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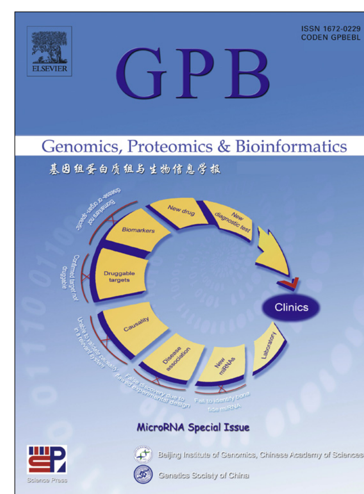

Roles, Functions, and Mechanisms of Long Non-coding RNAs in Cancer

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

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26 ## Abstract

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

34

35 **Keywords:** lncRNAs; Chromatin; Transcription regulation; Antisense oligonucleotides;
36 Cancer

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37

38 ## Introduction

39 Recent advances in sequencing technologies enabling more in-depth genomic and
40 transcriptomic analyses have revealed that as much as 85% of the human genome is
41 transcribed [1–3]. This was surprising, as studies of mammalian genomes have shown that a
42 drastically low population of RNA transcripts code for protein products. Notably, from the
43 recent Encyclopedia of DNA Elements (ENCODE) work, out of 41,204 called genes, only 56
44 genes (0.1%) showed mass spectrometric evidence consistent with protein expression,
45 suggesting that the majority of RNA transcripts are non-coding [4]. The generation of such a
46 large population of non-coding RNA (ncRNA) transcripts indicates that RNAs have a larger
47 and more diverse role in biological processes than initially anticipated. ncRNAs can be
48 roughly classified into two groups based on their size. One group includes short RNAs less
49 than 200 nucleotides (nt) in length, such as microRNAs (miRNAs) thatare small RNA
50 (sRNA) molecules around 21–24 nt in length, as well as other classes such as piwi-interacting
51 RNAs (piRNAs) [5]. The other group includes long ncNAs (lncRNAs) of around 200 nt or
52 more [6]. While miRNAs have been heavily studied and are well understood for their
53 function in gene regulation[7], lncRNAs in contrast are less understood.

54 Many lncRNAs have been functionally associated with human diseases, in particular,
55 cancers [8] (**Table 1**). Dysregulation of lncRNAs has been implicated in glioblastoma [9–11],
56 breast cancer [12], colorectal cancer [9,13], liver cancer [14,15], and leukaemia [9,16].
57 Commonly, dysregulation of lncRNAs exerts impacts on cellular functions such as cell
58 proliferation, resistance to apoptosis, induction of angiogenesis, promotion of metastasis, and
59 evasion of tumor suppressors [8,17].

60 An emerging view of lncRNAs is that they are fundamental regulators of transcription.
61 This view has led to an intense focus on elucidating the molecular mechanisms that underlie
62 their function [18]. Many lncRNAs have been characterized and several models of action
63 have been proposed, such as functioning as signal, decoy, scaffold, guide, enhancer RNAs,
64 and short peptides[19,20], as mentioned below . The main function of a signal lncRNA is to
65 serve as a molecular signal to regulate transcription in response to various stimuli. Thus its
66 production and presence can serve as an indicator of transcriptional activity [21]. Decoy
67 lncRNAs limit the availability of regulatory factors by presenting “decoy” binding sites.
68 These lncRNAs modulate transcription by sequestering regulatory factors including
69 transcription factors, catalytic proteins, subunits of larger chromatin modifying complexes, as

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70 well as miRNAs, thereby reducing their availability [22]. Transcripts from the scaffold class
71 of lncRNAs play a structural role by providing platforms forassembly of multiple-component
72 complexes, such as ribonucleoprotein (RNP) complexes [23]. In the case of RNP complexes,
73 once the complexeshave been fully assembled, either transcriptional activation or repression
74 could be conferred depending on the nature of proteins and RNAs present [24,25]. Guide
75 lncRNAs interact with RNPs and direct them to specific target genes. These guide lncRNAs
76 are essential for the proper localization of RNPs [26]. Next, enhancer RNAs (eRNAs) are
77 produced from enhancer regions and may work by influencing the 3-dimensional (3D)
78 organization of DNA, also known as "chromatin interactions". One hypothesized model of
79 action is that these lncRNAs may possibly work as "tethers" as they may not be released from
80 the enhancer regions when functioning, thus tethering the interacting proteins to enhancer
81 regions [19]. In addition, lncRNAs can encode short peptides, which may also have functions
82 [27]. It is likely that additional mechanisms will be discovered in the future.

83 This review introduces lncRNAs, focusing on lncRNAs with cancer-associated roles, and
84 discusses proposed mechanisms by which these lncRNAs function in chromatin remodeling,
85 chromatin interactions, and as competing endogenous RNAs (ceRNAs). In addition, we
86 highlight approaches for the identification and annotation of lncRNAs, cross-talk between
87 these mechanisms, and methods for perturbing specific lncRNAs, which could eventually
88 provide lncRNA-based therapies for diseases. We then conclude by highlighting challenges
89 and future research topics.

90

91 Characteristics of lncRNAs

92 lncRNAs are defined as RNA molecules with more than 200 nucleotides. This distinction,
93 while somewhat arbitrary and based on technical aspects of RNA isolation methods, serves to
94 distinguish lncRNAs from miRNAs and other sRNAs. lncRNAsare present in large numbers
95 in genome [28,29]. They typically do not possess functional open reading frames (ORFs).
96 However, this distinction is blurred by the discovery of bifunctional RNAs that can have both
97 protein-coding and coding-independent functions [30,31], raising the possibility that many
98 protein-coding genes may also have non-coding functions. Many lncRNAs are lowly
99 expressed [32], posing a challenge in terms of exploration of lncRNAs and explaining why
100 lncRNAs had beenthought to be only "transcriptional noise" until recently. RNA-seq studies
101 in different tetrapods show that most (81%) lncRNAs are poorly conserved in DNA sequence
102 and are primate-specific. However, it should be noted that several lncRNAs are ultra-

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103 conservedin DNA sequence — 3% of lncRNAs appear to have originated more than 300
104 million years ago and can be found from organisms ranging from *Xenopus* and chicken to
105 man [33]. It is possible that lncRNAs might be fast-evolving RNA species that can play key
106 roles in specifying lineages. In support of this idea,a comparison of matched tissues in
107 *Musmusculusdomesticus, Musmusculuscastaneus*, and *Rattusnorvegicus* shows that the
108 emergence or extinction of intergenic lncRNAs is associated with changes in transcription
109 levels of proximal protein-coding genes[34]. In addition, there are examples of lncRNAs
110 exhibiting conserved biological function but low sequence conservation, such as
111 megamind/TUNA, which is associated with brain development in zebrafish, mouse, and
112 human [35,36], as well asX-inactive specific transcript (*Xist*), which is involved in X-
113 inactivation [37]. It is possible that RNA molecules need less sequence conservation to retain
114 their function compared toproteins. Conversely, there is high sequence conservation of
115 lncRNA promoters, which is even higher than that of protein-coding gene promoters [29],
116 suggesting that regulation of lncRNA expression is important.

117 Many lncRNAs possess features reminiscent of protein-coding genes, such as 5' caps and
118 alternative splicing [32]. In fact, many lncRNA genes have two or more exons [32] and about
119 60% of lncRNAs have polyA+ tails [38] (**Figure 1**). Additionally, while there are long
120 intergenic RNAs (lincRNAs) [39] including eRNAs from gene-distal enhancers [40], the
121 majority of lncRNA genes are located within 10 kb of protein-coding genes [41] and many
122 lncRNAs are antisense to coding genes or intronic [42].

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

132

133 Acting mechanisms of lncRNAs

134 In cancer, lncRNAs work through multiple mechanisms such as chromatin remodeling,
135 chromatin interactions, ceRNAs, and natural antisense transcripts (NATs) (**Figure 2**).

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136 lncRNAs can interface with chromatin remodeling machinery in several ways, including
137 acting as signal lncRNAs or scaffold lncRNAs. Recently, another mechanism of action by
138 which lncRNAs function to regulate transcription has been posited. This involves the
139 production of lncRNAs from enhancer regions in the genome (eRNAs), which function to
140 stabilize as well as maintain chromatin loops [40,46–48]. Chromatin looping enables distally-
141 located enhancers (with some located several hundred kilobases away) to interact with their
142 target gene promoters [49–51]. In addition, several studies aimed at elucidating the biological
143 functions of miRNAs have previously used artificial miRNA sponges that compete with
144 native targets for interaction with the miRNAs of interest [52–54]. Similarly, studies have
145 also demonstrated that some lncRNAs modulate transcription by sequestering regulatory
146 factors, including transcription factors and catalytic proteins or subunits of larger chromatin-
147 modification complexes, as well as miRNAs [22]. They are known as ceRNAs [55–57].
148 Transcribed from the opposite DNA strand in relative to the sense transcripts, NATs overlap
149 with their sense transcripts [58] and are thought to regulate the expression of the sense
150 transcripts as well.We will discuss examples of these mechanisms in the next sections.
151 Nonetheless, it should be noted that the mechanisms described here are not exhaustive, and
152 future work is likely to provide new insights in this fast-moving field.

153

154 **lncRNAs in chromatin remodeling**

155 lncRNAs can act through chromatin remodeling to achieve transcriptional regulation. One
156 example is the potassium voltage-gated channel subfamily KT member 1 opposite
157 strand/antisense transcript 1(*KCNQ1OT1*), which is upregulated in colon cancer [59].
158 *KCNQ1OT1* acts as a signal lncRNA by recruiting G9a histone methyltransferases and
159 polycomb repressive complex 2 (PRC2) [21], which mediate the gene-silencing-associated
160 marks, *i.e.*, dimethylation of lysine 9 (H3K9me2) and lysine 27 on histone 3 (H3K27me3)
161 [60]. Through chromatin remodeling, *KCNQ1OT1* induces transcriptional silencing of genes,
162 which may occur in*cis* (for targets close by) or in *trans* (for distal targets).

163 As another example, *ANRIL*/*CDKN2B*, the antisense ncRNA in the *INK4* locus,works as a
164 scaffold in mediating transcriptional silencing of the *INK4b-ARF-INK4a* locus by recruiting
165 and interacting with PRC1 and PRC2 [24,25]. PRC2 recruited to this gene locus then
166 mediates the spread of the methylation marks that are distinct for transcriptionally-silent
167 genes. Several lncRNAs have been demonstrated to interact with chromosome-modification

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168 complexes and direct them to specific target genes. These guide lncRNAs are essential for the
169 proper localization of the chromosome-modification complexes including PRC2 and
170 subsequent repression of gene expression as demonstrated by the lncRNA fetal-lethal non-
171 coding developmental regulatory RNA (*FENDRR*), which serves to bring PRC2 in close
172 proximity to the promoters of genes associated with the formation and differentiation of the
173 lateral mesoderm lineage, such as forkheadbox F1 (*FOXF1*) and paired-like homeodomain2
174 (*PITX2*) genes [26]. We hypothesize that other guide lncRNAs may also function to regulate
175 transcription by targeting other chromatin-modification complexes, in addition to PRC2, to
176 their target genes.

177 Finally, the *HOX* transcript antisense RNA (*HOTAIR*)lncRNA functions cooperatively
178 with PRC2 in mediating the repression of the homeobox D cluster (*HOXD)* locus through
179 spreading H3K27me3 marks, which are associated with gene silencing [61]. *HOTAIR* forms
180 multiple double stem-loop structures that bind to lysine-specific demethylase 1 (LSD1) and
181 PRC2 histone-modification complexes [12]. Other lncRNAs also operate in a similar manner,
182 with as much as 20% of lncRNAs known to associate with PRC2 [62]. For example, the
183 lncRNA *TUG1* is associated with PRC2, and depletion of *TUG1* expression in the developing
184 mouse eye leads to the blockage of retinal development [62]. While PRC2 has been found to
185 interact with many lncRNAs, other chromatin remodelers have been implicated and it is
186 likely that more interactions with other chromatin remodelers remain to be discovered.
187 Moreover, given that cancers are associated with aberrant levels of PRC2, H3K27me3, and
188 mutated enhancer of zeste homolog 2 (EZH2), which is a component of the PRC2 complex
189 [63], we hypothesize that lncRNA-based mechanisms provide an explanation as to how these
190 alterations in PRC2 levels can give rise to cancer (**Table 1**). Taken together, it is becoming
191 increasingly clear that certain lncRNAs can associate with chromatin-modification complexes
192 to carry out cellular functions.
193

194 **lncRNAs in chromatin looping in cancers**

195 Another mechanism of action by which lncRNAs function to regulate transcription is via
196 enhancer lncRNAs (eRNAs) associated with chromatin loops. To study chromatin looping,
197 techniques including chromosome conformation capture (3C) [64] and fluorescence *in*
198 *situ*hybridization (FISH) [65], have been employed. More recent technical developments
199 have focused on higher-throughput analyses and these include circular chromosome
200 conformation capture (4C) [66,67], chromosome conformation capture carbon copy (5C)

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201 [68], combined 3C-ChIP-cloning (6C) [69], chromatin interaction analysis with paired-end
202 tag sequencing (ChIA-PET) [70], and Hi-C [71]. Using ChIA-PET, a correlation is revealed
203 between expression level of elncRNAs and estrogen receptor α (ERα)-associated chromatin
204 interactions [72]. The experimental downregulation of these eRNAs leads to a loss of
205 chromatin loops and a corresponding change inexpression of the genes targeted by ERα [46].
206 In addition, a relationship is found between the levels of eRNAs produced by upstream
207 enhancers of the prostate-specific antigen (*PSA*) gene and the actual levels of *PSA* gene
208 expression, suggesting a possible link between eRNAs and chromatin interactions [73].
209 Meloet al. show the existence of enhancer regions that bind the transcription factor p53.
210 These enhancer regions produce RNAs and display chromatin interactions with multiple
211 neighboring genes. Ablation of these eRNAs leads to reduced transcription at neighboring
212 genes and reduced p53-dependent cell cycle arrest [74]. More recently, a role for Integrator in
213 the biogenesis of eRNAs is demonstrated [75]. Integrator is a complex associated with RNA
214 polymerase II (RNAPII), and possesses RNA endonuclease activity, which is required for 3'
215 end processing of non-polyadenylated nuclear RNA genes [76]. These studies show that
216 depletion of Integratorleads to a decrease in the induction of eRNAs, which is accompanied
217 by the loss of enhancer-promoter chromatin looping [75].
218 One of the best examples of lncRNAs that regulate chromatin interactions is the *HOXA*
219 transcript at the distal tip (*HOTTIP). HOTTIP* is a lncRNA shown to regulate chromatin
220 interactions in the *HOX* cluster, from which it is produced. *HOTTIP* is necessary to
221 coordinate activation of *HOX* genes, through binding WD repeat-containing protein 5
222 (WDR5), an adaptor protein [77]. WDR5 interacts with the mixed lineage leukemia (MLL)
223 complexes for substrate recognition and genomic targeting. The MLL complexes catalyze
224 H3K4 methylation, which is a mark of actively transcribed genes [77]. *HOTTIP* is necessary
225 for the maintenance of a specific pattern of WDR5/MLL complexes across the *HOXA* locus
226 to facilitate gene transcription [77].
227 Additionally, there is cross-talk between the different mechanisms: a novel
228 RNAchromosome conformation capture (R3C) strategy demonstrated that *KCNQ1OT1*,
229 which regulates genes in the *KCNQ1* imprinting region, is involved in imprinting-associated
230 chromatin interactions [78]. EZH2, which catalyzes the deposition of gene silencing-
231 associated H3K27me3 marks, is recruited by the *KCNQ1OT1* [78]. Depletion of *KCNQ1OT1*
232 leads to depletion of the loop and loss of imprinting [78]. These examples all demonstrate a
233 role for eRNA-associated chromatin interactions in transcriptional suppression, enhancement,
234 and coordination of gene expression, as well as other functions such as imprinting. A

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235 hypothesized model of action is that eRNAs may help to tether different genomic regions
236 together, recruiting factors such as PRC2 and other proteins at the 5' end, while remaining
237 tethered to the location of production [79] **(Figure 3)**. Other factors might help to bring
238 together other DNA regions, or the length of the eRNA might enable it to tether and guide
239 other DNA regions together, thus forming a chromatin interaction. Other factorsmay bind to
240 the chromatin directly or indirectly through the bound proteins, stabilizing and maintaining
241 the chromatin even after the RNA may have been degraded. For example, cohesin has been
242 shown to play sucha role in the case of ERα-associated RNAs [46]. Adding support to this
243 idea is the finding that transcriptional repressor CCCTC-binding factor (CTCF), which is
244 involved in chromatin interactions, can bind to ncRNAs. For example, the steroid receptor
245 RNA activator (*SRA*) binds to CTCF and enhances its functioning [80].
246

247 **lncRNAs as ceRNAs in cancers**

248 The lncRNA *HULC* is highly upregulated in hepatocellular carcinoma (HCC) [81]. There are
249 multiple miR-372-binding sites present in *HULC*, and the overexpression of *HULC*can
250 reduce miR-372 expression. This leads to reduced translational repression of its target
251 transcript *PRKACB*, thereby inducing the phosphorylation of the cAMP-responsive
252 element(CRE)-binding protein (CREB) [81]. On the other hand, papillary thyroid carcinoma
253 susceptibility candidate 3 (*PTCSC3*) is highly downregulated in thyroid cancers and its
254 overexpression leads to reduced expression of oncogenic miR-574-5p, resulting in growth
255 inhibition, cell-cycle arrest, and increased apoptosis [82]. Studies identifying additional
256 molecular targets of decoy lncRNAs will add to our understanding of diseases while
257 presenting possible therapeutic interventions for which drugs may be designed.
258

259 **lncRNAs as NATs**

260 NATs are surprisingly common in the mammalian genome— over 20% of human transcripts
261 form sense-antisense pairs [83]. A later estimate in mice based on large RNA-seq datasets
262 shows that up to 72% of genes may have transcription-related activity on the opposite strand
263 [58]. NATs can be produced againstboth protein-coding and non-coding genes [58] and some
264 NATs are protein-coding geneswhile others are lncRNAs [58]. In addition, the genomic
265 organization of NATsin relative to their sense transcripts varieswith different configurations.
266 One common configuration, called "divergent",is that the promoter generates bidirectional
267 transcription [58,84]. Another common configuration, called "convergent", arises whereby

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268 the NAT starts from a different promoter, and transcribes a region on the opposite strand
269 from the protein-coding transcript [58]. For example, theNATcould start from the 3' end of
270 the sense transcript and transcribe towardits 5' end [58].

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

280 One example of a NAT in cancer is WD40-encoding RNA antisense to p53 (*Wrap53*)
281 [86].As a NAT of the important oncogene *TP53*, *Wrap53* can induce *TP53*expression by
282 targeting the 5' untranslated region of the *TP53* mRNA [86]. Blocking this interaction
283 between the *Wrap53* lncRNA and *TP53* mRNA reduces basal levels of *TP53* and prevents
284 induction of *TP53* after DNA damage [86]. *Wrap53*is overexpressed in cancer cell lines;
285 interestingly, overexpression of *Wrap53*results in cellular transformation while ablation leads
286 to apoptosis [87]. Additionally, *Wrap53* overexpression is correlated with poor prognosis in
287 head and neck squamous cell carcinoma [87]. Taken together, *Wrap53* is an oncogenic
288 lncRNA that regulates*TP53* expression.

289

290 **lncRNA networks and cross-talk in cancer**

291 An interesting aspect of lncRNAs is that there can be cross-talk between different
292 mechanisms, leading to the formation of complex networks, particularly in cancer. For
293 example, the transcription factor p53 regulates many genes [88], including lncRNAs such as
294 *lncRNA-p21*, *PANDA*, *H19*, and*loc285184*, which serve as effectors of p53 by leading to p53-
295 associated cellular functions such as cell cycle arrest and apoptosis [89]. p53is self-regulated
296 not only by well-known regulators such as MDM2, an E3 ubiquitin ligase, but also by
297 lncRNAs *MALAT1*, and*MEG3*, as well as the*TP53*NAT*Wrap53* as discussed in the previous
298 section [89]. *MALAT1* is upregulated in several cancers [90] and may be used to predict
299 survival and metastasis in non-small cell lung cancer [91]. And *MALAT1* is expressed in a
300 cell cycle-dependent manner and is required for G1/S and mitotic progression [92].

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301 Paradoxically, depletion of *MALAT1* leads to *TP53* activation [92]. However, the cells show
302 reduced oncogenic transcription factor B-MYB, leading to increased cellular proliferation via
303 B-MYB [92]. For more details on these lncRNAs, readers are referred to the excellent review
304 by Zhang et al. [89]. Interestingly, c-Myc, another important cancer-associated transcription
305 factor, has a similar network [93]. It remains anopen question whether other cancer-
306 associated transcription factors possess a similar network consisting of lncRNAs that lead to
307 altered cellular functions and ultimately cancer. Given that transcription factors are generally
308 considered difficult to target by small molecule inhibitors due to the intrinsically disordered
309 nature of their binding sites (meaning they lack stable secondary and/or tertiary structure
310 under physiological conditions *in vitro*) and their binding promiscuity [94], lncRNAs that
311 regulate the transcription factors may open up new avenues for targeting cancers with
312 aberrant transcription factor signaling.
313

314 Approaches for the identification and annotation of lncRNAs

315 In general, the expression levels of lncRNAs are low in abundance and lncRNAs are often
316 not polyA-tailed, posing several challenges for their identification and analysis because
317 previous methods of microarray and mRNA-seq rely on polyA+ isolation and tend to favor
318 the detection of transcripts that are abundant in expression. In spite of these challenges, a
319 number of effective methods have been developed for lncRNA identification and annotation
320 (**Table 2**). Today, RNA-seq is one of the most common methods for identifying novel
321 lncRNAs, because of the dropping sequencing costs and the single-nucleotide resolution
322 nature of RNA-seq [95]. RNA-seq involves converting RNA into cDNA, followed by
323 fragmentation, and ultra-high-throughput sequencing by methods such as Illumina HiSeq. As
324 lncRNAs have low expression levels, high sequencing depths of 100–150 million reads or
325 more are needed to discover these rare lncRNAs [96]. In addition, strand-specific sequencing
326 methods are also needed to distinguish antisense lncRNAs from sense transcripts and cDNA
327 conversion must be performed using random hexamers as opposed to oligo-dT methods [96].
328 RNA-seq experiments to detect lncRNAs have to be performed through rRNA-depletion to
329 enrich for mRNAs and lncRNAs or by sequencing both polyA+ and polyA- fractions [96].
330 Owing to the high sequencing depth, RNA-seq becomes expensive when investigating some
331 of the rarest lncRNAs. Hence researchers have also turned to custom lncRNA microarrays to
332 screen through many lncRNAs in many different samples [97], as well as custom tiling
333 microarrays to identify lncRNAs in specific regions of the genome [61]. Other than that,

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334 microarray capture followed by sequencing is also used for quantitative gene profiling and
335 annotation of specific lncRNAs [98].

336 Several new sequencing methods are becoming available, such as Pacific Biosciences
337 (PacBio) sequencing, which features extremely long (on the order of kilobases) reads [99,
338 100]. PacBio sequencing allows for read-through of complete gene sequences, facilitating the
339 understanding of alternative splicing. While this method is currently only cost-effective for
340 small genomes such as bacteria, in the near-future, it will probably be applicable to analysis
341 of human samples.

342 There is a battery of experimental methods for annotating lncRNAs. RNA
343 immunoprecipitation (RIP)-Seq involves sequencing RNAs that are associated with a
344 particular RNA-binding protein of interest with immunoprecipitation [101]. A related
345 method, chromatin isolation by RNA purification (ChIRP)-Seq involves the design of
346 multiple biotin-tagged oligonucleotide probes that recognize a particular RNA [46,102].
347 Cross-linked chromatin complexes with RNA of interest are then isolated using streptavidin
348 magnetic beads recognizing the biotin tag [46,102]. DNA, RNA, and protein can be isolated
349 from these complexes and subjected to sequencing or mass spectrometry for identification
350 [46,102]. Another method, RNA-FISH, involves designing fluorescent probes to RNA and
351 performing the hybridization in cells followed by microscopic imaging. It is used to visualize
352 the location of the lncRNAs in different cellular organelles and investigate how lncRNA
353 localization is altered in response to different stimuli [103]. In addition, RNA-3C involves
354 double-stranded cDNA synthesis using biotinylated oligonucleotides to obtain chromatin
355 complexes with biotinylated cDNA, followed by digestion and proximity ligation, generating
356 DNA-cDNA constructs. These constructs can then be pulled down using the biotin group and
357 analyzed by PCR. RNA-3C has been used to investigate associations between lncRNAs and
358 3-D genome organization [78].

359 In addition, there are a variety of sequencing methods for analyzing the secondary
360 structure of RNA. These include selective 2'-hydroxyl acylation analyzed by primer
361 extension sequencing (SHAPE-Seq), whereby RNA is isolated, barcoded, and folded *in vitro*
362 [104]. A SHAPE reagent, 1M7, is added to the isolated, barcoded, and folded RNA, which
363 blocks the reverse transcriptase reaction whereby the 1M7 is included in the RNA, and hence
364 leads to a series of truncated products to be sequenced, allowing for reconstruction of the
365 original structure [104]. A similar method, parallel analysis of RNA structure (PARS)
366 involves digestion of RNAs using RNAses specific for double-stranded and single-stranded
367 RNAs. The fragments are then reversely transcribed and compared with each other, allowing

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368 for deduction of RNA structures *in vivo* [105,106]. A third method, fragmentation sequencing
369 (FRAG-Seq), is similar to PARS except that it uses P1 nuclease instead of RNAses [107].
370 Perturbing lncRNA expression levels can be achieved in different ways,
371 includingclustered regularly interspaced short palindromic repeat (CRISPR) genomic editing
372 of lncRNA genes [108,109]. In addition, new antisense oligonucleotide (ASO) approaches
373 can be applied to perturb both cytoplasmic and nuclear lncRNAs, which will be discussed in
374 more details in the next section. CRISPR genomic editing comes from the immune defenses
375 of bacteria and archaea, which use short RNA to degrade invading nucleic acids [110, 111].
376 Being one component of the CRISPR immune defense system, Cas9 effector nuclease is the
377 first known nuclease that is capable of binding to specific short RNA and thereby directing
378 cleavage at complementary genomic loci [110]. CRISPR/Cas9 represents a giant leap forward
379 in terms of ease of use and efficiency in comparison with traditional methods for excising
380 DNA such as homologous recombination and zinc finger nucleases (ZFNs) [110]. This
381 method has since been widely adopted by many labs around the world, in a wide range of
382 different cell types and organisms [110]. CRISPR editing can also be used to excise lncRNAs
383 for functionalstudies. In addition, CRISPR can be modified forother purposes such as
384 upregulating gene expression by combining a Cas9 unable to cleave nucleic acids with
385 transcription activators such as VP64 activator domains [110,112,113].This system could be
386 used to upregulate lncRNA expression levels to understand their functions.
387 There are several databasesavailable for lncRNA research (**Table 3**). ENCODE [28],
388 FANTOM [114], and TCGA [115] have all embarked on massive RNA-seq efforts in
389 different tissues including patient samples, which have yielded an unprecedented collection
390 of lncRNAs. In addition, several groups have developed curated lists of lncRNAs by mining
391 the existing literature and by predictions. For example, lncRNome [116] can serve as a
392 general resource, while LncRNA Disease [117] presents lncRNA−disease associations. Other
393 groups have also developed bioinformatics tools to infer functions of lncRNAs. These
394 include looking for co-expressed genes and lncRNAs as featured in the lncRNAtor database
395 [118], looking at functional similarity patterns (motifs) and predicting RNA structures as
396 featured in LNCipedia and lncRNome [116,119], and integrating epigenomic information as
397 featured in lncRNome [116]. For a detailed discussion and comparison of lncRNA databases,
398 we refer readers to the article by Fritah et al. [120].
399

400 ## ASOs for modulation of lncRNA

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401 The importance of lncRNAs in health and diseases (Table 1) indicates a need to find methods
402 for modulating lncRNAs. To do this, ASOs are used (**Figure 4**), which are short DNA
403 sequences and complementary to an RNA of interest. The oligonucleotide works by
404 hybridizing to the RNA, which then blocks the action of the RNA. While unmodified
405 oligonucleotides are available, certain chemical modifications such as 2′-*O*-(2-methoxy) ethyl
406 oligonucleotides increase the lifespan of the oligonucleotide in the complex milieu of
407 nucleases within the cell, and reduce degradation products, which may also have effects on
408 the cells [121]. Two ASOs have been approved as drugs by the Food and Drug
409 Administration (FDA) of the United States: fomivirsen, which is used to treat
410 cytomegalovirus retinitis [122], and mipomersen, which is used to treat homozygous familial
411 hypercholesterolemia [123]. ASOs can enter the nucleus and knock down nuclear lncRNAs
412 [124], enabling all classes of lncRNAs to be explored. In contrast, siRNA and shRNA are not
413 effective in targeting nuclear lncRNAs because the RNAi machinery is located in the
414 cytoplasm.

415 A major interest for antisense therapy is to upregulate gene expression. Several lncRNAs
416 work as NATs to genes of therapeutic interest. Classic small molecule drugs work by
417 inhibiting gene expression; therefore genes that need to be upregulated have been almost
418 undruggable, although enzyme replacement therapy has been used as a strategy, such as
419 lysosomal enzymes in the case of lysosomal storage diseases [125].Antisense targeting of
420 natural antisense lncRNAs, or “antagoNATs”, provides a very specific way for gene
421 upregulation [126,127]. Taken together, ASOs constitute a very promising toolthat can be
422 used to translate basic discoveries about lncRNAs to the clinic. For instance, Opko-Curna is a
423 company taking forward this technology into the clinic [126].

424 Similarly, company RaNa Therapeutics aims to upregulate gene expression, but using a
425 different approach [128]. Using RIP-Seq, thousands of lncRNAs were found to interact with
426 PRC2, which represses transcription of the genes targeted by lncRNAs [101]. Notably,
427 specific PRC2-lncRNA interactions can be disrupted using locked nucleic acids, resulting in
428 upregulation of the target genes [129]. Locked nucleic acids are RNA derivatives similar in
429 principle to ASOs [130].Therefore, the company is keen to de-repress the expression of genes
430 of interest by using locked nucleic acids against specific lncRNAs that interact with PRC2.

431 Given that lncRNAs have been found to act in other pathways, such as eRNAs, theyhave
432 additional potential in terms of therapeutic mechanisms that can be targeted by ASOs. For
433 example, transcription factors, chromatin modulators, and chromatin interactions are difficult
434 pathways to target, as these factors are in the nucleus, which is difficult for small molecule

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435 inhibitors to enter. ASOs against lncRNAs, which interact with factors that can enter the
436 nucleus, might offer a feasiblemechanism for future targeting of these important pathways.
437

438 Future directions

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

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

456 Moreover, it is unclear whether there exists correlation between particular functioning
457 mechanisms of lncRNAs and their roles in cancer. For example, *XIST* is alncRNA with a
458 well-established role in dosage compensation in the fruitfly [135] and mammals [136]. *XIST*
459 lncRNA is exclusively produced by the *XIST* gene located on the inactive X-chromosome
460 [137]. Notably,such inactivation is mediated by the ability of *XIST* to recruit chromatin-
461 modification complexes [37]. At the same time, aberrant expression of *XIST* has been linked
462 to a variety of cancers [138]. One mechanism that*XIST* may function through is as a miRNA
463 sponge for miR-152 [11]. This raises interesting questions such as whether this is the only
464 mechanism of action for*XIST* in cancer? Does the role of *XIST* in dosage compensation also
465 have anything to do with its role in cancer?

466 Another direction is to understand how different lncRNAs cross talk with each other, and
467 how aberrantcross-talk may be regulated in cancer. Laying the ground for further work are

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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 aetiology 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 healthcare 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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Figure legends

854 **Figure 1 Characteristics of lncRNAs**

855

856 **Figure 2 Actingmechanisms of lncRNAs**

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

859

860 **Figure 3 Hypothesizedacting mechanism for eRNAsto initiate chromatin interactions**

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

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863 **Figure 4 Targeting lncRNAs for therapeutic applications**
864 NAT, natural antisense transcript; PRC2, polycomb repressive complex 2; ASO, antisense
865 oligonucleotide; eRNA, enhancer RNA.
866
867 **Tables**
868 **Table 1 Examples of lncRNAs in cancer**
869 **Table 2 Examples of key technologies and tools for identifying and annotating lncRNAs**
870 **Table 3 Key databases of lncRNAs**
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Figure 1

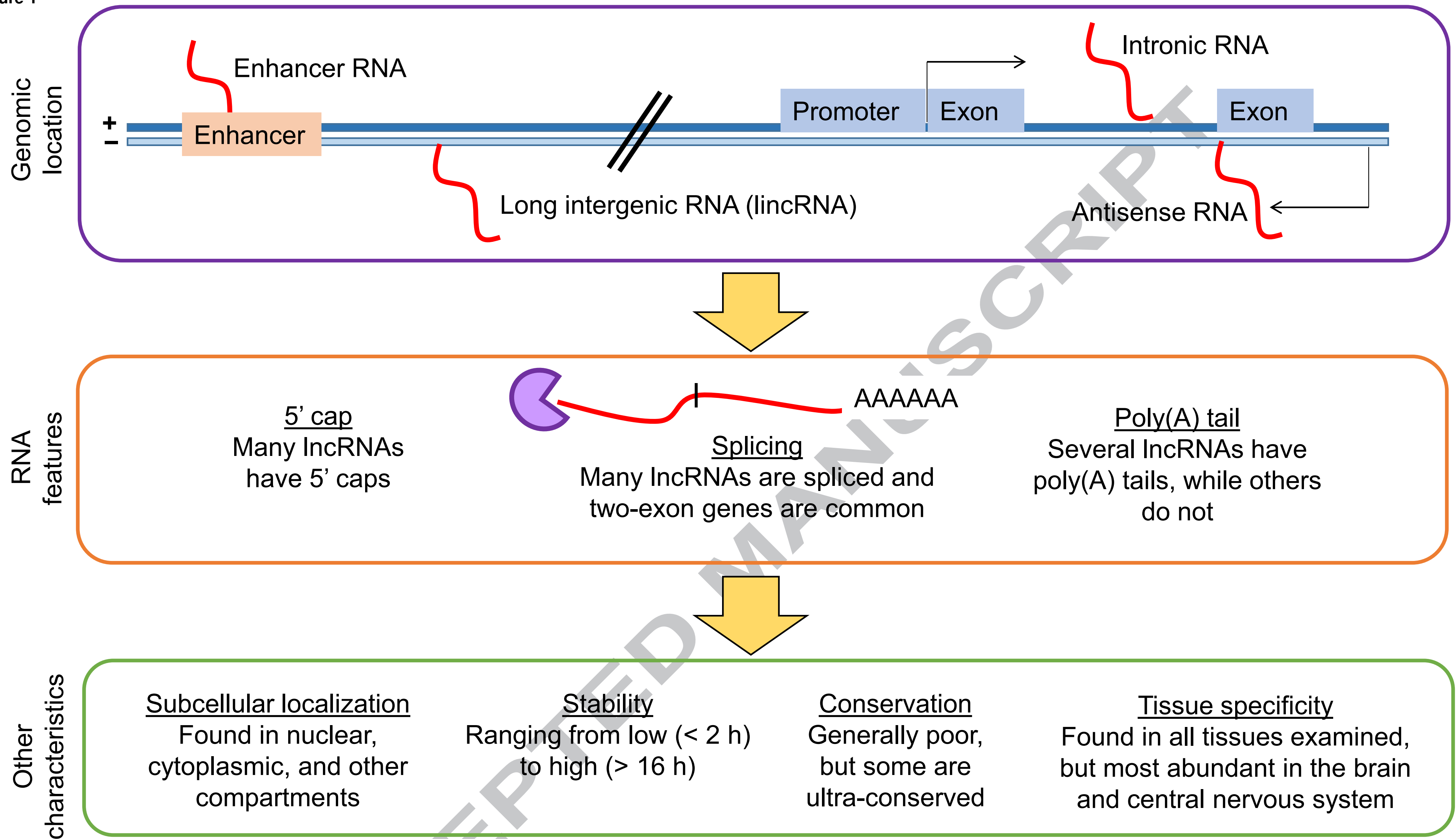

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Figure 2

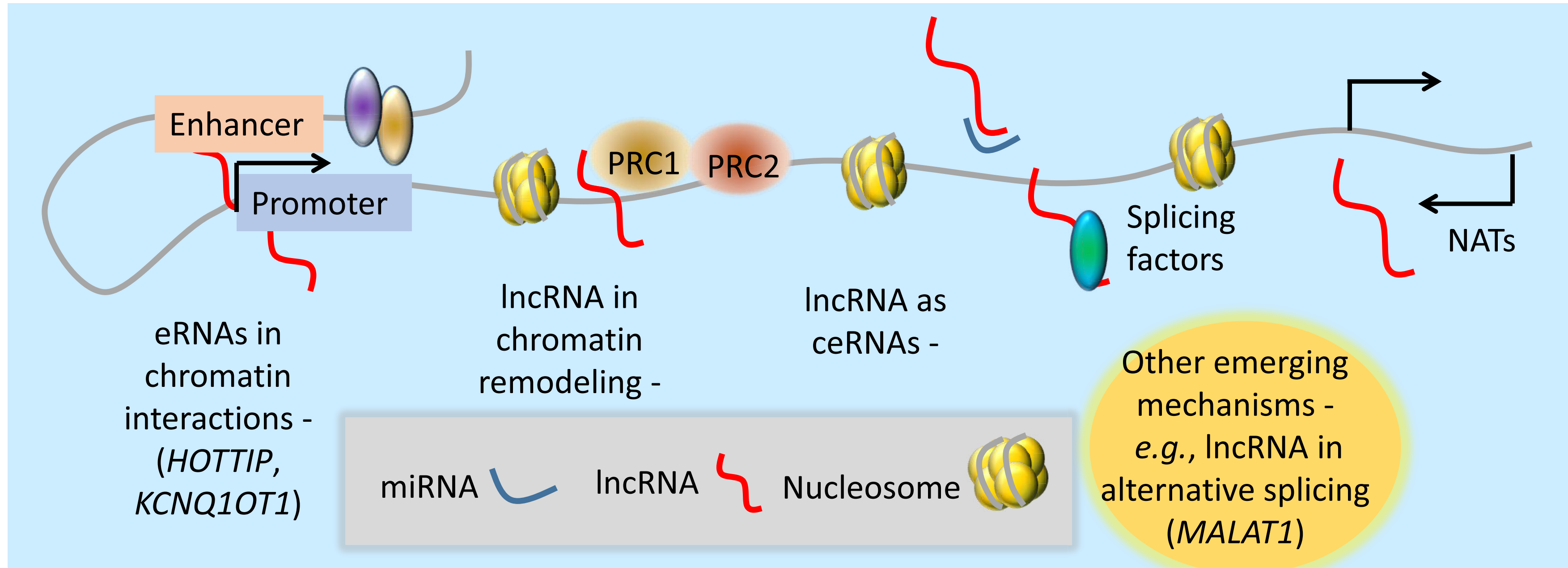

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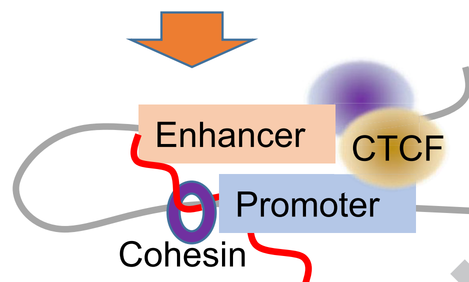
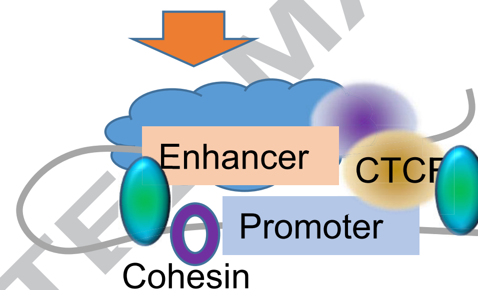
Figure 3

A transcription factor binds to an enhancer, leading to production of eRNAs



The eRNA may tether and/or guide other factors and DNA regions to form a chromatin interaction



Other factors may be recruited to stabilize the chromatin interaction even in the absence of the RNA

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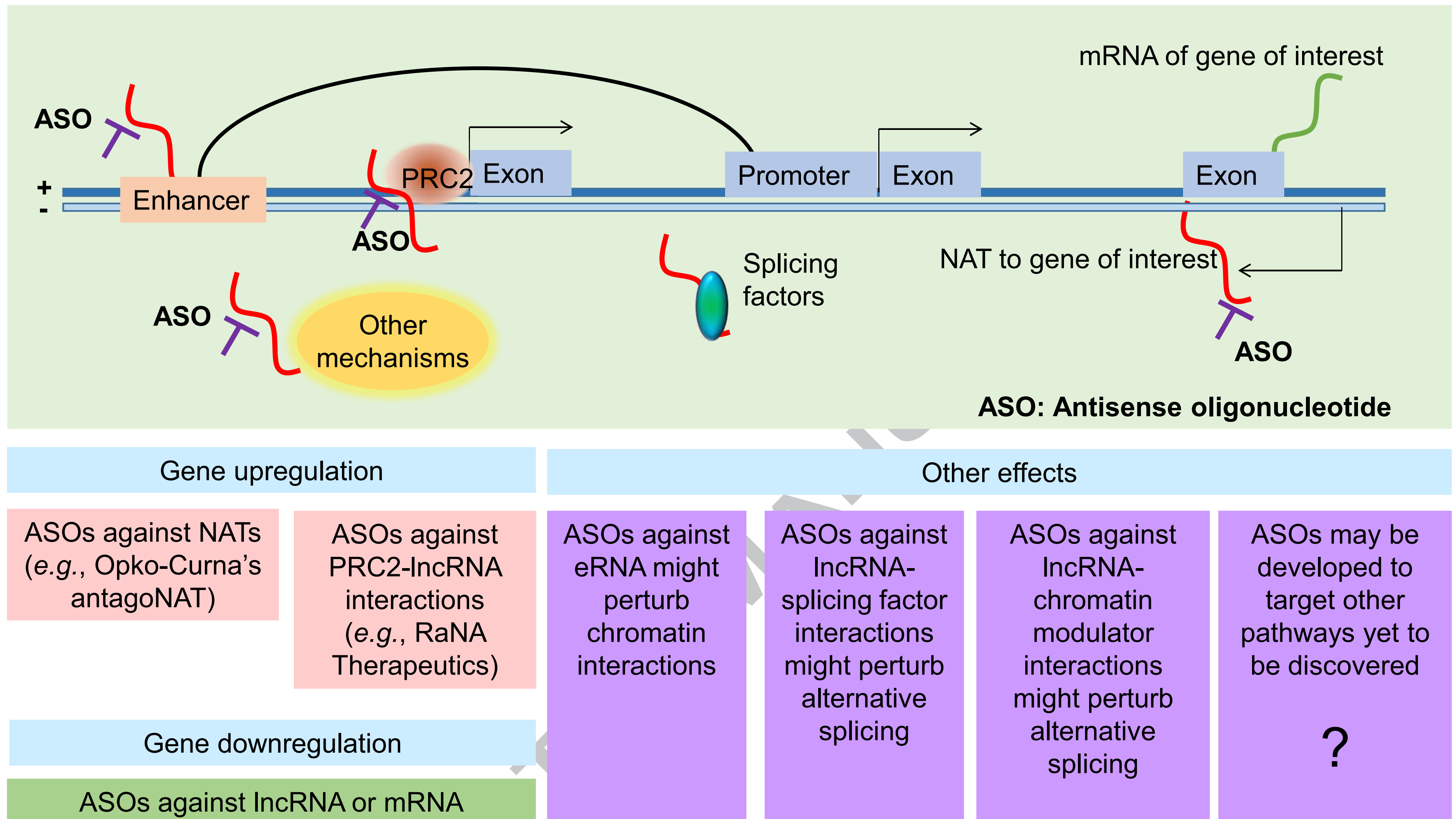
Figure 4



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874 **Table 1 Examples of lncRNAs in cancer**

lncRNA	Description	Refs.
PTCSC3	Downregulated in thyroid cancers	[82]
HULC	Upregulated in hepatocellular carcinoma	[81,139–141]
XIST	Dysregulated in various cancers	[16,142]
GAPLINC	Associated with poor prognosis in gastric cancer	[143]
MALAT1	Associated with poor prognosis and metastasis in liver, lung and colorectal cancers	[144–147]
HOTAIR	Associated with metastasis in colorectal, liver, pancreatic, breast and gastric cancers	[12–14,148–150]
ANRIL	Upregulated in prostate cancer	[151]
KCNQ1OT1	Upregulated in colorectal cancer	[59]
PRNCR1	Upregulated in prostate cancer	[152]
H19	Highly expressed in hepatocellular carcinoma	[153]

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880 **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]

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

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Table 3 Key databases of lncRNAs

Database	Description	Web link	Ref.
Databases with RNA-seq and other primary datasets			
ENCODE	Produced many RNA-seq and complementary datasets for a certain set of human cell lines	https://genome.ucsc.edu/ENCODE/	[28]
FANTOM	Produced many RNA-seq and complementary datasets from mice	http://fantom.gsc.riken.jp/	[114]
TCGA	Contains lncRNAs from primary patient tumors	http://cancergenome.nih.gov/	[115]
Curated lists of lncRNAs			
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	http://genome.igib.res.in/lncRNome/	[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	http://www.lncipedia.org/	[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	http://www.cuilab.cn/lncrnadisease	[117]
Annotators			
LncRNAtor	Co-expression between mRNAs and lncRNAs in various tissues	http://lncrnator.ewha.ac.kr/index.htm	[118]

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