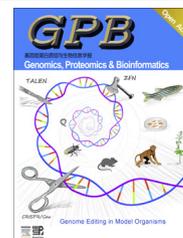




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### REVIEW

# Roles, Functions, and Mechanisms of Long Non-coding RNAs in Cancer

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**Abstract** Long non-coding RNAs (**lncRNAs**) play important roles in **cancer**. They are involved in **chromatin** remodeling, as well as transcriptional and post-transcriptional regulation, through a variety of chromatin-based mechanisms and via cross-talk with other RNA species. **lncRNAs** can function as decoys, scaffolds, and enhancer RNAs. This review summarizes the characteristics of **lncRNAs**, including their roles, functions, and working mechanisms, describes methods for identifying and annotating **lncRNAs**, and discusses future opportunities for lncRNA-based therapies using **antisense oligonucleotides**.

### Introduction

Recent advances in sequencing technologies enabling more in-depth genomic and transcriptomic analyses have revealed that as much as 85% of the human genome is transcribed [1–3]. This was surprising, as studies of mammalian genomes have shown that a drastically low population of RNA transcripts code for protein products. Notably, from the recent Encyclopedia of DNA Elements (ENCODE) work, out of 41,204 called genes, only 56 genes (0.1%) showed mass spectrometric

evidence consistent with protein expression, suggesting that the majority of RNA transcripts are non-coding [4]. The generation of such a large population of non-coding RNA (ncRNA) transcripts indicates that RNAs have a larger and more diverse role in biological processes than initially anticipated. ncRNAs can be roughly classified into two groups based on their size. One group includes short RNAs less than 200 nucleotides (nt) in length, such as microRNAs (miRNAs) that are small RNA (sRNA) molecules around 21–24 nt in length, as well as other classes such as piwi-interacting RNAs (piRNAs) [5]. The other group includes long ncRNAs (lncRNAs) of around 200 nt or more [6]. While miRNAs have been heavily studied and are well understood for their function in gene regulation [7], lncRNAs in contrast are less understood.

Many lncRNAs have been functionally associated with human diseases, in particular, cancers [8] (Table 1). Dysregulation of lncRNAs has been implicated in glioblastoma [9–11],

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**Table 1** Examples of lncRNAs in cancer

lncRNA	Description	Refs.
<i>PTCSC3</i>	Downregulated in thyroid cancers	[82]
<i>HULC</i>	Upregulated in hepatocellular carcinoma	[81,139–141]
<i>XIST</i>	Dysregulated in various cancers	[16,142]
<i>GAPLINC</i>	Associated with poor prognosis in gastric cancer	[143]
<i>MALAT1</i>	Associated with poor prognosis and metastasis in liver, lung and colorectal cancers	[144–147]
<i>HOTAIR</i>	Associated with metastasis in colorectal, liver, pancreatic, breast and gastric cancers	[12–14,148–150]
<i>ANRIL</i>	Upregulated in prostate cancer	[151]
<i>KCNQ1OT1</i>	Upregulated in colorectal cancer	[59]
<i>PRNCRI</i>	Upregulated in prostate cancer	[152]
<i>H19</i>	Highly expressed in hepatocellular carcinoma	[153]

breast cancer [12], colorectal cancer [9,13], liver cancer [14,15], and leukemia [9,16]. Commonly, dysregulation of lncRNAs exerts impacts on cellular functions such as cell proliferation, resistance to apoptosis, induction of angiogenesis, promotion of metastasis, and evasion of tumor suppressors [8,17].

An emerging view of lncRNAs is that they are fundamental regulators of transcription. This view has led to an intense focus on elucidating the molecular mechanisms that underlie their function [18]. Many lncRNAs have been characterized and several models of action have been proposed, such as functioning as signal, decoy, scaffold, guide, enhancer RNAs, and short peptides [19,20], as mentioned below. The main function of a signal lncRNA is to serve as a molecular signal to regulate transcription in response to various stimuli. Thus its production and presence can serve as an indicator of transcriptional activity [21]. Decoy lncRNAs limit the availability of regulatory factors by presenting “decoy” binding sites. These lncRNAs modulate transcription by sequestering regulatory factors including transcription factors, catalytic proteins, subunits of larger chromatin modifying complexes, as well as miRNAs, thereby reducing their availability [22]. Transcripts from the scaffold class of lncRNAs play a structural role by providing platforms for assembly of multiple-component complexes, such as ribonucleoprotein (RNP) complexes [23]. In the case of RNP complexes, once the complexes have been fully assembled, either transcriptional activation or repression could be conferred depending on the nature of proteins and RNAs present [24,25]. Guide lncRNAs interact with RNPs and direct them to specific target genes. These guide lncRNAs are essential for the proper localization of RNPs [26]. Next, enhancer RNAs (eRNAs) are produced from enhancer regions and may work by influencing the 3-dimensional (3D) organization of DNA, also known as “chromatin interactions”. One hypothesized model of action is that these lncRNAs may possibly work as “tethers” as they may not be released from the enhancer regions when functioning, thus tethering the interacting proteins to enhancer regions [19]. In addition, lncRNAs can encode short peptides, which may also have functions [27]. It is likely that additional mechanisms will be discovered in the future.

This review introduces lncRNAs, focusing on lncRNAs with cancer-associated roles, and discusses proposed mechanisms by which these lncRNAs function in chromatin remodeling, chromatin interactions, and as competing endogenous RNAs (ceRNAs). In addition, we highlight approaches for the identification and annotation of lncRNAs, cross-talk between these mechanisms, and methods for perturbing speci-

fic lncRNAs, which could eventually provide lncRNA-based therapies for diseases. We then conclude by highlighting challenges and future research topics.

### Characteristics of lncRNAs

lncRNAs are defined as RNA molecules with more than 200 nucleotides. This distinction, while somewhat arbitrary and based on technical aspects of RNA isolation methods, serves to distinguish lncRNAs from miRNAs and other sRNAs. lncRNAs are present in large numbers in genome [28,29]. They typically do not possess functional open reading frames (ORFs). However, this distinction is blurred by the discovery of bifunctional RNAs that can have both protein-coding and coding-independent functions [30,31], raising the possibility that many protein-coding genes may also have non-coding functions. Many lncRNAs are lowly expressed [32], posing a challenge in terms of exploration of lncRNAs and explaining why lncRNAs had been thought to be only “transcriptional noise” until recently. RNA-seq studies in different tetrapods show that most (81%) lncRNAs are poorly conserved in DNA sequence and are primate-specific. However, it should be noted that several lncRNAs are ultra-conserved in DNA sequence—3% of lncRNAs appear to have originated more than 300 million years ago and can be found from organisms ranging from *Xenopus* and chicken to man [33]. It is possible that lncRNAs might be fast-evolving RNA species that can play key roles in specifying lineages. In support of this idea, a comparison of matched tissues in *Mus musculus domesticus*, *Mus musculus castaneus*, and *Rattus norvegicus* shows that the emergence or extinction of intergenic lncRNAs is associated with changes in transcription levels of proximal protein-coding genes [34]. In addition, there are examples of lncRNAs exhibiting conserved biological function but low sequence conservation, such as megamind/TUNA, which is associated with brain development in zebrafish, mouse, and human [35,36], as well as X-inactive specific transcript (*Xist*), which is involved in X-inactivation [37]. It is possible that RNA molecules need less sequence conservation to retain their function compared to proteins. Conversely, there is high sequence conservation of lncRNA promoters, which is even higher than that of protein-coding gene promoters [29], suggesting that regulation of lncRNA expression is important.

Many lncRNAs possess features reminiscent of protein-coding genes, such as 5' cap and alternative splicing [32]. In

fact, many lncRNA genes have two or more exons [32] and about 60% of lncRNAs have polyA+ tails [38] (Figure 1). Additionally, while there are long intergenic RNAs (lincRNAs) [39] including eRNAs from gene-distal enhancers [40], the majority of lncRNA genes are located within 10 kb of protein-coding genes [41] and many lncRNAs are antisense to coding genes or intronic [42].

In line with a wide range of functions that lncRNAs are involved in, lncRNAs can be found in many tissues, although the brain and central nervous system appear to have the highest diversity of expressed lncRNAs [43]. Also, lncRNAs can be found in different cellular compartments including both the cytoplasm and nucleus to which they seem to predominantly localize [32]. lncRNAs have been generally thought to be unstable due to their low expression levels and the existence of known classes of unstable transcripts such as the promoter upstream transcripts (PROMPTs) [44]. Interestingly, a recent study indicates that only a minority (240 out of 823 lncRNAs; 29%) of lncRNAs are unstable with half-lives less than 2 h, while 51 (6%) were extremely stable with half-lives of over 12 h [45].

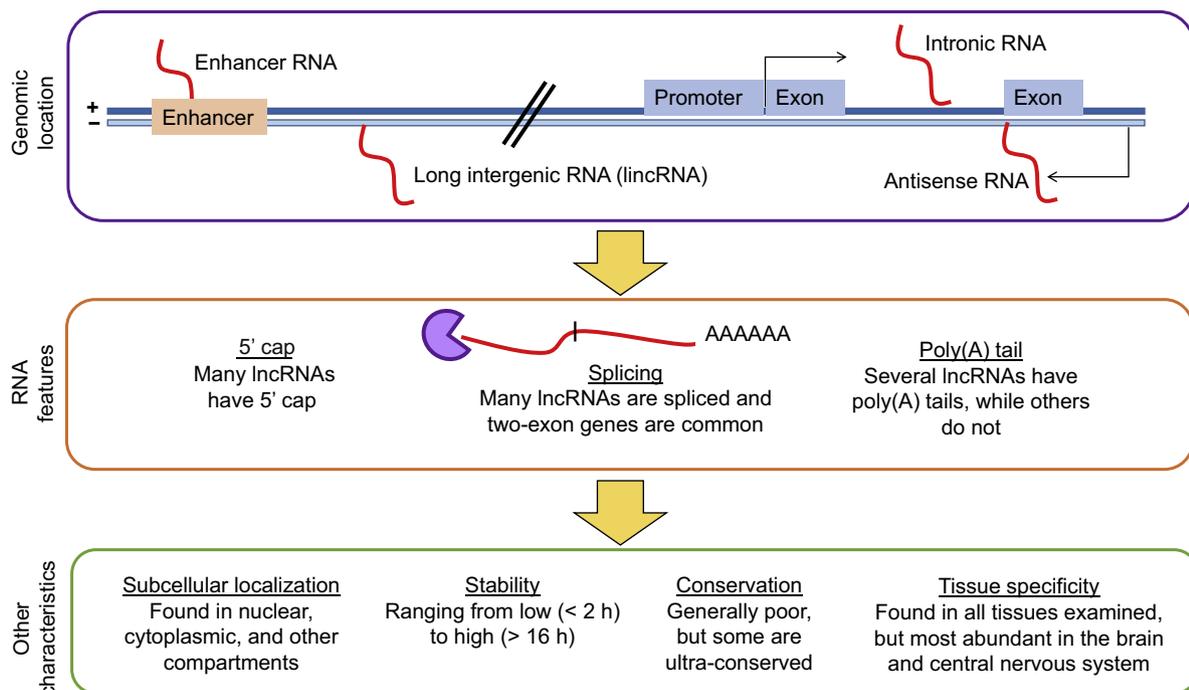
### Acting mechanisms of lncRNAs

In cancer, lncRNAs work through multiple mechanisms such as chromatin remodeling, chromatin interactions, ceRNAs, and natural antisense transcripts (NATs) (Figure 2). lncRNAs can interface with chromatin remodeling machinery in several ways, including acting as signal lncRNAs or scaffold lncRNAs. Recently, another mechanism of action by which lncRNAs function to regulate transcription has been posited. This involves the production of lncRNAs from enhancer regions in the genome (eRNAs), which function to stabilize as well as maintain chromatin loops [40,46–48]. Chromatin

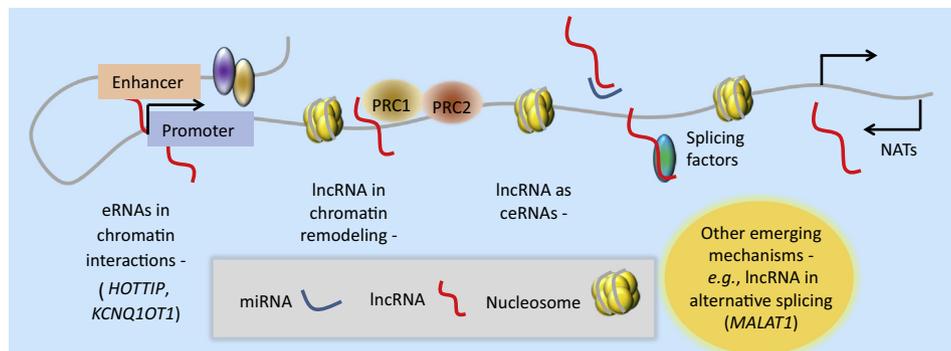
looping enables distally-located enhancers (with some located several hundred kilobases away) to interact with their target gene promoters [49–51]. In addition, several studies aimed at elucidating the biological functions of miRNAs have previously used artificial miRNA sponges that compete with native targets for interaction with the miRNAs of interest [52–54]. Similarly, studies have also demonstrated that some lncRNAs modulate transcription by sequestering regulatory factors, including transcription factors and catalytic proteins or subunits of larger chromatin-modification complexes, as well as miRNAs [22]. They are known as ceRNAs [55–57]. Transcribed from the opposite DNA strand in relative to the sense transcripts, NATs overlap with their sense transcripts [58] and are thought to regulate the expression of the sense transcripts as well. We will discuss examples of these mechanisms in the next sections. Nonetheless, it should be noted that the mechanisms described here are not exhaustive, and future work is likely to provide new insights in this fast-moving field.

### lncRNAs in chromatin remodeling

lncRNAs can act through chromatin remodeling to achieve transcriptional regulation. One example is the potassium voltage-gated channel subfamily KT member 1 opposite strand/antisense transcript 1 (*KCNQ1OT1*), which is upregulated in colon cancer [59]. *KCNQ1OT1* acts as a signal lncRNA by recruiting G9a histone methyltransferases and polycomb repressive complex 2 (PRC2) [21], which mediate the gene-silencing-associated marks, *i.e.*, dimethylation of lysine 9 (H3K9me2) and lysine 27 on histone 3 (H3K27me3) [60]. Through chromatin remodeling, *KCNQ1OT1* induces transcriptional silencing of genes, which may occur in *cis* (for targets close by) or in *trans* (for distal targets).



**Figure 1** Characteristics of lncRNAs



**Figure 2 Acting mechanisms of lncRNAs**

eRNA, enhancer RNA; ceRNA, competing endogenous RNA; NAT, natural antisense transcript; PRC1, polycomb repressive complex 1.

As another example, *ANRIL/CDKN2B*, the antisense ncRNA in the *INK4* locus, works as a scaffold in mediating transcriptional silencing of the *INK4b-ARF-INK4a* locus by recruiting and interacting with PRC1 and PRC2 [24,25]. PRC2 recruited to this gene locus then mediates the spread of the methylation marks that are distinct for transcriptionally-silent genes. Several lncRNAs have been demonstrated to interact with chromosome-modification complexes and direct them to specific target genes. These guide lncRNAs are essential for the proper localization of the chromosome-modification complexes including PRC2 and subsequent repression of gene expression as demonstrated by the lncRNA fetal-lethal non-coding developmental regulatory RNA (*FENDRR*), which serves to bring PRC2 in close proximity to the promoters of genes associated with the formation and differentiation of the lateral mesoderm lineage, such as forkhead box F1 (*FOXF1*) and paired-like homeodomain2 (*PITX2*) genes [26]. We hypothesize that other guide lncRNAs may also function to regulate transcription by targeting other chromatin-modification complexes, in addition to PRC2, to their target genes.

Finally, the *HOX* transcript antisense RNA (*HOTAIR*) lncRNA functions cooperatively with PRC2 in mediating the repression of the homeobox D cluster (*HOXD*) locus through spreading H3K27me3 marks, which are associated with gene silencing [61]. *HOTAIR* forms multiple double stem-loop structures that bind to lysine-specific demethylase 1 (LSD1) and PRC2 histone-modification complexes [12]. Other lncRNAs also operate in a similar manner, with as much as 20% of lncRNAs known to associate with PRC2 [62]. For example, the lncRNA *TUG1* is associated with PRC2, and depletion of *TUG1* expression in the developing mouse eye leads to the blockage of retinal development [62]. While PRC2 has been found to interact with many lncRNAs, other chromatin remodelers have been implicated and it is likely that more interactions with other chromatin remodelers remain to be discovered. Moreover, given that cancers are associated with aberrant levels of PRC2, H3K27me3, and mutated enhancer of zeste homolog 2 (*EZH2*), which is a component of the PRC2 complex [63], we hypothesize that lncRNA-based mechanisms provide an explanation as to how these alterations in PRC2 levels can give rise to cancer (Table 1). Taken together, it is becoming increasingly clear that certain lncRNAs can associate with chromatin-modification complexes to carry out cellular functions.

## lncRNAs in chromatin looping in cancers

Another mechanism of action by which lncRNAs function to regulate transcription is via enhancer lncRNAs (eRNAs) associated with chromatin loops. To study chromatin looping, techniques including chromosome conformation capture (3C) [64] and fluorescence *in situ* hybridization (FISH) [65], have been employed. More recent technical developments have focused on higher-throughput analyses and these include circular chromosome conformation capture (4C) [66,67], chromosome conformation capture carbon copy (5C) [68], combined 3C-ChIP-cloning (6C) [69], chromatin interaction analysis with paired-end tag sequencing (ChIA-PET) [70], and Hi-C [71]. Using ChIA-PET, a correlation is revealed between expression level of eRNAs and estrogen receptor  $\alpha$  ( $ER\alpha$ )-associated chromatin interactions [72]. The experimental downregulation of these eRNAs leads to a loss of chromatin loops and a corresponding change in expression of the genes targeted by  $ER\alpha$  [46].

In addition, a relationship is found between the levels of eRNAs produced by upstream enhancers of the prostate-specific antigen (*PSA*) gene and the actual levels of *PSA* gene expression, suggesting a possible link between eRNAs and chromatin interactions [73]. Melo et al. show the existence of enhancer regions that bind the transcription factor p53. These enhancer regions produce RNAs and display chromatin interactions with multiple neighboring genes. Ablation of these eRNAs leads to reduced transcription at neighboring genes and reduced p53-dependent cell cycle arrest [74]. More recently, a role for Integrator in the biogenesis of eRNAs is demonstrated [75]. Integrator is a complex associated with RNA polymerase II (RNAPII), and possesses RNA endonuclease activity, which is required for 3' end processing of non-polyadenylated nuclear RNA genes [76]. These studies show that depletion of Integrator leads to a decrease in the induction of eRNAs, which is accompanied by the loss of enhancer-promoter chromatin looping [75].

One of the best examples of lncRNAs that regulate chromatin interactions is the *HOXA* transcript at the distal tip (*HOTTIP*). *HOTTIP* is a lncRNA shown to regulate chromatin interactions in the *HOX* cluster, from which it is produced. *HOTTIP* is necessary to coordinate activation of *HOX* genes, through binding WD repeat-containing protein 5 (*WDR5*), an adaptor protein [77]. *WDR5* interacts with

the mixed lineage leukemia (MLL) complexes for substrate recognition and genomic targeting. The MLL complexes catalyze H3K4 methylation, which is a mark of actively transcribed genes [77]. *HOTTIP* is necessary for the maintenance of a specific pattern of WDR5/MLL complexes across the *HOXA* locus to facilitate gene transcription [77].

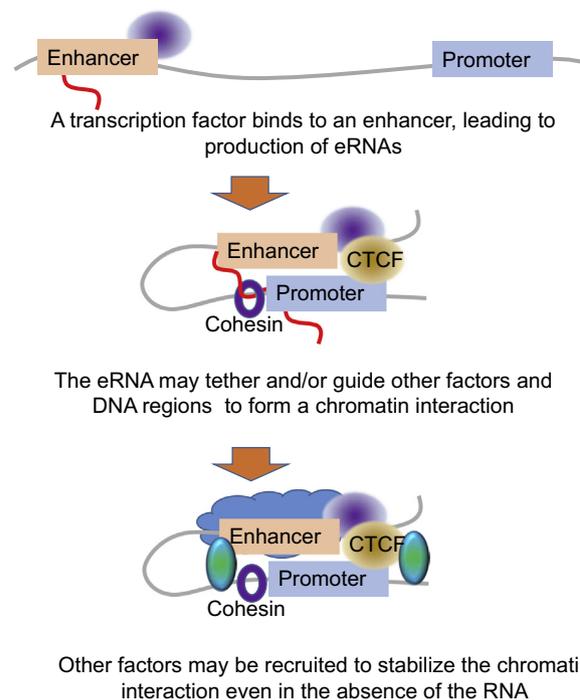
Additionally, there is cross-talk between the different mechanisms: a novel RNA chromosome conformation capture (R3C) strategy demonstrated that *KCNQ1OT1*, which regulates genes in the *KCNQ1* imprinting region, is involved in imprinting-associated chromatin interactions [78]. EZH2, which catalyzes the deposition of gene silencing-associated H3K27me3 marks, is recruited by the *KCNQ1OT1* [78]. Depletion of *KCNQ1OT1* leads to depletion of the loop and loss of imprinting [78]. These examples all demonstrate a role for eRNA-associated chromatin interactions in transcriptional suppression, enhancement, and coordination of gene expression, as well as other functions such as imprinting. A hypothesized model of action is that eRNAs may help to tether different genomic regions together, recruiting factors such as PRC2 and other proteins at the 5' end, while remaining tethered to the location of production [79] (Figure 3). Other factors might help to bring together other DNA regions, or the length of the eRNA might enable it to tether and guide other DNA regions together, thus forming a chromatin interaction. Other factors may bind to the chromatin directly or indirectly through the bound proteins, stabilizing and maintaining the chromatin even after the RNA may have been degraded. For example, cohesin has been shown to play such a role in the case of ER $\alpha$ -associated RNAs [46]. Adding support to this idea is the finding that transcriptional repressor CCCTC-binding factor (CTCF), which is involved in chromatin interactions, can bind to ncRNAs. For example, the steroid receptor RNA activator (*SRA*) binds to CTCF and enhances its functioning [80].

### lncRNAs as ceRNAs in cancers

The lncRNA *HULC* is highly upregulated in hepatocellular carcinoma (HCC) [81]. There are multiple miR-372-binding sites present in *HULC*, and the overexpression of *HULC* can reduce miR-372 expression. This leads to reduced translational repression of its target transcript *PRKACB*, thereby inducing the phosphorylation of the cAMP-responsive element (CRE)-binding protein (CREB) [81]. On the other hand, papillary thyroid carcinoma susceptibility candidate 3 (*PTCSC3*) is highly downregulated in thyroid cancers and its overexpression leads to reduced expression of oncogenic miR-574-5p, resulting in growth inhibition, cell-cycle arrest, and increased apoptosis [82]. Studies identifying additional molecular targets of decoy lncRNAs will add to our understanding of diseases while presenting possible therapeutic interventions for which drugs may be designed.

### lncRNAs as NATs

NATs are surprisingly common in the mammalian genome—over 20% of human transcripts form sense–antisense pairs [83]. A later estimate in mice based on large RNA-seq datasets shows that up to 72% of genes may have transcription-related activity on the opposite strand [58]. NATs can be produced



**Figure 3 Hypothesized acting mechanism for eRNAs to initiate chromatin interactions**

eRNA, enhancer RNA; CTCF, CCCTC-binding factor.

against both protein-coding and non-coding genes [58] and some NATs are protein-coding genes while others are lncRNAs [58]. In addition, the genomic organization of NATs relative to their sense transcripts varies with different configurations. One common configuration, called “divergent”, is that the promoter generates bidirectional transcription [58,84]. Another common configuration, called “convergent”, arises whereby the NAT starts from a different promoter, and transcribes a region on the opposite strand from the protein-coding transcript [58]. For example, the NAT could start from the 3' end of the sense transcript and transcribe toward its 5' end [58].

NATs exert varied influence on their sense transcripts, either suppression or activation. The expression levels of sense/antisense pairs are generally concordant, but reciprocal expression is also observed [58]. NATs can work through a variety of mechanisms. Other than employing mechanisms similar to other lncRNAs such as by scaffolding proteins, NATs that overlap with sense transcripts can work through a particular mechanism, that is, transcriptional collision. RNAPII complexes transcribing on opposite DNA strands cannot bypass each other [85]. Head-to-head collision results in stalling of RNAPII and subsequent removal of collided RNAPII by ubiquitin-directed proteolysis [85]. Therefore, convergent sense and antisense transcripts could lead to sense strand suppression.

One example of a NAT in cancer is WD40-encoding RNA antisense to p53 (*Wrap53*) [86]. As a NAT of the important oncogene *TP53*, *Wrap53* can induce *TP53* expression by targeting the 5' untranslated region of the *TP53* mRNA [86]. Blocking this interaction between the *Wrap53* lncRNA and *TP53* mRNA reduces basal levels of *TP53* and prevents induction of *TP53* after DNA damage [86]. *Wrap53* is overexpressed

in cancer cell lines; interestingly, overexpression of *Wrap53* results in cellular transformation while ablation leads to apoptosis [87]. Additionally, *Wrap53* overexpression is correlated with poor prognosis in head and neck squamous cell carcinoma [87]. Taken together, *Wrap53* is an oncogenic lncRNA that regulates *TP53* expression.

### lncRNA networks and cross-talk in cancer

An interesting aspect of lncRNAs is that there can be cross-talk between different mechanisms, leading to the formation of complex networks, particularly in cancer. For example, the transcription factor p53 regulates many genes [88], including lncRNAs such as *lncRNA-p21*, *PANDA*, *H19*, and *loc285184*, which serve as effectors of p53 by leading to p53-associated cellular functions such as cell cycle arrest and apoptosis [89]. p53 is self-regulated not only by well-known regulators such as MDM2, an E3 ubiquitin ligase, but also by lncRNAs *MALAT1*, and *MEG3*, as well as the *TP53NAT-Wrap53* as discussed in the previous section [89]. *MALAT1* is upregulated in several cancers [90] and may be used to predict survival and metastasis in non-small cell lung cancer [91]. And *MALAT1* is expressed in a cell cycle-dependent manner and is required for G1/S and mitotic progression [92]. Paradoxically, depletion of *MALAT1* leads to *TP53* activation [92]. However, the cells show reduced oncogenic transcription factor B-MYB, leading to increased cellular proliferation via B-MYB [92]. For more details on these lncRNAs, readers are referred to the excellent review by Zhang and colleagues [89]. Interestingly, c-Myc, another important cancer-associated transcription factor, has a similar network [93]. It remains an open question whether other cancer-associated transcription factors possess a similar network consisting of lncRNAs that lead to altered cellular functions and ultimately cancer. Given that transcription factors are generally considered difficult to target by small molecule inhibitors due to the intrinsically disordered nature

of their binding sites (meaning they lack stable secondary and/or tertiary structure under physiological conditions *in vitro*) and their binding promiscuity [94], lncRNAs that regulate the transcription factors may open up new avenues for targeting cancers with aberrant transcription factor signaling.

### Approaches for the identification and annotation of lncRNAs

In general, the expression levels of lncRNAs are low in abundance and lncRNAs are often not polyA-tailed, posing several challenges for their identification and analysis because previous methods of microarray and mRNA-seq rely on polyA+ isolation and tend to favor the detection of transcripts that are abundant in expression. In spite of these challenges, a number of effective methods have been developed for lncRNA identification and annotation (Table 2). Today, RNA-seq is one of the most common methods for identifying novel lncRNAs, because of the dropping sequencing costs and the single-nucleotide resolution nature of RNA-seq [95]. RNA-seq involves converting RNA into cDNA, followed by fragmentation, and ultra-high-throughput sequencing by methods such as Illumina HiSeq. As lncRNAs have low expression levels, high sequencing depths of 100–150 million reads or more are needed to discover these rare lncRNAs [96]. In addition, strand-specific sequencing methods are also needed to distinguish antisense lncRNAs from sense transcripts and cDNA conversion must be performed using random hexamers as opposed to oligo-dT methods [96]. RNA-seq experiments to detect lncRNAs have to be performed through rRNA-depletion to enrich for mRNAs and lncRNAs or by sequencing both polyA+ and polyA– fractions [96]. Owing to the high sequencing depth, RNA-seq becomes expensive when investigating some of the rarest lncRNAs. Hence researchers have also turned to custom

**Table 2** Examples of key technologies and tools for identifying and annotating lncRNAs

Technique	Purpose	Refs.
RNA-seq	Used to characterize and annotate RNAs, including lncRNAs. It provides the abundance and exonic structure of the RNAs, allowing for better understanding of alternative splicing	[95]
Custom arrays	Custom lncRNA microarrays used to screen through many lncRNAs in many different samples; custom tiling microarrays used to identify lncRNAs in specific regions of the genome; custom microarrays used to perform sequence capture	[98]
RIP-Seq	Used to characterize RNAs that bind to a particular protein of interest	[101]
ChIRP-Seq	Used to characterize DNA or RNA or proteins from chromatin complexes	[46,102]
RNA-FISH	Used to visualize the location of the lncRNAs in different cellular organelles	[103]
RNA-3C	Used to investigate associations between lncRNAs and 3-D genome organization	[78]
Structure-Seq	Used to infer the secondary structure of RNA with several structure determination methods available, such as SHAPE-Seq, PARS, and FRAG-Seq	[104–107]
CRISPR	Used to engineer knock-outs or knock-ins or other constructs at the genomic loci of lncRNAs	[108,109]
ASO	Used to perturb particular lncRNAs and their interactions with other proteins, DNAs, or RNAs	[121–123]

*Note:* RIP-Seq, RNA immunoprecipitation-Seq; ChIRP-Seq, chromatin isolation by RNA purification-Seq; RNA-FISH, RNA-fluorescence *in situ* hybridization; RNA-3C, RNA-chromosome conformation capture; SHAPE-Seq, selective 2'-hydroxyl acylation analyzed by primer extension sequencing; PARS, parallel analysis of RNA structure; FRAG-Seq, fragmentation sequencing; CRISPR, clustered regularly interspaced short palindromic repeat; ASO, antisense oligonucleotide.

lncRNA microarrays to screen through many lncRNAs in many different samples [97], as well as custom tiling microarrays to identify lncRNAs in specific regions of the genome [61]. Other than that, microarray capture followed by sequencing is also used for quantitative gene profiling and annotation of specific lncRNAs [98].

Several new sequencing methods are becoming available, such as Pacific Biosciences (PacBio) sequencing, which features extremely long (on the order of kilobases) reads [99,100]. PacBio sequencing allows for read-through of complete gene sequences, facilitating the understanding of alternative splicing. While this method is currently only cost-effective for small genomes such as bacteria, in the near-future, it will probably be applicable to analysis of human samples.

There is a battery of experimental methods for annotating lncRNAs. RNA immunoprecipitation (RIP)-Seq involves sequencing RNAs that are associated with a particular RNA-binding protein of interest with immunoprecipitation [101]. A related method, chromatin isolation by RNA purification (ChIRP)-Seq involves the design of multiple biotin-tagged oligonucleotide probes that recognize a particular RNA [46,102]. Cross-linked chromatin complexes with RNA of interest are then isolated using streptavidin magnetic beads recognizing the biotin tag [46,102]. DNA, RNA, and protein can be isolated from these complexes and subjected to sequencing or mass spectrometry for identification [46,102]. Another method, RNA-FISH, involves designing fluorescent probes to RNA and performing the hybridization in cells followed by microscopic imaging. It is used to visualize the location of the lncRNAs in different cellular organelles and investigate how lncRNA localization is altered in response to different stimuli [103]. In addition, RNA-3C involves double-stranded cDNA synthesis using biotinylated oligonucleotides to obtain chromatin complexes with biotinylated cDNA, followed by digestion and proximity ligation, generating DNA-cDNA constructs. These constructs can then be pulled down using the biotin group and analyzed by PCR. RNA-3C has been used to investigate associations between lncRNAs and 3-D genome organization [78].

In addition, there are a variety of sequencing methods for analyzing the secondary structure of RNA. These include selective 2'-hydroxyl acylation analyzed by primer extension sequencing (SHAPE-Seq), whereby RNA is isolated, barcoded, and folded *in vitro* [104]. A SHAPE reagent, 1M7, is added to the isolated, barcoded, and folded RNA, which blocks the reverse transcriptase reaction whereby the 1M7 is included in the RNA, and hence leads to a series of truncated products to be sequenced, allowing for reconstruction of the original structure [104]. A similar method, parallel analysis of RNA structure (PARS) involves digestion of RNAs using RNAses specific for double-stranded and single-stranded RNAs. The fragments are then reversely transcribed and compared with each other, allowing for deduction of RNA structures *in vivo* [105,106]. A third method, fragmentation sequencing (FRAG-Seq), is similar to PARS except that it uses P1 nuclease instead of RNAses [107].

Perturbing lncRNA expression levels can be achieved in different ways, including clustered regularly interspaced short palindromic repeat (CRISPR) genomic editing of lncRNA genes [108,109]. In addition, new antisense oligonucleotide (ASO) approaches can be applied to perturb both cytoplasmic and nuclear lncRNAs, which will be discussed in more

details in the next section. CRISPR genomic editing comes from the immune defenses of bacteria and archaea, which use short RNA to degrade invading nucleic acids [110,111]. Being one component of the CRISPR immune defense system, Cas9 effector nuclease is the first known nuclease that is capable of binding to specific short RNA and thereby directing cleavage at complementary genomic loci [110]. CRISPR/Cas9 represents a giant leap forward in terms of ease of use and efficiency in comparison with traditional methods for excising DNA such as homologous recombination and zinc finger nucleases (ZFNs) [110]. This method has since been widely adopted by many labs around the world, in a wide range of different cell types and organisms [110]. CRISPR editing can also be used to excise lncRNAs for functional studies. In addition, CRISPR can be modified for other purposes such as upregulating gene expression by combining a Cas9 unable to cleave nucleic acids with transcription activators such as VP64 activator domains [110,112,113]. This system could be used to upregulate lncRNA expression levels to understand their functions.

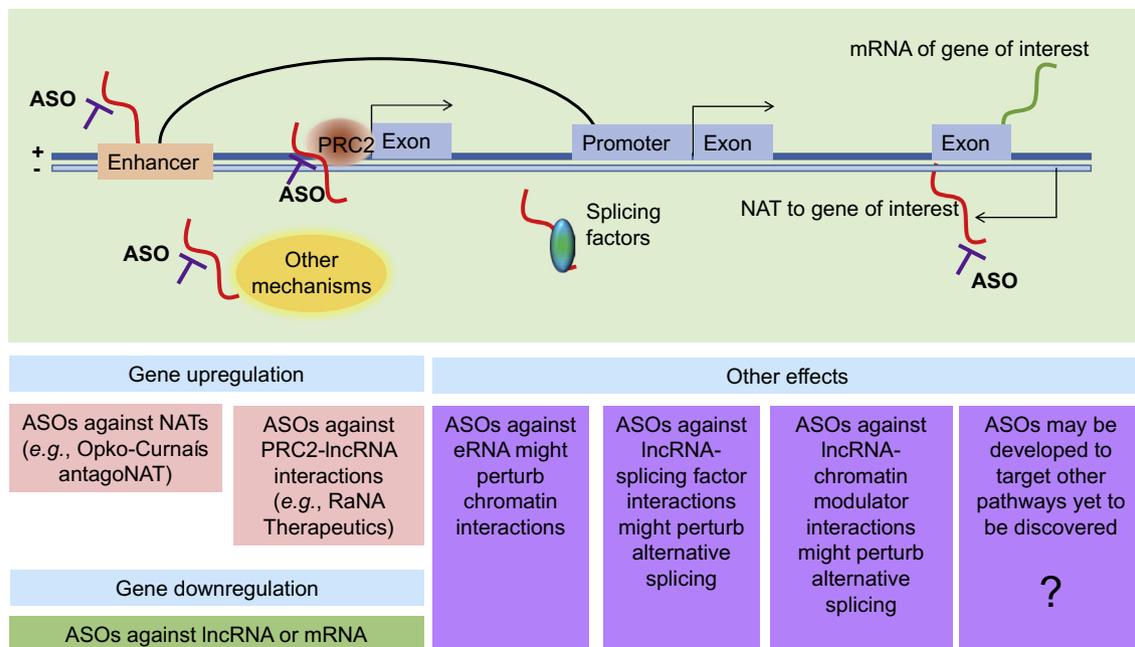
There are several databases available for lncRNA research (Table 3). ENCODE [28], FANTOM [114], and TCGA [115] have all embarked on massive RNA-seq efforts in different tissues including patient samples, which have yielded an unprecedented collection of lncRNAs. In addition, several groups have developed curated lists of lncRNAs by mining the existing literature and by predictions. For example, lncRNome [116] can serve as a general resource, while lncRNA Disease [117] presents lncRNA-disease associations. Other groups have also developed bioinformatics tools to infer functions of lncRNAs. These include looking for co-expressed genes and lncRNAs as featured in the lncRNator database [118], looking at functional similarity patterns (motifs) and predicting RNA structures as featured in LNCipedia and lncRNome [116,119], and integrating epigenomic information as featured in lncRNome [116]. For a detailed discussion and comparison of lncRNA databases, we refer readers to the article by Fritah and colleagues [120].

## ASOs for modulation of lncRNA

The importance of lncRNAs in health and diseases (Table 1) indicates a need to find methods for modulating lncRNAs. To do this, ASOs are used (Figure 4), which are short DNA sequences and complementary to an RNA of interest. The oligonucleotide works by hybridizing to the RNA, which then blocks the action of the RNA. While unmodified oligonucleotides are available, certain chemical modifications such as 2'-O-(2-methoxy) ethyl oligonucleotides increase the lifespan of the oligonucleotide in the complex milieu of nucleases within the cell, and reduce degradation products, which may also have effects on the cells [121]. Two ASOs have been approved as drugs by the Food and Drug Administration (FDA) of the United States: fomivirsin, which is used to treat cytomegalovirus retinitis [122], and mipomersen, which is used to treat homozygous familial hypercholesterolemia [123]. ASOs can enter the nucleus and knock down nuclear lncRNAs [124], enabling all classes of lncRNAs to be explored. In contrast, siRNA and shRNA are not effective in targeting nuclear lncRNAs because the RNAi machinery is located in the cytoplasm.

**Table 3** Key databases of lncRNAs

Database	Description	Web link	Ref.
<b>Databases with RNA-seq and other primary datasets</b>			
ENCODE	Produced many RNA-seq and complementary datasets for a certain set of human cell lines	<a href="https://genome.ucsc.edu/ENCODE/">https://genome.ucsc.edu/ENCODE/</a>	[28]
FANTOM	Produced many RNA-seq and complementary datasets from mice	<a href="http://fantom.gsc.riken.jp/">http://fantom.gsc.riken.jp/</a>	[114]
TCGA	Contains lncRNAs from primary patient tumors	<a href="http://cancergenome.nih.gov/">http://cancergenome.nih.gov/</a>	[115]
<b>Curated lists of lncRNAs</b>			
lncRNome	A curated list of >17,000 annotated lncRNAs with information on chromosomal locations, biological functions, diseases associations, and the types of the lncRNAs. Datasets on protein-lncRNA interactions and genomic variations in lncRNA loci are also accessible from this database	<a href="http://genome.igib.res.in/lncRNome/">http://genome.igib.res.in/lncRNome/</a>	[116]
LNCipedia	An integrated database with a curated list of 111,685 annotated lncRNAs obtained from different sources. This database provides transcript and structure information on the lncRNAs as well as statistics for secondary structure information, protein coding potential, and microRNA binding sites	<a href="http://www.lncipedia.org/">http://www.lncipedia.org/</a>	[119]
LncRNADisease	A curated list of >1000 lncRNA-disease associated data from ~500 publications, as well as predictions of novel lncRNA-disease associations of 1564 human lncRNAs	<a href="http://www.cuilab.cn/lncrnadisease">http://www.cuilab.cn/lncrnadisease</a>	[117]
<b>Annotators</b>			
LncRNAtor	Co-expression between mRNAs and lncRNAs in various tissues	<a href="http://lncrnator.ewha.ac.kr/index.htm">http://lncrnator.ewha.ac.kr/index.htm</a>	[118]

**Figure 4** Targeting lncRNAs for therapeutic applications

NAT, natural antisense transcript; PRC2, polycomb repressive complex 2; ASO, antisense oligonucleotide; eRNA, enhancer RNA.

A major interest for antisense therapy is to upregulate gene expression. Several lncRNAs work as NATs to genes of therapeutic interest. Classic small molecule drugs work by inhibiting gene expression; therefore genes that need to be upregulated have been almost undruggable, although enzyme replacement therapy has been used as a strategy, such as lysosomal enzymes in the case of lysosomal storage diseases [125]. Antisense targeting of natural antisense lncRNAs, or “antagoNATs”, provides a very specific way for gene

upregulation [126,127]. Taken together, ASOs constitute a very promising tool that can be used to translate basic discoveries about lncRNAs to the clinic. For instance, Opko-Curna is a company taking forward this technology into the clinic [126].

Similarly, company RaNa Therapeutics aims to upregulate gene expression, but using a different approach [128]. Using RIP-Seq, thousands of lncRNAs were found to interact with PRC2, which represses transcription of the genes targeted by

lncRNAs [101]. Notably, specific PRC2-lncRNA interactions can be disrupted using locked nucleic acids, resulting in upregulation of the target genes [129]. Locked nucleic acids are RNA derivatives similar in principle to ASOs [130]. Therefore, the company is keen to de-repress the expression of genes of interest by using locked nucleic acids against specific lncRNAs that interact with PRC2.

Given that lncRNAs have been found to act in other pathways, such as eRNAs, they have additional potential in terms of therapeutic mechanisms that can be targeted by ASOs. For example, transcription factors, chromatin modulators, and chromatin interactions are difficult pathways to target, as these factors are in the nucleus, which is difficult for small molecule inhibitors to enter. ASOs against lncRNAs, which interact with factors that can enter the nucleus, might offer a feasible mechanism for future targeting of these important pathways.

## Future directions

A major challenge to work on lncRNAs is that despite their importance, the molecular mechanisms underlying their functions are not yet fully understood. Further insight into the biological significance and functioning of lncRNAs will require additional studies to be conducted, which may lead to the discovery of yet more mechanisms of action. Several functions are just starting to be appreciated, such as roles in alternative splicing. For example, *MALAT1* controls alternative splicing by regulating the phosphorylation and distribution of serine/arginine splicing factors in nuclear speckle domains [131]. It is likely that other lncRNAs will be found that regulate alternative splicing through other mechanisms. Moreover, this review did not cover novel mechanisms and forms of lncRNAs such as circular RNA [132]. These novel forms will need to be explored in more details, and their possible relevance to cancer pathways will also need to be examined.

Further confounding factors in our understanding of lncRNAs is that lncRNAs can have more than one mechanisms of action to confer transcriptional activation or repression of their target genes. For example, *KCNQ1OT1* can function as both a signal lncRNA [133] and a guide lncRNA [21]. In addition, *HOTAIR* acts via at least three mechanisms namely signal, decoy, and guide [134]. These studies suggest that for many lncRNAs, even lncRNAs that have been characterized, new mechanisms may yet be uncovered.

Moreover, it is unclear whether there exists correlation between particular functioning mechanisms of lncRNAs and their roles in cancer. For example, *XIST* is a lncRNA with a well-established role in dosage compensation in the fruitfly [135] and mammals [136]. *XIST* lncRNA is exclusively produced by the *XIST* gene located on the inactive X-chromosome [137]. Notably, such inactivation is mediated by the ability of *XIST* to recruit chromatin-modification complexes [37]. At the same time, aberrant expression of *XIST* has been linked to a variety of cancers [138]. One mechanism that *XIST* may function through is as a miRNA sponge for miR-152 [11]. This raises interesting questions such as whether this is the only mechanism of action for *XIST* in cancer? Does the role of *XIST* in dosage compensation also have anything to do with its role in cancer?

Another direction is to understand how different lncRNAs cross talk with each other, and how aberrant cross-talk may be

regulated in cancer. Laying the ground for further work are new technologies, for example new sequencing methods that can directly sequence RNA and RNA modifications without the need for reverse transcription. In addition, miniaturization of sequencing devices could enable the development of hand-held RNA-seq devices that rapidly analyze miRNAs and lncRNAs of interest in patient blood samples or biopsies, quickly providing insights into RNA biology that may be dysregulated in individual patients, thereby helping pave the way to an era of precise, personalized evaluation of patient health and disease.

In conclusion, enhanced understanding of lncRNAs in cancer will shed light into disease etiology and will help guide future diagnosis as well as therapeutic options. In future, with the potential therapeutic options for modulating lncRNAs in the form of ASOs as well as other technologies that may arise, lncRNA-based therapies could become an important health-care strategy for consideration.

## Competing interests

MJF is a co-inventor on 2 patents related to chromatin interactions. There are no other conflicts of interest to be declared.

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