISPRa system can target transcription start sites (TSS) to induce transcriptional activation. (H) LncRNA sequences (cDNA) can be cloned into plasmid either with native promoter or a constitutively active promoter (*i.e.* EF1a, CMV). The different tools are compared base on criteria described in the subheadings of each column. 'Target' describes what aspects of the lncRNA's gene expression are targeted, for example transcriptional (CRISPRi, C) or posttranscriptional silencing (RNAi, A). 'Reversible loss-of-function?' is the silencing effect of this tool reversible in a cell? In general, only the nuclease active Cas9 activity (D) is not reversible since the regions of the loci are actually deleted. 'Type of phenotype' refers to whether the tool completely shuts off expression of the lncRNAs. Hypomorphic refers to the fact that some level of expression remains despite the level of knock-down. Only compete removal of the locus completely abolishes its expression. Knock-down of nuclear lncRNAs? Touches on the observation made by many groups, which have demonstrated that RNAi is not very active in the nuclear (in contrast to the other technology). 'Time to phenotype' refers to the amount of time from planning experiment to obtaining phenotype. *Short:* transfection of RNAi and ASO (A and B) remain the quickest route to phenotype since they don't require cloning or modification to the cell. *Medium:* CRISPRi/a (C, G) requires design and cloning guide RNAs to a specific locus in addition to generation of a functional CRISPRi/a cell line. Medium/Long: Both CRISPR mediated deletions and adding poly(A) signal (D and E) require screening for cells (making single cell clones) to identify those with successful modifications.

immunity in mice and was specifically required for the expression of Il12 mRNA [150]. The exact mechanism of how this peptide drives Il12 expression is yet to be elucidated.

4. LncRNA expression manipulation

4.1. RNA interference (RNAi)

The laborious process of functionally characterizing lncRNAs has remained a major limitation to lncRNA functional discovery. For example, while ~16,000 long noncoding RNAs (lncRNAs) have been identified in the human genome, only 3% of all validated lncRNAs have an ascribed function [47,91,151]. For more than two decades since its discovery, RNA interference (RNAi) has been the method of choice for loss of function studies. The ease and versatility of RNAi make it appealing for use, requiring short complementary small RNAs transfected into cells that can then utilize the endogenous cellular machinery to target specific transcripts [152,153] (Fig. 3A). Additionally, short hairpin RNAs (shRNAs) can be expressed in a lentiviral context to accomplish stable RNAi in cells [154]. RNAi is most active in the cytoplasm, which makes it most useful for targeting mRNAs and lncRNAs that reside in the cytoplasm [155]. Success in knocking down some lncRNAs, for example lincRNA-Cox2, has been attributed to the fact that this lncRNA is expressed both in the cytoplasm and the nucleus (perhaps cycling between both compartments) and hence is susceptible to RNAi [42]. However, many lncRNAs are thought to be nuclear-restricted where RNAi has been demonstrated to have limited efficiency [156] (Fig. 3).

4.2. Antisense oligonucleotides (ASOs)

Antisense oligonucleotides (ASOs) can be either RNA or DNA-based and can be used to target complementary sequences within a transcript. Unlike RNAi, ASOs do not engage the cellular RNAi machinery [157]. Instead, ASOs function by hybridizing to the target RNA and inhibiting its function by either inducing the RNase H pathway or by steric inhibition. The RNase H enzyme is part of a cellular pathway that normally functions to resolve unwanted DNA: RNA interactions that can

occur during replication and/or transcription [158]. One of the most widely used types of ASOs for targeting lncRNAs are gapmers which contain a "hybrid" modified/unmodified configuration consisting of ~10 nt DNA core flanked by 2′-*O*-Methyl or LNA-modified synthetic nucleotides (Fig. 3B). Gapmers offer the benefit of modifications for stability and reduced toxicity, while still allowing engagement of the RNase H pathway (Fig. 3B) [159].

Efficient depletion of nuclear lncRNAs has been demonstrated using modified DNA anti-sense oligonucleotides [155]. Ilott et al. used gapmers to successfully knock-down TLR-induced enhancer RNA, as they were unable to knock-down these same eRNAs with 4 different siRNAs [56]. Recent *in vivo* applications for DNA oligonucleotides have also proven successful as a possible treatment of a neural degenerative disease called Angelman's syndrome. Angelman's syndrome is a monogenic disorder caused by mutations in the E3 ubiquitin ligase 3A (UBE3A) [160,161]. As UBE3A is a paternally-imprinted gene, a mutation in the maternal allele is sufficient to lead to disease [160,161]. Paternal imprinting of UBE3A requires the expression of an antisense lncRNA called UBE3A-ATS. Therefore, targeting the paternal lncRNAs UBE3A-ATS using ASOs leads to de-repression of expression of paternal UBE3A, allowing the rescue of the defective maternal copy [161]. In a mouse model, they showed that even partial restoration of the UBE3A protein expression ameliorated some cognitive deficits associated with the disease [161].

Mechanistically, it is important to dissect what features of a lncRNA are important for its function — for example, determining whether the lncRNA transcript is required or whether the mere act of transcription is the important feature needed to mediate its function (discussed further below). siRNAs and ASOs are thought to inhibit gene expression at the posttranscriptional level, albeit by different mechanisms [162,163]. Nevertheless, there are examples of siRNAs (reviewed in [164]) mediating transcriptional inhibition. Targeting sequences near the 5′ end of the transcript can induce the "torpedo-effect," resulting in pre-mature termination of transcription [165,166]. Therefore, when targeting lncRNAs with ASOs or siRNAs, it's important to consider where along with the transcript you are targeting to ensure biological interpretation of the ablation is correct.

4.3. CRISPR/Cas technology

The introduction of CRISPR/Cas9 technology has revolutionized the field of functional genomics by providing a novel tool for interrogating gene function. CRISPR/Cas9 is a deoxyribose nuclease (DNase) that can be specifically targeted to genomic regions *via* a guide RNA (gRNA) [167,168]. Targeting of Cas9 to a region results in a blunt double-stranded DNA break that engages the cellular Non-Homologous End-Joining (NHEJ) DNA repair pathway, which promotes imprecise repair, yielding small deletions in the repaired sequence. These small deletions result in a frame-shift that disrupts the ORFs within coding genes, thereby disrupting protein synthesis. LncRNAs do not contain ORFs and span tens of kilobases of sequence in size. As such, targeting them with a single gRNA is thought to be insufficient to disrupt their function [169]. An alternative application of Cas9 that has proven effective for targeting lncRNAs involves using two gRNAs flanking the lncRNA region of interest to induce deletion of the entire locus (Fig. 3E). The main advantage of deleting lncRNAs is obtaining complete loss-of-function, as demonstrated in a recent study that performed a CRISPR-mediated deletion-screen to identify lncRNAs that positively and negatively regulate cancer growth [170]. On the other hand, deletion of the DNA sequence may result in an inability to resolve whether a phenotype is due to loss of lncRNA production or loss of DNA sequence (discussed below) [171]. In an alternative approach, Liu and colleagues recently performed a CRISPR screen targeting the splice sites of over 10-thousand lncRNAs and identified 230 lncRNAs that proved essential for viability (Fig. 3D) [172]. Targeting of splice sites has also been shown to induce exon skipping within coding genes [173]. LncRNAs have many of the same regulatory elements as coding genes. Hence future studies may opt to target TF binding sites, secondary structure and/or polyadenylation sites as a way to more finely dissect the functional portions of lncRNAs.

The ease in which Cas9 can be targeted to specific genomic regions sparked the development of a modified (catalytically inactivated) version of the protein fused to the KRAB (Krüppel associated box) chromatin-silencing domain termed CRISPRi [174,175] (Fig. 3C). CRISPRi can be used to target both coding and noncoding genes (such as lncRNAs), triggering localized heterochromatin-silencing at the transcription start site (TSS) [176]. Unlike RNAi, the transcription-based inhibition by CRISPRi offers the ability to efficiently target lncRNAs regardless of their localization in the cell. Gilbert et al. have shown maximum knock-down efficiency when targeting regions +50 to +500 nucleotides relative to the transcription start site (TSS) [174]. However, CRISPRi is limited by the availability of an accurately annotated TSS, particularly for lncRNAs. Incorporating CAGE-seq, Gro-seq and/or ChIP-seq data into gRNA design may improve efficient targeting of a lncRNA of interest [177]. Nevertheless, a recent study from the Weissman and Lim groups utilized CRISPRi to target 16,401 lncRNA loci in 7 diverse cell lines and identified hundreds of lncRNAs required for cell growth [96]. Therefore, despite its caveats, it appears that CRISPRi can be a useful and powerful tool for interrogating lncRNA biology.

An alternative approach to gene ablation is overexpression, gain-of-function. Plasmid-based overexpression systems have been used for decades to overexpress specific coding genes [178]. Plasmid-based over-expression of lncRNAs is possible but is limited by the size (max size: 6-8 kb) of the specific lncRNA (unpublished observations). However, because lncRNAs can function in *cis*, it's important that there is the significant mechanistic characterization of the candidate lncRNA to justify its cloning into a plasmid.

The same catalytically inactivated Cas9 has also been fused to a transcriptional activation domain, for example, the VP16, a strong transcriptional activator derived from herpesviruses [179,180]. This strategy allows CRISPR-based transcriptional gene activation that can be used to study gain-of-function phenotypes. The Zhang group recently performed a CRISPR activation screen targeting over 10-thousand lncRNAs [181]. They identified 11 lncRNAs that upon activation, mediated BRAF inhibitor resistance in melanoma cells [181]. One of the advantages of using a CRISPRa library is that the same library can be used across different cell types, which makes it more cost effective. Nevertheless, several caveats to the CRISPRa system, include the possibility that high expression of lncRNAs may create non-physiological conditions leading to incorrect conclusions of specific biology.

LncRNAs have been shown to mediate functions *via* binding to specific proteins. Hence over-expression of lncRNAs, without its protein partner, may result in the inability to identify important biology. In conclusion, it's important to understand the advantages and disadvantages of the loss- or gain-of-function methods used to modulate lncRNA expression. All methods have their caveats, and these are important to consider when deciding which method to use.

4.4. Dissecting the complex functions of lncRNAs in vivo

LncRNAs can span large stretches of DNA sequence and can contain important regulatory regions, such as enhancers, that are functionally independent of the lncRNA product [182]. LncRNA promoters have been proposed to also function as enhancers, promoting the recruitment of transcriptional-activating factors that can affect the local nucleosome environment and ultimately the expression of neighboring genes [118,183,184]. Nevertheless, *in vivo* assessment of lncRNA function has predominantly relied on assessing the consequence of deleting entire lncRNA loci [171,185]. Numerous studies involving deletion of lncRNA loci have been unable to rescue the deletion phenotype using a transgene approach, making it difficult to attribute the phenotype to the

lncRNA product itself [186,187]. While deleting the entire lncRNA is a useful first step to establish a phenotype, this approach can make it difficult to identify which component of the lncRNA is important for the observed phenotype [171,185,188–190].

There are many approaches to generating a lncRNA knockout/knockdown mouse. For instance, the complete knockout is the easiest first step to take, which can be designed to remove the entire gene. After this approach, one can use a more fine-tuned approach to reveal exactly how the gene is working, including deleting specific regions of the gene or inserting a poly-adenylation cassette before the exon1 of the gene. A well-documented example of this phenomena is *Fendrr*, which was shown to have a lethal phenotype in two independent studies, but the importance of the lncRNA in development differed due to the mouse ablation strategy. One *in vivo* study generated a knockout mouse by removing the genomic loci completely and replacing it with LacZ while leaving the native promoter intact [185]. This study identified *Fendrr* as a key regulator of lung development and mesenchymal differentiation. However this study did not attempt to rescue the ablated *Fendrr* allele *via* a transgene [185]. An additional group generated a *Fendrr* knockdown mouse using an alternative approach. Instead of disrupting the chromatin architecture or removing any possible DNA enhancer regions, they inhibited the transcription of *Fendrr* through the insertion of a poly-A cassette into exon1 [191]. Using this lncRNA knockdown mouse design, the scientists determined that loss of *Fendrr* led to heart and body wall defects, which is slightly different from the phenotype in the KO mouse study. Importantly, the heart and body wall defects caused by the terminator insertion were rescued by a transgene of *Fendrr in vivo*, further confirming that the phenotype is due to the RNA while the KO phenotype could be due elements within the genomic DNA [191]. *Fendrr* is located ~4 kb downstream of Foxf1 and ~12 kb upstream of Irf8. The observed phenotype of the KO mouse is possibly due to the deletion of an enhancer element that could impact the expression of protein-coding genes, which could have roles in lung development [192,193].

We recently used multiple genetic mouse models to dissect both *cis* and *trans* functions of lincRNA-Cox2 *in vivo* [46]. Working with a complete lincRNA-Cox2 knockout [185], in which the gene is replaced with a LacZ cassette, we observed a strong *cis* defect on the neighboring protein-coding gene Ptgs2. From these studies, we concluded that lincRNA-Cox2 functions *in cis* through an enhancer RNA mechanism to regulate its neighboring gene Ptgs2 [46]. In order to determine how lincRNA-Cox2 functions *in trans* to regulate genes independent of its cis effects, we generated a mutant/intronless mouse by targeting the splice sites of lincRNA-Cox2 using CRISPR/Cas9. This mouse represents a knockdown mouse, and because there is a low level of transcription of lincRNA-Cox2, Ptgs2 levels are the same as WT. LincRNA-Cox2 is not inducible in the mutant mouse, probably because of transcript instability due to the lack of splicing, enabling us to study its trans regulatory roles. Similar to our early *in vitro* work we observed using an LPS shock model that many genes are both up and downregulated in the serum of mutant mice indicating that lincRNA-Cox2 can indeed function *in trans* to regulate immune genes *in vivo* [46].

To prove genetically that a lncRNA is functioning in *trans,* a *trans* rescue experiment can be performed using transgenic mice that constitutively express a lncRNA. This rescue strategy has been utilized in some studies including *Evf2* [194,195], *Jpx* [196], as well as *Pnky* [197] which both demonstrate successful rescue experiments where the phenotype from germline ablation of a lncRNA is rescued through generation and crossing with a transgenic animal.

5. Understanding the mechanism of action of a lncRNA

5.1. Subcellular localization and binding partners

LncRNAs are immensely adaptable molecules that are capable of working through RNA-RNA, RNA-DNA, or RNA-protein interactions. RNA-directed technologies such as Chromatin Isolation by RNA Purification (ChIRP) [198,199] or RNA antisense purification (RAP) [200,201], will help uncover lncRNA interactomes for RNA, genomic or protein partners for highly expressed candidates. If a candidate is lowly expressed, one can exogenously introduce a biotinylated form of the lncRNA using the RNA pull-down method, which we have successfully used to identify binding partners for lincRNA-Cox2. This has been performed for many lncRNAs [42,202,203].

Functions of lncRNAs are associated with their subcellular fates. Web servers can assist in quickly assessing experimentally determined or predictive RNA-RNA interactions [204] or even RNA-protein interactions [205,206]. Depending on the subcellular or extracellular compartmental localization of a lncRNA, this patterning will elude to the regulatory role of the gene, as well as how a lncRNA might execute its function [207–209]. Some LncRNAs that are localized to the nucleus or chromatin have been experimentally shown to function *in cis* to regulate the transcriptional expression of a neighboring gene, or *in trans* to regulate the transcriptional regulation of a subclass of genes through the interactions between heterogeneous nuclear ribonucleoproteins (hnRNPs) [42,54,60,210]. In the cytosol, some lncRNAs have been shown to interact with RNAs and proteins to carry out their molecular functions [42,79,211,212].

Cellular localization of lncRNAs can be predicted using a publicly available user-friendly web server established at http://lin-group.cn/server/iLoc-LncRNA [213]. This can be the first step by a researcher to attempt to predict the localization of your candidate based off of its sequence. iLoc-LncRNA predicts subcellular location of a lncRNA by utilizing the 8-tuple nucleotide features into the general PseKNC (Pseudo K-tuple Nucleotide Composition) and rigorous tests show the overall accuracy achieved by the new predictor is 86.72%, which is over 20% better than previous algorithms [213]. In addition to prediction methods, there are also publicly available sequencing datasets that have performed RNA sequencing on fractionated cells. Bhatt et al. utilized murine bone marrow derived macrophages, with and without inflammatory stimulation, and fractionated these cells into chromatin, nuclear, and cytoplasmic compartment to determine RNA localization [74]. This data set can now be utilized to investigate the localization of any murine candidate expressed in macrophages. A recent study took this question a step further by performing RNA sequencing on nine separate locations with a cell including: nucleus, nucleolus, nuclear lamina, nuclear pore, cytosol, endoplasmic reticulum membrane (ERM), outer mitochondrial membrane (OMM), mitochondrial matrix (MITO), and endoplasmic reticulum lumen [214]. This exciting study utilized an APEX-sequencing method, where the peroxidase enzyme APEX2 was localized to these nine separate locations in nine separate Human embryonic kidney (HEK) 293T cell lines. APEX2 can biotinylate nearby RNA molecules allowing for streptavidin-based immunoprecipitation and RNA sequencing. The APEX-seq datasets will provide a powerful resource for referencing localization of specific lncRNA candidates that are expressed in HEK293T cells [214].

There are a few commonly used experimental approaches that can be used to validate and determine the localization of a lncRNA. Subcellular chromatin, nuclear and cytoplasmic fractionations of any primary or immortalized cells can be prepared using previously published procedures [74,215], followed by RNA isolation and RT-qPCR to assess the localization. If additional compartmental fractionation is desired other compartments can be enriched, such as mitochondria [216,217]. Another standard gold technique is to visually determine cellular localization by RNA FISH [42]. RNA localization can also be directly visualized by microscopy [218]. SeqFISH techniques have recently been pioneered for imaging thousands of cellular RNAs at once using barcoded oligonucleotides [219]. The drawbacks of these *in-situ* fluorescence hybridization (FISH) based approaches, however, are the need for cell fixation and permeabilization, which can re-localize or extract cellular components [220]. In addition to the difficulty of assigning RNAs to specific organelles or cellular landmarks due to spatial

resolution limits, some of these difficulties can be overcome with the addition of stains for markers of specific organelles.

5.2. Determining structure or motifs within a lncRNA

Conventionally, lncRNAs display poor sequence conservation across species, with the exception of finite regions of conserved bases surrounded by large seemingly unconstrained sequences [221]. While sequence conservation does not constrain lncRNA genes, lncRNA function is found to be conserved across species when identifying motifs or structure. A great example of this phenomena is represented in the study of human maternally expressed gene 3 (MEG3), which utilized the computer program *mfold* and multiple *ex vivo* and *in vitro* chemical probing techniques to identify common motifs critical for retained function in orangutan, rat, mouse and pig [222,223]. Another study highlighting the marsupial Rsx lncRNA was initially found to have no linear sequence similarity with the lncRNA Xist. However, it shared substantial levels of non-linear conservation within k-mer repeats that share functionally analogous protein-binding domains [224]. Publicly available web servers can be utilized to determine the RNA structure of a lncRNA depending on the size. The caveats to these web servers are they do not work efficiently with large transcripts. To overcome this, one can attempt to identify the critical sequence within the lncRNA that could be functional using RIP-sequencing databases, which allow you to input a gene ID to identify possible RNA binding proteins (RBPs). Additionally, if a lncRNA has already been identified to bind to a protein (s), RIP-sequencing databases can elucidate the specific binding location(s). Knowing the location of RNA-protein interaction narrows down the sequence input, that can be used for many web servers, which could enhance the elucidation of a predictive structure. If there is no information about potential protein binding partners, then these RNA structure webservers will be of little use.

An alternative approach to further investigate structure is to utilize bioinformatic tools which now include parameters for covariation. Covariation analysis identifies the positions in an RNA molecule that have similar patterns of variation and the purpose of this covariance is due to structural constraints initially shown for ribosomal RNA [225] and now also for lncRNAs [123,226]. This study predicts structures for MALAT1, using over 130 vertebrate sequences, as well as lncRNAs RepA and HOTAIR [227]. These powerful tools allow scientists to predict structures in an RNA molecule based on covariance, which in turn drive the next steps of experimentally validating these findings.

Fortunately, there are several *in vitro* and *in vivo* experimental techniques used to assess RNA structure for any size, even up to 17,000 nt (XIST). Dimethyl sulfate (DMS) probing uses a base-specific reagent that can bind and alter the methylation state of unpaired adenosine and cytosine nucleotides [228,229]. DMS "footprinting" is optimized for structural analysis of RNA. Protein binding to RNA will generate a "footprint" that can be traced due to alterations in the RNA structure. The transcript size that can be evaluated is rather small (< 500 nt) but this method can be performed both *in vitro* and *in vivo* as DMS can easily penetrate the cell membrane, shown to work for lncRNAs from 590 nt, Braveheart (Brvht) [230]. Xue et al. utilized DMS and SHAPE to determine the multiple smaller order structures of Brvht, including an AGIL motif and a 90-degree turn [231]. This AGIL motif is critical for transcription factor binding, which specifies the cardiovascular lineage. Targeting Structure-Seq relies on RNA methylation by DMA being performed *in vivo*. Using this method, structural models of elements within Xist were developed [232]. SHAPE (selective 2′hydroxyl acylation by primer extension), as well as the modified SHAPE-MaP [233], In-cell SHAPE-Seq [234] and icSHAPE-seq [235], can interrogate the RNA structure both *in vitro* and *in vivo* using the chemical NMIA and its derivatives to detect flexible regions in RNA secondary structure [236,237]. This method has been proven valuable for Xist [238], RepA [239], and Rox1/2 [240]. PARIS (psoralen analysis of RNA interactions and structures) was recently developed to determine both RNA structure and interactions *in vivo* [241]. Using this approach, a model for the higher order structure of Xist was interrogated [241]. These approaches are critical for identifying structural motifs and enhance conservation studies, as well as these identified elements, can be used as novel targets for further exploration in precise intervention suitable for therapeutic applications.

5.3. Alternative splicing of lncRNAs

Alternative splicing (AS) significantly impacts the diversity of RNA isoforms produced, which in turn impacts the protein isoforms produced and can affect many aspects of the protein's biology including binding, intracellular localization, enzymatic activity, stability, post-translational modifications [242]. AS also impacts lncRNA genes which can have multiple isoforms depending on the cell/tissue, age, and disease state [243–245]. The UCSC genome browser [125], as well as NONCODEV5 [92], have all the annotated isoform transcripts for each gene. These tools will identify annotated transcript isoforms while RNA sequencing will provide information on which of these splicing events is utilized in a given cell or biological state. To date, there have only been a small number of papers focusing on the role that alternative splicing plays in controlling the immune system. One recent global study has shown that widespread shortening of 3′ untranslated regions and increased exon inclusion are evolutionarily conserved features of innate immune responses in primary human macrophages following *Listeria monocytogenes* and *Salmonella typhimurium* infection [78]. This is a transformative study for mRNAs but can be reanalyzed to examine AS and possible contributions from lncRNAs.

Publicly available tools for tissue isoform expression specificity is available on GTEx [97] and XENA [98] for human genes. Tabula Muris [100], a murine specific dataset, is now available as a UCSC genome browser track on mm10 and can be used to view cell-type specific splicing events and isoform expression. In order to identify isoforms in illumina RNA sequencing datasets, several tools can be utilized. MISO (Mixture of Isoforms) is software for a probabilistic model for RNA seq will identify specific the 5′ splice sites used for each isoform [246]. Two other tools highly used for splicing analysis are JuncBASE [247], MAJIQ-SPEL [248], and DRIMSeq [249]. These tools can be used to define if your candidate gene undergoes any alternative splicing (alternative start site, exon inclusion/exclusion or alternative last exon) during a specific biological process for example following inflammatory activation. These tools are limited because of their dependence on a fully annotated transcriptomes. Therefore if a lncRNA is unannotated or has unannotated transcriptional isoforms, these events will not be captured. To overcome the limitations of incomplete transcriptomes, researchers can perform *de novo* transcriptome assembly using short RNA-Seq reads [250–252].

The future of RNA sequencing is headed towards long read sequencing, which is being met by Pacific Biosciences Single Molecule Real Time sequencing technology (PacBio) and Oxford Nanopore Technology (ONT) [253,254]. While both powerful technologies perform long read sequencing, their platforms are very different. PacBio technology is dependent on sequencing-by-synthesis. A DNA polymerase incorporates nucleotides that each have a corresponding conjugated fluorescent dye. The DNA polymerase works at a rate of 1000 bp/s, which is beyond the capabilities of current technologies. However, by circularizing the DNA PacBio has overcome this limitation through continuous long read sequencing, resulting in ability to generate 500k-4million reads at an error rate of below 1% [255,256]. On the other hand, ONT's approach relies on a pore embedded in a membrane. As a long cDNA or RNA strand translocates through the nanopore at single nucleotide precision from enzymatic regulation, the ionic current across the membrane is recorded. This technology can sequence full-length transcripts and can yield up to 10 million reads on the MinION or up to 60 million reads on the PromethION for cDNA [257,258]. An initial limitation of ONT was the 5–10% per read error

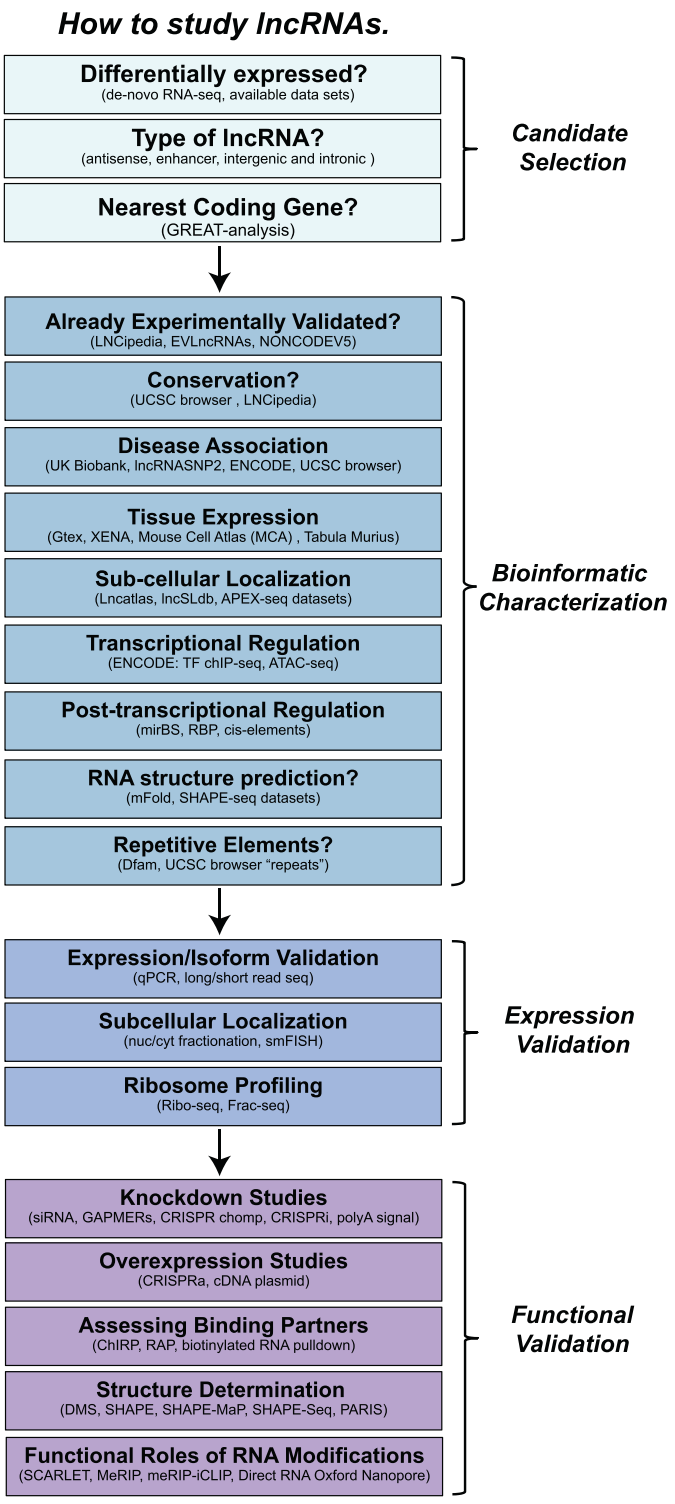


Fig. 4. How to study lncRNAs?.
The flow-chart provides a "beginning to end" guide to study lncRNAs. Not all suggested databases will be appropriate for all lncRNAs being studied. A. Selection of candidate lncRNAs should factor in changes in expression, type of lncRNA and nearby coding genes. B. Bioinformatic characterization of lncRNAs can be done using a variety of online databases including: LNCipedia to assess conservation or using the Mouse Cell Atlas (MCA) to assess tissue specific expression. C. Expression validation: This includes validation of expression and confirming that the lncRNA is in-fact non-protein coding. D. Functional validation dives into the final stage of mechanistic characterization of a lncRNAs, which involved manipulating its expression, as well as uncovering the specific cis-elements within the transcript important for its function.

rate, which has been overcome with a new technology called Rolling Circle to Concatemeric Consensus (R2C2) bringing the error rate down to 2.5% by increasing the read coverage. Overall, both of these technologies have overcome the transcriptome assembly and isoform identification limitations of short-read Illumina sequencing [259].

As stated above, ONT and PacBio can perform long read cDNA sequencing [260] and even more exciting, both technologies can perform direct long read RNA sequencing [261,262]. The beauty and simplicity that ONT and PacBio offers is the ability to sequence the full expressed isoform (cDNA and direct RNA), without worries of misidentifying a complex splicing pattern, RNA cleavage events and also not relying on transcript annotation files will lead to the identification of novel isoforms which are problems faced using short-read Illumina sequencing. For lncRNAs there have been a couple of studies that focus on isoform specificity and function for a given lncRNA. Neat1 [263] and lncRNA-PXN-AS1 [264] are two studies that show how one gene can have different functions depending on the RNA isoform expressed. While these studies are not immunology specific, this field is still at the early stages and we anticipate it becoming more prevalent in future studies.

5.4. RNA modifications of lncRNAs

RNA modifications are widespread and diverse in chemical nature, as well as highly conserved in their occurrence and function throughout species. RNA modifications function to affect RNA stability, localization, alternative site of poly-adenylation, and more [265]. Since lncRNAs can function as decoys and scaffolds, which are highly dependent on RNA structure, a single modification can enhance or eradicate this RNA-protein interaction. As you study a lncRNA, the mechanism of this molecule could be dependent on a modified nucleotide.

There are many techniques used to determine a single RNA modification in a cell type and biology of choice. Site-specific Cleavage and Radioactive-labeling followed by ligation-assisted extraction and TLC (SCARLET) Technology give scientists the ability to probe for N6-methyladenosine RNA (m6A) modification status at single nucleotide resolution in mRNA and long noncoding RNA [266]. The significance of RNA modifications to the control of the immune response is beginning to be appreciated. A study by Winkler et al. showed that m6A modification controls the innate immune response to infection by targeting type I interferons [267]. A few recent studies have shown that lncRNAs do have RNA modifications such as MALAT1 containing m6A modifications [268,269], HOTAIR containing m5C and m6A [270,271] and XIST containing ψ, m6A and m5C modifications [269]. A study by Zhou et al. showed that the RNA modification, m6A, acts as a structural 'switch' in Malat1. When there is a modification at site 2515, it results in an increased ability to bind hnRNPG, while a modification at 2577 leads to an increase in binding to hnRNPC [269]. In clinical research, lncRNA RP11-139J23.1 is highly expressed in colorectal cancer cells (CRC), and this specific upregulation was controlled by m6A methylation [272]. The study showed that m6A could regulate the lncRNA, which in turn triggered the dissemination of CRC cells *via* post-translation upregulation of the protein Zeb1. This novel study, connecting the interplay of RNA modifications and lncRNAs, has paved the way for a novel predictive biomarker or therapeutic target in CRC [272].

There are over 160 identified RNA modifications, while only a few have been studied to any extent [273]. Of these RNA modifications, the way they are enriched for in analysis is through an assortment of techniques including methylated RNA immunoprecipitation (MeRIP), MeRIP-iCLIP (crosslinking and immunoprecipitation), Suicide enzyme trap and Clickable chemicals (Reviewed in [274]). These techniques have many limitations and biases, but hopefully, future studies using direct RNA nanopore sequencing will overcome all these pitfalls. In a recent study, direct RNA sequencing using nanopore technology showed detection of m6A modifications with a 97% accuracy with the design of synthetic sequences [275]. As the performance of the algorithm increases, use of this tool will be extremely insightful when

analyzing myeloid or lymphoid primary cells with and without a treatment to understand how RNA modifications are regulated in innate immunity and specifically as it relates to our long noncoding transcriptome.

6. Conclusions and future insights for the field

LncRNAs, including XIST and H19, have been studied intensely for decades [275,276]. At the time we had no idea that these genes would represent the largest family of RNA genes produced in the genome. As Louis Pasteur once said, "Chance favors the prepared mind," and this is especially true following the development of next-generation sequencing. RNA-sequencing provided an unprecedented insight into the human genome. We did not identify new proteins, instead we found a wealth of noncoding RNA transcripts. The lncRNA field is growing at a blistering pace with labs from all aspects of biology, and now immunology branching out to include questions about the regulatory impact of these pervasive long noncoding gene species. As detailed in this review, there are many publicly available datasets and web servers that will streamline how to begin a lncRNA project, from how to pick a lncRNA candidate by interrogating published RNA-sequencing data, to determine the best tools to use to study the function and mechanism of a candidate (Fig. 4). Since this field is still at an early stage in its development, there are some shortcomings, including poorly annotated lncRNA transcripts. However, this will be overcome with direct RNA sequencing using ONT and PacBio technology. These technologies will enable us to determine the exact isoforms of transcripts expressed in a particular cell and begin to catalog the different RNA modifications that exist basally and during a biological process such as activation of inflammation. Since lncRNAs are cell-type specific in their expression patterns continued development of single-cell sequencing technologies will provide a complete catalog of lncRNAs in the genome. As the list of annotated lncRNAs grows, characterizing the function of all these genes has become a definite bottle-neck in the field. However, high-throughput CRISPR screening provides an approach to quickly identify functional lncRNAs in a particular biological system. Utilizing all the tools outlined here should enable researchers to develop this field rapidly. For our research focus, gaining a better understanding of the role of lncRNAs in regulating immune responses will provide novel insights into the molecular mechanisms governing inflammation. This data will be critical for identifying new avenues for therapeutic intervention for infectious and inflammatory disease.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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