Identification and characterization of functional long noncoding RNAs in cancer

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Abstract
Long noncoding RNAs (lncRNAs) have emerged as key regulators in a variety of cellular processes that influence disease states. In particular, many lncRNAs are genetically or epigenetically deregulated in cancer. However, whether lncRNA alterations are passengers acquired during cancer progression or can act as tumorigenic drivers is a topic of ongoing investigation. In this review, we examine the current methodologies underlying the identification of cancer-associated lncRNAs and highlight important considerations for evaluating their biological significance as cancer drivers.

Abbreviations: AFAP1, actin filament-associated protein 1; AFAP1-AS1, AFAP1 antisense RNA 1; ANRIL, antisense noncoding RNA in the INK4 locus; APC, adenomatous polyposis coli protein; AR, androgen receptor; ARLNC1, AR-regulated long noncoding RNA 1; ASO, antisense oligonucleotide; BANCR, BRAF-activated non-protein coding RNA; BC, breast cancer; BCAR4, breast cancer anti-estrogen resistance 4; CARLo-5, cancer-associated region long noncoding RNA 5; CASC14, cancer susceptibility candidate-14; CCAT1, colon cancer-associated transcript 1; CCAT2, colon cancer-associated transcript 2; CDKN2A, cyclin-dependent kinase inhibitor 2A; CDKN2B, cyclin-dependent kinase inhibitor 2B; CNV, copy number variation; CRISPR, clustered regular interspaced short palindromic repeats; CRISPRa, CRISPR activation; CRISPRi, CRISPR inhibition; CRNDE, colorectal neoplasia differentially expressed; CTLs, cytotoxic T cells; DANCHR, differentiation antagonizing non-protein coding RNA; DINO, DNA, damage-induced noncoding; DMBA, 7,12-Dimethylbenz[a]anthracene; EPIC1, epigenetically induced lncRNA 1; ER, estrogen receptor; ERAR, ER agitation-related; FAL1, focally amplified lncRNA 1; FALEC, focally amplified long noncoding RNA in epithelial cancer; gadd7, growth-arrested DNA damage-inducible gene 7; GAPLINC, gastric adenocarcinoma predictive long intergenic noncoding RNA; GAS5, growth arrest-specific 5; GATA6, GATA-binding protein 6; GEMM, genetically engineered mouse model; GOF, gain-of-function; gRNA, guide RNA; GWAS, genome-wide associated study; HOTAIR, HOX transcript antisense RNA; HOTTIP, HOXA distal transcript antisense RNA; HPV, human papillomavirus; HULC, highly upregulated in liver cancer; Isb, inhibitor of kappa B; LAST, lncRNA-assisted stabilization of transcripts; LED, lncRNA activator of enhancer domains; LINC-PINT, long intergenic non-protein coding RNA, p53-induced transcript; LNA, locked nucleic acid; IncGATA6, IncRNA GATA6; IncPRESS1, IncRNA p53-regulated and ESC-associated; lncRNA, long noncoding RNA; LOF, loss-of-function; LOH, loss of heterozygosity; LSAMP, limbic system-associated membrane protein; LUAD, lung adenocarcinoma; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MAPK, mitogen-activated protein kinase; MaTARs, mammary tumor-associated RNAs; MCL1, myeloid cell leukemia sequence 1; MEFs, mouse embryonic fibroblasts; MEG3, maternally expressed 3; MITF, microphthalmia-associated transcription factor; MMTV-PyMT, mouse mammary tumor virus-polyomavirus middle T antigen; MYCLos, MYC-regulated lncRNAs; NBAT-1, neuroblastoma-associated transcript-1; nRNA, noncoding RNA; NEAT1, nuclear enriched abundant transcript 1; NF-kB, nuclear factor-kappa B; NKILA, NF-kB interacting long noncoding RNA; OIS1, oncogene-induced senescence 1; ORF, open reading frame; ORL1, oncogenic RAS-induced lncRNA 1; p53BERS, p53-bound enhancer regions; p53RE, p53 response element; PANDAR, promoter of CDKN1A antisense DNA damage activated RNA; PanINs, pancreatic intraepithelial neoplasias; PAS, polyadenylation signal; PCa, prostate cancer; PCAT1, prostate cancer-associated transcript 1; PCAT19, prostate cancer-associated transcript 19; PCATs, prostate cancer-associated ncRNAs transcripts; PCGEM1, prostate cancer gene expression marker 1; PDAC, pancreatic ductal adenocarcinoma; PDFX, patient-derived xenograft; PINCR, p53-induced noncoding RNA; PRAL, p53 regulation-associated IncRNA; PR- IncRNAs, p53-regulated IncRNAs; PRNCR1, prostate cancer-associated noncoding RNA 1; PTENP1, phosphatase and tensin homolog pseudogene 1; PURPL, p53 upregulated regulator of p53 levels; PVT1, plasmaacytoma variant translocation 1; RNAi, RNA interference; SALNR, senescence-associated IncRNA; SAMMSON, survival-associated mitochondrial melanoma-specific oncogenic noncoding RNA; SCC, squamous cell carcinoma; SCNV, somatic copy number variation; SNHG15, small nucleolar RNA host gene 15; SNP, single nucleotide polymorphism; SPRY4, sprouty RTK signaling antagonist 4; SPRY4-IT1, SPRY4 intronic transcript 1; TCGA, The Cancer Genome Atlas; THOR, testis-associated highly conserved oncogenic long noncoding RNA; TPA, 12-O-tetradecanoylphorbol-13-acetate; XIST, X-inactive specific transcript.
1 | INTRODUCTION

Cancer is a disease of aberrant cell growth arising from a complex genetic landscape of inherited and sporadic mutations and environmental factors. Historically, cancer research has prioritized examining alterations to protein-coding genes in molecular pathways influencing the hallmarks of cancer.\(^1,2\) While these analyses have provided extensive insights into key players in tumorigenesis, protein-coding sequences account for only 2% of the genome.\(^3\) Both the pervasive transcription of the human genome\(^4\) and the presence of cancer-associated mutations in noncoding regions\(^5\) have suggested a potential wealth of unexplored cancer targets. Notably, the heterogeneous class of long noncoding RNAs (lncRNAs) occupies a significant space within the noncoding transcriptome, with recent estimates suggesting the existence of over 100,000 human lncRNA transcripts.\(^6-9\)

LncRNAs are operationally defined as RNA molecules exceeding 200 nucleotides in length that lack protein-coding potential.\(^10,11\) Able to dynamically fold into intricate secondary structures,\(^12\) to interact with DNA, proteins, and other RNAs, lncRNAs are diverse in their structure, localization, and pattern of expression, enabling them to regulate the flow of cellular information at many levels.\(^13\) Frequently the targets of transcriptional programs, lncRNAs influence many fundamental cellular processes including cell division, genome maintenance, and pluripotency.\(^14-16\)

As lncRNAs are expressed with exquisite cell-type and disease-state specificity, they are ideally positioned to act as biomarkers for a number of pathologies, including different cancers.\(^17-19\) Identifying lncRNA expression changes, or their association with recurrent copy number variations (CNVs) or cancer susceptibility single nucleotide polymorphisms (SNPs) have the potential to become useful tools in cancer diagnosis and treatment planning. Beyond their diagnostic and prognostic utility, over the past decade, individual lncRNAs have been mechanistically and functionally dissected, revealing critical roles in cancer-related pathways at the cellular and organismal levels. These studies have pointed to lncRNAs as operators within proto-oncogenic and tumor suppressive networks, suggesting that lncRNAs themselves may play active roles in promoting or limiting tumor development.\(^20-22\)

Despite growing data supporting the involvement of lncRNAs in tumorigenesis, it is often difficult to surmise whether changes in individual lncRNAs are bona fide drivers of human cancer development and whether targeting altered lncRNAs in patients would be expected to produce therapeutic benefit. Here, we present an overview of how functional lncRNAs in cancer are identified. We highlight promising therapeutic targets based on patient data and on experimental evidence from in vitro and in vivo cancer models. We also discuss important discrepancies to suggest a best-practice roadmap for further characterization of the roles of lncRNAs in cancer.

2 | IDENTIFICATION OF CANCER-ASSOCIATED LNCRNAS

2.1 | Mining global human cancer genomic and transcriptomic data

Integrating genomic and transcriptomic data from diverse human cancers has provided a starting point for the identification of lncRNAs with functional roles in cancer. In particular, recurrent genetic alterations have implicated many genes involved in oncogenesis, and the capacity to identify such genes has expanded in the last several years due to rapid advances in sequencing technologies. These studies have uncovered that many recent somatic copy number variations (SCNVs) map to noncoding regions.\(^23\) Notably, analysis of 5000 human tumor samples across 13 cancer types from The Cancer Genome Atlas (TCGA) revealed that, on average, as many as one quarter of all lncRNAs manifest frequent cancer-related copy number gains or losses.\(^19\) A more recent study probed the copy number of over 10,000 lncRNAs in 80 cancer cell lines across 11 cancer types, identifying 136 lncRNAs involved in focal SCNVs.\(^24\) Importantly, 76 of these lncRNAs lacked copy number changes in flanking protein-coding genes, suggesting potential lncRNA-driven genomic alterations in cancer. Cancer risk SNPs in noncoding loci can also point to a potential role for specific lncRNAs in tumorigenesis. One study identified nearly 4000 lncRNAs overlapping disease-associated SNPs, while another estimated that roughly 12% of all cancer-associated SNPs mapped within 5 Kb of lncRNA loci (compared to 55% mapping near protein-coding genes).\(^18,19\)

Apart from harboring genomic alterations, lncRNAs have also been found to exhibit differential expression patterns in tumor samples compared to normal tissues. A comprehensive meta-analysis of over 7000 gene expression datasets, including a range of normal and cancer samples, identified as many as 60,000 lncRNAs with altered expression.\(^18\) Notably, many previously unannotated lncRNAs were found in disease-associated regions and the expression of roughly 8000 lncRNAs clustered with specific
cancer or cell lineages, suggesting the potential for lncRNAs to execute cancer-specific functions. Along similar lines, an analysis of seven cancer types revealed that, on average, 26% of expressed lncRNAs were significantly deregulated in at least one cancer type (15% upregulated and 11% downregulated) with 60% of these altered lncRNAs demonstrating cancer specificity. In addition, a recent study of lncRNA-associated epigenetic alterations across 20 different cancers identified over 2000 lncRNAs either epigenetically activated or silenced in at least one cancer type. Altogether, these studies led to the consensus that, as a class, lncRNAs are subject to frequent genetic and epigenetic alterations in cancer.

2.2 | LncRNA loci with recurrent SCNVs in cancer

In addition to global patterns of lncRNA deregulation in cancer, several individual lncRNAs have been identified based on frequent large-scale genomic alterations. One of the first cancer-associated lncRNAs was identified in murine lymphomas due to the frequent translocations and viral insertions involving the as yet uncharacterized PVT1 (Plasmacytoma Variant Translocation 1) lncRNA, located approximately 72 Kb downstream of the MYC (Myelocytomatosis) proto-oncogene. Later studies extended these results to human cancer and demonstrated a correlation between PVT1 genomic amplification and poor prognosis in acute myeloid leukemia and in breast and ovarian cancers, among others (reviewed in). Significantly, PVT1 amplification is observed frequently in a range of cancer types including in 33% of ovarian cancers, 20% of esophageal cancers, 13% of invasive breast carcinomas, and 7% of lung adenocarcinomas based on TCGA data. Moreover, PVT1 alterations are associated with a significant reduction in overall and disease-free survival.

Another prominent example of a lncRNA initially characterized by genomic alterations is FAL1 (Focally Amplified LncRNA 1, also known as FALEC) located on chromosome 1q21. FAL1 copy number gains have been observed across many cancer types, including in approximately 10% of liver cancers, invasive breast carcinomas, and lung adenocarcinomas according to TCGA data. FAL1 amplification and overexpression are associated with late stage tumors and with decreased survival of patients with ovarian cancer. Similarly, the lncRNA SAMMSON (Survival-Associated Mitochondrial Melanoma-Specific Oncogenic Noncoding RNA) was identified in a region of focal amplification on chromosome 3p13-14 in 10% of melanomas. High SAMMSON copy number and expression levels are correlated with a reduction in disease-free survival of melanoma patients and associated with resistance to MAPK (Mitogen Activated Protein Kinase) inhibitors.

The locus of the lncRNA LOC285194 on chromosome 3q, moreover, is subject to recurrent monoallelic deletions in as many as 80% of osteosarcomas, often followed by loss of heterozygosity (LOH). Loss of LOC285194 is associated with decreased survival in osteosarcoma patients. The focal deletion of PRAL (p53 Regulation-Associated LncRNA) on chromosome 17p in hepatocellular carcinoma has also been associated with reduced survival. Similarly, recurrent loss of the 9p21 locus, where the lncRNA ANRIL (Antisense Noncoding RNA in the INK4 Locus) resides, is observed in over 50% of glioblastomas, more than 40% of mesotheliomas, and roughly 30% of bladder cancers. Interestingly, a 403 Kb germline deletion encompassing the ANRIL locus is associated with a strong hereditary predisposition to melanoma development.

Many regions of recurrent SCNVs, however, harbor both lncRNAs and protein-coding genes. Therefore, determining the specific contribution of the lncRNA has been challenging. For example, the presence of multiple overlapping transcripts in the ANRIL locus, including the p15INK4B (CDKN2B), p16INK4A (CDKN2A), and p19ARF tumor suppressors, has confounded the role of ANRIL. Analogously, PVT1 is co-amplified with MYC and the PVT1 gene body contains DNA regulatory elements, which promote MYC expression. Likewise, SAMMSON is expressed near MITF (Microphthalmia-Associated Transcription Factor), a key factor in melanocyte differentiation, whereas the commonly amplified genomic region in which FAL1 resides contains the proto-oncogene MCL1 (Myeloid Cell Leukemia Sequence 1). Finally, the LOC285194-associated region of deletion also harbors the tumor suppressor LSAMP (Limbic System-Associated Membrane Protein). Given the complex chromatin architecture and transcriptional profiles in these loci, further studies are needed to deconvolve the specific roles of the lncRNAs and to determine whether lncRNAs act in cooperation with or independently of their neighboring protein-coding genes.

2.3 | LncRNA loci with cancer-associated SNPs

The link between inherited germline variants in lncRNA loci and cancer predisposition or prognosis has been probed extensively in large-scale genome-wide associated studies (GWAS). These studies have identified a plethora of lncRNA-linked SNPs associated with altered cancer risk or patient prognosis.

As an example, the 2 Mb region mapping to 8q24 has emerged as a major hotspot for over a 100 SNPs strongly associated with multiple diseases, including cancers of the
Many of these SNPs are significantly correlated with cancer development and highly predictive of poor patient outcome. While MYC is the dominant oncogene in the locus, many of the cancer risk SNPs are linked to the expression of lncRNAs in the surrounding region, including PVT1, CCAT1 (Colon Cancer-Associated Transcript 1, also known as CARLo-5), CCAT2 (Colon Cancer-Associated Transcript 2), PCAT1 (Prostate Cancer-Associated Transcript 1), PCAT19 (Prostate Cancer-Associated Transcript 19), and PRNCR1 (Prostate Cancer-Associated Noncoding RNA 1). The ANRIL locus is another example of a hotspot harboring more than 10 cancer risk SNPs, some of which are correlated with ANRIL expression. Other lncRNAs linked to cancer SNPs include HOTAIR (HOX Transcript Antisense RNA), HOTTIP (HOXA Distal Transcript Antisense RNA), MALAT1 (Metastasis-Associated Lung Adenocarcinoma Transcript 1), HULC (Highly Upregulated in Liver Cancer), MEG3 (Maternally Expressed 3), H19, GAS5 (Growth Arrest-Specific 5), and PTENP1 (Phosphatase And Tensin Homolog Pseudogene 1). Mechanistic investigations of SNPs associated with lncRNAs have suggested that the risk variants may, in some cases, affect regulatory DNA sequences, thereby resulting in altered lncRNA expression. For example, the PCAT1-linked risk variant rs7463708 was found to increase the activity of a distal enhancer, resulting in increased PCAT1 expression, whereas the PCAT19-linked SNP rs11672691 was proposed to perturb transcription factor binding sites, resulting in the increased expression of a pro-metastatic PCAT19 isoform. Finally, a high-risk neuroblastoma-associated SNP rs693940 on chromosome 6p22 was found to contribute to differential CpG methylation and decreased expression of NBAT-1 (Neuroblastoma-Associated Transcript-1, also known as CASC14), a lncRNA with tumor suppressor properties. Apart from these intriguing examples, however, the majority of lncRNA-associated SNPs lack experimental support that would robustly link the cancer susceptibility variants with deregulation of lncRNA levels or function, and have thus had limited impact on the identification and characterization of functional lncRNAs in cancer.

2.4 LncRNAs differentially expressed in cancer

Global gene expression analyses of normal and cancer samples have also led to the identification of numerous differentially expressed lncRNAs hypothesized to contribute to disease development. Some of the initial analyses revealed frequent upregulation of lncRNAs, such as the imprinted lncRNA H19 in Wilms’ tumors and lung cancer, the prostate cancer-specific lncRNA PCGEM1 (Prostate Cancer Gene Expression Marker 1), the lung metastasis-promoting lncRNA MALAT1, and the hepatocellular carcinoma over-expressed lncRNA HULC.

The differential expression of some of these lncRNAs has been associated with clinical outcomes. For example, altered H19 expression correlates with poor clinical outcomes across various cancer types including breast cancer, non-small cell lung cancer, and acute myeloid leukemia. Moreover, increased expression of PCGEM1 in normal prostate tissue is a prostate cancer risk factor. At the same time, a large body of literature has cemented the strong correlation between high MALAT1 expression levels and poor patient prognosis across over 20 cancer types. Finally, high expression of HULC is associated with poor overall survival and distant metastases.

Notably, integrated analysis of gene expression and methylation datasets has also led to the identification of differentially expressed lncRNAs arising from cancer-associated epigenetic changes, including AFAP1-ASI (AFAP1 Antisense RNA 1), and EPIC1 (Epigenetically Induced lncRNA1), both identified as hypomethylated and overexpressed in Barrett’s esophagus and esophageal adenocarcinoma, and breast cancer, respectively.

Gene expression profiling in cohorts of cancer patients have further fueled the discovery of lncRNAs associated with specific cancer types. Transcriptome sequencing across a cohort of prostate cancer patients identified PCAT1 among 121 unannotated prostate cancer-associated ncRNA (non-coding RNA) transcripts (PCATs). Similarly, comprehensive lncRNA profiling in colorectal carcinoma led to the identification of CCAT1, CCAT2, and other CCAT family members, whereas the lncRNA GAPLINC (Gastric Adenocarcinoma Predictive Long Intergenic Noncoding RNA) stood out as aberrantly overexpressed in gastric tumors. A different set of analyses led to the identification of stage-specific lncRNAs, such as the lncRNA CRNDE (Colorectal Neoplasia Differentially Expressed), a marker of early stages of colorectal cancer development, although the protein-coding capacity of CRNDE remains an open question. Transcriptome profiling of breast cancer subtypes, moreover, highlighted the sets of lncRNAs which are either differentially expressed in tumor samples compared to normal tissues or uniquely enriched in specific stages or subtypes of breast cancer. Examples include MALAT1, HOTAIR, and BCAr4 (Breast Cancer Anti-Estrogen Resistance 4). In parallel, mouse models of cancer were recently employed for the identification of 30 murine MaTArS (Mammary Tumor-Associated RNAs), many of which were found to have human counterparts (hMaTArS) with potential clinical significance determined based on differential expression and correlation with cancer subtype and/or hormone receptor.
2.5 | LncRNAs in cancer pathways

In addition to profiling tumor samples, many researchers have undertaken diverse functional approaches to identify novel lncRNAs, including dissecting tumor suppressive and pro-oncogenic transcriptional networks, analyzing various cancer-related cellular states and processes, and performing genome-wide functional screens.

Analysis of the p53 (also known as Trp53) transcriptional network, in particular, has revealed a wealth of lncRNAs with potential tumor suppressor functions. By comparing gene expression profiles and p53-binding patterns in the absence and in the presence of genotoxic or oncogenic stress, known to activate the p53 pathway, as well as in p53-proficient and -deficient cells, researchers have identified multiple direct lncRNA targets of p53. These included *lincRNA-p21*,88 *PANDAR* (Promoter Of CDKN1A Antisense DNA Damage-Activated RNA, also known as *PANDA*);89 p53BERs (p53-Bound Enhancer Regions);90 *Linc-Pint* (Long Intergenic Non-Protein Coding RNA, P53-Induced Transcript);91 *LED* (LncRNA Activator of Enhancer Domains);92 *PR-lncRNAs* (P53-Regulated lncRNAs);93,94 *DINO* (Damage-Induced Noncoding);95 *lncPRESS1* (LncRNA P53-Regulated And ESC-Associated 1);96 *NEAT1* (Nuclear Enriched Abundant Transcript 1);97-99 *PURPL* (P53 Upregulated Regulator Of P53 Levels);100 *PINCR* (P53-Induced Noncoding RNA);101 *GUARDIN*;102 and an isoform of *Pvt1*, *Pvt1b*.103 Functional characterizations have suggested that many of these lncRNAs contribute to p53 tumor suppressor activities.

Other lncRNAs have been identified downstream of oncogenic signaling networks, giving insight into their potential functions. For example, *Orilnc1* (Oncogenic RAS-induced IncRNA 1) was identified as a target of oncogenic RAS signaling with a proposed role in promoting cell growth.104 *LncRNA-OIS1* (Oncogene-Induced Senescence 1) was found to modulate senescence induced by activation of oncogenic RAS105 whereas *BANCR* (BRAF-Activated Non-Protein Coding RNA) was identified as a transcript induced upon the expression of oncogenic BRAFV600E.106 Analogously, investigation of estrogen receptor (ER) signaling targets identified 33 ER agitation-related (ERAR) lncRNAs and suggested potential roles in ER-positive breast cancer.107 A similar study was performed to examine lncRNAs regulated by androgen receptor (AR) signaling, which identified *ARlnc1* (AR-Regulated Long Noncoding RNA 1) as both a downstream target and upstream effector of AR signaling during prostate cancer progression.108 MYC-regulated lncRNAs have also been identified, including a set of *MYCLos* (MYC-regulated LncRNAs),78 *LAST* (LncRNA-Assisted Stabilization of Transcripts),109 *DANCR* (Differentiation Antagonizing Non-Protein Coding RNA),110 and *SNHG15* (Small Nucleolar RNA Host Gene 15).111

Alterations of cancer hallmarks that enable tumorigenesis have also been linked to the functions of specific lncRNAs (reviewed in112). Examples include lncRNA *gadd7* (growth-arrested DNA damage-inducible gene 7) with a proposed role in suppressing cell cycle progression,113 *SPRY4-IT1* (SPRY4 Intronic Transcript 1) with a proposed role in inhibiting apoptosis in melanoma,114 and *SALNR* (Senescence-Associated lncRNA), proposed to regulate senescence.115

Finally, genome-wide functional screens for lncRNAs involved in promoting or inhibiting specific cellular outcomes important in cancer have aimed to identify candidates for further study. A CRISPR/Cas9-based genome editing approach used a paired guide RNA (gRNA) strategy to target for deletion a set of 700 human lncRNAs, identifying 51 lncRNAs able to regulate cancer cell growth.116 Alternatively, CRISPRi (CRISPR inactivation) and CRISPRa (CRISPR activation) screens, involving a nuclelease-dead Cas9 to tether transcriptional repressors or activators to lncRNA loci have provided effective epigenetic loss-of-function and gain-of-function approaches to query on a genome-wide level the role of lncRNAs in processes such as cellular proliferation or therapeutic resistance.117-120

3 | FUNCTIONAL CHARACTERIZATION OF LNCRNAS IN CANCER

3.1 | Common approaches and limitations

For the 100 or so lncRNAs identified in the approaches described above, the pressing question has become how to accurately distinguish functional lncRNAs from lncRNAs that are subject to passenger genetic and epigenetic alterations in cancer. RNA interference (RNAi)-mediated downregulation of lncRNAs has been a common approach for functional characterization. In parallel, antisense oligonucleotides (ASOs) have provided a convenient and efficient loss-of-function alternative. While RNAi is most effective for lncRNAs exported to the cytoplasm, ASOs lend broader efficacy by triggering RNase H-mediated co-transcriptional RNA cleavage and degradation, in some cases accompanied by transcriptional repression.121,122 Frequently, RNAi and ASO approaches have been performed in parallel with exogenous lncRNA overexpression. Regrettably, few studies have complemented RNAi or ASO loss-of-function experiments with knockdown-resistant lncRNA rescue mutants, missing an important opportunity to both demonstrate specificity and establish a system to investigate the sequence
basis for lncRNA function. CRISPR-based epigenetic inhibition (CRISPRi) and activation (CRISPRa) have also been employed as successful loss-of-function and gain-of-function approaches, respectively.

Genetically engineered mouse models (GEMMs) of lncRNAs and CRISPR-based editing of lncRNA loci in cell lines have also brought important insights. In contrast to protein-coding genes, where genetic approaches aim to perturb the open reading frame (ORF) and, therefore, the functional output of the transcript, methods to target lncRNAs have been, by necessity, more diverse and creative (reviewed in\textsuperscript{123}). Some loss-of-function studies have undertaken deletion of the entire gene body, the promoter region, or narrower functional regions, while others have employed introduction of a premature polyadenylation signal (PAS) or polyadenylation cassette (STOP) to terminate transcription. Conversely, gain-of-function studies in animal models have involved the introduction of a transgenic lncRNA sequence or amplification of an entire lncRNA locus.

Strikingly, for many lncRNAs, observed phenotypes have varied with the use of alternative approaches. For example, initial RNAi knockdown of the p53-regulated lncRNA, lincRNA-p21, suggested that it acts globally to modulate the expression of multiple p53 target genes, whereas subsequent genetic deletion of its promoter in the mouse revealed a more restricted role in promoting the expression of the neighboring p21/CDKN1A gene.\textsuperscript{88,124} Further investigation involving a locus deletion genetic approach, however, raised doubts about whether the lncRNA plays any functional role at all.\textsuperscript{125} The metastasis-promoting lncRNA HOTAIR has provided additional examples of the complexity in developing lncRNA models. While ectopic expression of HOTAIR in breast cancer cell lines induced global gene expression changes and increased metastases in a xenograft mouse model, supporting an oncogenic function,\textsuperscript{84} loss-of-function models, including RNAi-mediated knockdown, a 4 Kb gene body deletion, and a 140 Kb locus deletion have led to significant discrepancies.\textsuperscript{126-129} The differences between alternative models have highlighted the need to use multiple independent and complementary approaches to investigate the functional roles of lncRNAs in cancer biology.

### 3.2 Multi-pronged approaches to lncRNA characterization

In this section, we focus on a small set of lncRNAs for which work from multiple groups or involving an array of in vitro and in vivo approaches has revealed exciting functional insights and provided starting points for further exploration of their contributions to tumor development.

#### 3.2.1 MALAT1

MALAT1 remains one of the most studied cancer-associated lncRNAs, with proposed roles in influencing nuclear speckles,\textsuperscript{130} pre-mRNA splicing,\textsuperscript{131} and epigenetically regulating gene transcription.\textsuperscript{132} While initial studies pointed to a pro-metastatic function,\textsuperscript{65} further characterization resulted in discrepancies (Figure 1). Three different loss-of-function GEMMs, including an insertion of a LacZ reporter and polyadenylation cassette 69 nucleotides downstream of the Malat1 transcription start site, a 3 Kb deletion of the 5′ end and promoter region of Malat1, and a conditional deletion of 7 Kb encompassing the entire Malat1 gene body, revealed that Malat1 is dispensable for organismal development and viability.\textsuperscript{133-135} Strikingly, none of the mouse models showed effects on global gene expression, nuclear speckle formation, or alternative pre-mRNA splicing. This opposed previous findings using RNAi to downregulate MALAT1 levels in cancer cell lines in vitro,\textsuperscript{131,132} perhaps suggesting a cancer-specific function. Furthermore, different in vivo models have yielded conflicting results about the function of MALAT1 in cancer. On the one hand, crossing the promoter deletion model\textsuperscript{135} to the MMTV-PyMT (mouse mammary tumor virus-polyomavirus middle T antigen) mouse model of breast cancer resulted in reduced metastases to the lung, without affecting primary tumor burden, an effect largely recapitulated by ASO-depletion of Malat1 in vivo.\textsuperscript{82} This pro-metastatic function was also observed in a mouse xenograft model of lung cancer where MALAT1 knockout human lung tumor cells formed fewer tumor nodules.\textsuperscript{136} In this model, targeting MALAT1 with ASOs after tumor implantation prevented metastasis formation, pointing to MALAT1 as a viable therapeutic target.\textsuperscript{136} On the other hand, crossing the Malat1 premature termination model\textsuperscript{134} to the MMTV-PyMT breast cancer model led to a significant increase in the number and area of metastatic nodules in the lungs.\textsuperscript{137} This surprising tumor suppressive effect could be rescued with a Malat1 transgene expressed from the Rosa26 locus.\textsuperscript{137} A similar effect was observed in vitro in human breast cancer cells, with the expression of MALAT1 from an exogenous construct rescuing the increased metastatic ability conferred by MALAT1 knockout in clonal cell populations.\textsuperscript{137} The debate surrounding the precise contribution of MALAT1 to cancer development is ongoing. It is unclear whether the phenotypic differences arising from MALAT1 loss might be due to differences in experimental setup, such as mouse strain or knockout approach, or reflect the complex biology of MALAT1. Altogether, investigations of MALAT1 using in vitro and in vivo approaches have highlighted the biological and technical complexities associated with studying the functional roles of lncRNAs in cancer.\textsuperscript{138,139}
3.2.2 NEAT1

Similarly to MALAT1, several studies have examined the role of NEAT1 during cancer development, leading to opposing views (Figure 2). Initial studies suggested that NEAT1 levels were elevated in a variety of human cancers relative to normal tissues and correlated with worse prognosis, suggesting a pro-oncogenic role for NEAT1 (reviewed in). This conclusion was supported by a study of Neat1 knockout mice subjected to chemical induction of skin squamous cell carcinoma with the carcinogen DMBA and the pro-inflammatory agent TPA. While Neat1-deficient animals displayed no obvious phenotypes in the absence of stress, loss of Neat1 conferred resistance to chemically induced squamous cell carcinoma. Interestingly, studies have also suggested that NEAT1 may...
be a target of the p53 pathway and, therefore, may have tumor suppressive activities in some contexts. Indeed, tumor suppressive functions of Neat1 were unveiled in primary mouse embryonic fibroblasts (MEFs), where Neat1 knockout led to increased colony formation in an EIA; HrasG12V transformation experiment, as well as in an autochthonous mouse model of pancreatic cancer, where Neat1 deficiency increased the occurrence of premalignant lesions, known as pancreatic intraepithelial neoplasias (PanINs). Interestingly, Malat1 and Neat1 are neighboring genes and studies have suggested that genomic deletion of either lncRNA may impact the epigenetic organization and transcriptional profiles of the entire locus, raising questions about the specificity of each approach.
3.2.3 | PVT1

As one of the lncRNAs strongly associated with advanced disease and poor patient prognosis, PVT1 has been the subject of extensive investigation (Figure 3). In keeping with the finding that PVT1 is frequently co-amplified with the MYC proto-oncogene, Myc-Pvt1 co-amplification in a mouse model of breast cancer was found to be more tumorigenic than Myc amplification alone. This study suggested that PVT1 acts in trans to promote MYC protein stability, based on evidence that a 300 Kb genomic deletion of the PVT1 locus in a human colorectal carcinoma cell line resulted in

**FIGURE 3** Identification and functional characterization of PVT1. PVT1 was identified in murine lymphomas following the observation of translocations, viral insertions, and amplifications involving the Pvt1 locus. Functional characterization of PVT1 has utilized various loss-of-function (LOF) and gain-of-function (GOF) models including amplification genetically engineered mouse models (GEMMs) (Myc/Pvt1AMP, MycAMP145), locus deletion (PVT1Δ), tumor-specific mutagenesis of the Pvt1-associated p53 Response Element (p53RE) (Δp53RE, 103), transcript degradation with RNAi and ASO, and CRISPR-mediated epigenetic activation and inhibition (CRISPRa/i). The increased tumor growth observed in a Myc/Pvt1 co-amplification GEMM (Myc/Pvt1AMP) compared to Myc amplification alone (MycAMP) when crossed to the MMTV-Neu BC (breast cancer) GEMM suggests an oncogenic function for Pvt1 (red box,145). However, the increased tumor growth in Pvt1-associated p53RE mutagenized lung tumors following Cre-mediated tumor initiation in a Kras-driven lung adenocarcinoma (LUAD) GEMM suggests a tumor suppressor function (green box,103)
decreased MYC protein levels. However, later studies found evidence for MYC enhancers within the region of deletion, raising questions about the role of the PVT1 locus and its associated RNA in MYC regulation. Subsequent studies confirmed the presence of DNA regulatory elements in the locus but challenged the understanding of PVT1 as a strictly pro-oncogenic lncRNA. On the one hand, deletion of a ~600 bp region containing a p53-binding site and mapping to the 5’ end of PVT1 led to defects in p53-mediated MYC repression, although the contribution of PVT1 to the p53 response was unclear. On the other hand, CRISPRi-based inhibition of PVT1 in breast cancer cell lines revealed a role for the PVT1 promoter as a DNA tumor suppressor boundary element that limits MYC promoter accessibility to enhancers within the PVT1 gene body, resulting in restricted MYC expression. In this setting, the PVT1 RNA appeared to be dispensable. In contrast, our group identified a stress-induced, p53-dependent isoform of Pvt1, Pvt1b, which is both necessary and sufficient to repress Myc transcription. These findings were recapitulated in vitro using a genetic loss-of-function approach to mutate the p53-binding site required for Pvt1b expression. Importantly, mutagenesis of the Pvt1-associated p53-binding site at the time of tumor initiation in an autochthonous mouse model of lung cancer led to larger tumors and indicated a key role for Pvt1b in restraining tumor growth downstream of p53. In the future, it would be interesting to deconvolve the oncogenic and tumor suppressive elements in the PVT1 locus and to differentiate between DNA elements and RNA isoforms with potentially distinct functions.

3.2.4 | XIST

With a critical role in X-chromosome inactivation and dosage compensation that has been investigated for decades (reviewed in), the potential role of XIST (X-Inactive Specific Transcript) in tumorigenesis has intrigued researchers. Historically, it has been observed that altered chromosome copy numbers and inappropriate dosage compensation are frequently associated with human cancer. Notably, men with Klinefelter syndrome characterized by an extra X-chromosome have an increased risk of many malignancies including breast cancer and non-Hodgkin lymphoma, and loss of X-chromosome inactivation has been observed in breast cancer cell lines and testicular germ cell tumors. In support of these correlative observations, a conditional Xist deletion model in mouse blood cell lineages led to aggressive myeloproliferative neoplasm and myelodysplastic syndrome with complete penetrance, likely as the result of widespread gene expression changes. The tumor suppressive role of XIST was recapitulated in RNAi and overexpression studies in breast cancer cell lines as well as by crossing the Xist knockout to the MMTV-PyMT mouse model of breast cancer. Further studies should determine the prevalence of XIST and X-inactivation perturbations in human cancer and investigate the possibility of targeting this pathway as a therapeutic strategy.

3.2.5 | ANRIL

High ANRIL expression in tumor tissues has been linked to aggressive pathological features and poor overall survival. In initial studies, targeted deletion of a 70 Kb region in the Anril locus, which harbors multiple cancer and coronary artery disease-associated SNPs, led to viable progeny but showed increased mortality during development and as adults. Primary cultures of smooth muscle cells, isolated from mutant mice, exhibited excessive proliferation and diminished senescence, cellular phenotypes consistent both with accelerated coronary disease pathogenesis and increased cancer risk. Mechanistic investigation revealed that the effects were mediated in cis through the reduced expression of Cdkn2a and Cdkn2b and led to the conclusion that the risk region contained key regulatory elements. Subsequent investigation using exogenous overexpression of ANRIL in primary human fibroblasts suggested that the lncRNA may be responsible for CDKN2A/2B repression through the locus-specific recruitment of the repressive PRC1 complex. Unfortunately, little progress has been made over the past decade in determining whether ANRIL transcription or transcript accumulation is required for its cis-regulatory function, in part due to the limited conservation of ANRIL sequence and exonic structure between human and mouse.

3.3 | Promising lncRNA candidates warranting further investigation

In this section we examine exciting, albeit limited, initial studies of lncRNAs with putative cancer functions, the validation of which could benefit from the development of alternative approaches and further characterization.

3.3.1 | SAMMSON

To investigate the role of SAMMSON as a lineage addiction oncogene in melanoma, researchers employed ASO-mediated knockdown and exogenous overexpression as loss-of-function and gain-of-function tools. They observed that SAMMSON amplification and increased expression led to altered mitochondrial metabolism and homeostasis. In turn, this caused increased melanoma cell viability and clonogenic potential and resulted in sensitization of melanoma.
cells to MAPK targeting therapeutics in vitro and in patient-derived xenograft (PDX) models in vivo. Further mechanistic studies clarified the role of SAMMSON in balancing mitochondrial translation rates. The generation of genetic models of SAMMSON may reveal further insights into its role in melanoma development.

### 3.3.2 | NKILA

NKILA (NF-κB Interacting Long Noncoding RNA) was identified as both a target and negative modulator of the NF-κB signaling pathway, with low NKILA levels observed in metastatic breast cancer cell lines and correlated with decreased disease-free survival in a cohort of breast cancer patients. Mechanistically, a series of deletion mutants demonstrated that NKILA interacts directly and stably with the NF-κB:IkB complex in the cytoplasm to prevent IkB phosphorylation and suppress activation of the NF-κB pathway, suggesting a tumor suppressive role for NKILA in limiting inflammatory processes in cancer. A different study from the same group showed that RNAi downregulation of NKILA in cytotoxic T cells (CTLs) led to increased tumor infiltration and reduced tumor volume in a breast cancer PDX mouse model, implicating NKILA as a potential target in the field of cancer immunotherapy.

### 3.3.3 | LncGata6

LncGata6 (LncRNA GATA6) was identified as a divergent transcript expressed from the promoter of Gata6, which is specifically enriched in a subset of intestinal stem cells. Deletion of exons 2-4 of LncGata6 in the mouse did not affect Gata6 levels but resulted in decreased intestinal regeneration due to decreased proliferative capacity of intestinal stem cells. Consistent with the key role of stem cells in intestinal tumorigenesis, genetic and ASO-mediated depletion of LncGata6 in intestinal adenoma and in a PDX model were found to impair tumor growth in the APCmin mouse model of intestinal adenoma and in a PDX model. Future studies should focus on elucidating the mechanism by which LncGATA6 is upregulated in colorectal cancer and on determining the extent to which it contributes to aberrant Wnt signaling, a known colorectal cancer driver.

### 3.3.4 | DINO

The p53 target lncRNA DINO binds to and stabilizes p53 in a positive feedback loop, enhancing the activation of p53 target genes. Importantly, RNAi knockdown of DINO in human fibroblasts and a deletion of the Dino promoter in MEFs led to impaired cell cycle arrest following genotoxic stress. Interestingly, ectopic expression of DINO in HPV-positive cervical cancer cells, which suppress p53 stabilization and express DINO at low levels, led to reactivation of dormant p53, resulting in sensitization of the cancer cells to chemotherapeutic agents and vulnerability to metabolic stress. To date, however, evidence that DINO acts as a tumor suppressor in human cancer is limited.

### 3.3.5 | LINC-PINT

Like DINO, linc-Pint was also identified as a p53 target. A knockout mouse generated by replacing the linc-Pint locus with a LacZ reporter cassette yielded smaller pups, suggesting a role for linc-Pint in early development. Characterization of LINC-PINT function in cancer suggested a role in limiting cell invasion, with LINC-PINT overexpression leading to decreased liver metastases in a mouse model. In a transwell migration and invasion assay, invasiveness increased following treatment with LINC-PINT-targeting ASOs or following CRISPR-mediated deletion of a highly conserved LINC-PINT sequence element. Analysis of the previously generated linc-Pint knockout mouse in a cancer background could help to support these results. However, the potential role of the LINC-PINT RNA may be confounded by the identification of a peptide with a function in suppressing cell proliferation encoded by a circular form of LINC-PINT.

### 3.3.6 | THOR

While examples of alternative organismal models for lncRNA function in cancer are limited, in part due to low evolutionary conservation of lncRNAs, investigation of the highly conserved lncRNA THOR (Testis-Associated Highly Conserved Oncogenic Long Noncoding RNA) in human and zebrafish cancer models has implicated this lncRNA in promoting melanoma development (Figure 4). THOR expression is normally restricted to the testis, but has been found aberrantly overexpressed in multiple cancer types, including lung adenocarcinoma, lung squamous carcinoma, and melanoma. Knockdown of THOR via RNAi and ASOs in lung adenocarcinoma and melanoma cell lines led to decreased proliferation and reduced colony formation. These findings were corroborated in two independently derived lung adenocarcinoma cell lines harboring approximately 3 Kb CRISPR-mediated deletions within the THOR gene body. Conversely, THOR overexpression gave the opposite phenotype, leading to increased proliferative capacity and anchorage-independent growth. Importantly, ectopic expression of human THOR in zebrafish cooperated
with oncogenic NRAS and p53 loss to promote melanoma development, whereas knockout of THOR in zebrafish embryos delayed mutant NRAS-induced melanoma formation. Further studies may reveal the potential of using THOR expression as a biomarker or targeting THOR as a therapeutic strategy.

**FIGURE 4** Identification and functional characterization of THOR THOR was identified as a testis-specific ultra-conserved IncRNA aberrantly expressed in cancer tissues. Hosono and colleagues generated several in vitro and in vivo loss-of-function (LOF) and gain-of-function (GOF) models to functionally characterize THOR. LOF models included transcript degradation with RNAi and ASO, and THOR partial locus deletion (THOR−/−) in both human cells injected in severe combined immunodeficiency disease (SCID) mice and in a genetically engineered zebrafish model (THOR−/−) embryonically injected with NRAS to induce melanoma. GOF models included THOR overexpression (OE) in vitro and OE of human THOR (hTHOR) in p53-deficient zebrafish (p53−/−) embryonically injected with NRAS to induce melanoma. Overexpression of THOR plays an oncogenic role (red box) in cancer by binding to IGF2BP1 and increasing the stability of its mRNA targets to promote cancer progression.

**4 | FUTURE PERSPECTIVES**

Identification of IncRNAs that are genetically or epigenetically perturbed in cancer has risen sharply over the past decade. The precipitous increase in the number of cancer-associated IncRNAs has been accompanied by a growing
excitement that many lncRNAs may act as novel drivers of cancer development. Yet, lagging understanding of how lncRNAs function in physiologic and pathologic contexts has limited our insights into the roles of lncRNAs in tumorigenesis. The current literature points to many lncRNAs acting as both oncogenes and tumor suppressors. While these seemingly contradictory findings may stem from differences in experimental models, they may also be reflective of complex and context-dependent lncRNA biology, analogous to the dual oncogenic and tumor suppressor roles played by cancer-associated protein-coding genes.166 Future studies should prioritize the identification and validation of true dual functions from technical inconsistencies.

LncRNAs make attractive drug targets, particularly in diseases where protein candidates are not amenable to pharmacological inhibition.167 Both siRNA- and ASO-mediated lncRNA degradation as well as locked nucleic acid (LNA)-mediated interference with lncRNA function have emerged as clinic-ready approaches.168,169 The successful deployment of these approaches in cancer, however, is predicated upon robust functional characterization. In the future, it would be essential to develop in vitro and in vivo models that closely recapitulate the recurrent genetic or epigenetic changes of lncRNAs observed in human cancer. In parallel, experiments that uncover the functional elements of perturbed lncRNA loci will inform whether motives or structural features of the lncRNA molecules, the act of their transcription, or underlying DNA elements mediate their roles in disease development. These questions will be best answered through the integration of diverse and complementary approaches and by corroboration from multiple independent studies.

5 | COMPETING INTEREST

The authors declare no competing financial interest.

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AUTHOR CONTRIBUTIONS

C. Olivero and N. Dimitrova wrote and edited the manuscript. C. Olivero created the figures.

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