Uncovering the role of genomic “dark matter” in human disease

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The human genome encodes thousands of long noncoding RNAs (lncRNAs). Although most remain functionally uncharacterized biological “dark matter,” lncRNAs have garnered considerable attention for their diverse roles in human biology, including developmental programs and tumor suppressor gene networks. As the number of lncRNAs associated with human disease grows, ongoing research efforts are focusing on their regulatory mechanisms. New technologies that enable enumeration of lncRNA interaction partners and determination of lncRNA structure are well positioned to drive deeper understanding of their functions and involvement in pathogenesis. In turn, lncRNAs may become targets for therapeutic intervention or new tools for biotechnology.

Introduction

RNA is now recognized as an important regulator of biological systems. While its primary sequence can encode protein, RNA can also fold into non–protein-coding structural motifs that perform catalysis (1), bind small molecules (2), or serve as protein scaffolds (3). Noncoding RNAs can conditionally govern gene expression (4) and have impressive regulatory capacity, as small noncoding RNAs may modulate the expression of greater than 60% of human coding genes (5). Built upon the growing number of well-characterized regulatory RNAs, novel RNA-based control systems are now being applied to problems in microbial (6, 7) and mammalian biotechnology. Gene networks have been programmed to recognize and respond to cancer-associated miRNA profiles (8), shRNA-based genetic switches may support gene therapy applications (9), and drug-responsive RNA sensors have been developed for T cell therapy (10).

Despite the remarkable progress in characterizing RNA-based regulation and the promise of RNA biotechnology, much of the human transcriptome remains functionally uncharacterized. Although less than 1.5% of the human genome codes for proteins, a much larger fraction of the human genome appears to code for RNA. Some studies indicate that greater than 90% of the human genome is transcribed (11), although others have argued that technical artifacts or biological noise explain much of this pervasive transcription (12). Increasingly sensitive RNA sequencing approaches (13) are helping to address this controversy and have confirmed the existence of many novel transcripts (14).

Ongoing efforts are focused on classifying these presumed noncoding transcripts, although care and additional experiments are necessary to exclude their protein coding potential (15). Considerable attention has focused on short noncoding RNAs (lncRNAs), which are distinguished from short regulatory RNAs by having a length greater than 200 nucleotides. Recent studies have classified more than 8,000 intergenic lncRNAs (16), which are often transcribed at lower abundance than mRNAs by RNA polymerase II in a tissue-specific manner. Many lncRNAs map to regions associated with disease by genome-wide association studies (GWAS) (16), and the number of papers discussing lncRNA disease associations grows each year (17).

Here, we explore the rapidly expanding literature by discussing lncRNAs that exert epigenetic, transcriptional, and posttranscriptional regulation in gene networks that are relevant to human health, including tumor suppression and development. At each level of control, we highlight lncRNA regulatory mechanisms and putative roles in human disease. Finally, we discuss new technologies that are well-positioned to drive deeper mechanistic understanding of lncRNA function.

Epigenetic control

Despite having identical genomes, the different cell types within an individual exhibit unique and heritable gene expression patterns. Heritable variation (−genetic) must be encoded in molecular signatures beyond (epi-) the DNA sequence itself (18). These epigenetic signatures can be written to chromatin, the structural housing of genetic information in which DNA is wrapped around repeating octamers of histone proteins. Methylation of cytosines residues in DNA and posttranslational histone modifications can specify the state of chromatin, resulting in transcriptional activation or silencing. In mammalian systems, the chromatin-remodeling components that write and erase these epigenetic signatures generally lack domains to specify DNA localization (19) and depend upon ancillary factors in order to target specific loci. lncRNAs can serve as these factors, recruiting chromatin-remodeling components to their point of synthesis as nascent transcripts in cis or acting as diffuse scaffolds that guide these components to distant loci in trans. cis- and trans-acting lncRNAs facilitate epigenetic control over diverse processes, including tumor suppression and development.

Cell cycle control and tumor suppression. Senescence is a state of cell-cycle arrest that guards the cell against unrestrained proliferation and tumor progression. Spanning an approximately 42-kb region on human chromosome 9p21, the INK4a (p19)–ARF (p14)–INK4b (p16) locus is an important regulator of cellular senescence, as it codes for three tumor suppressors: p15 and p16 promote retinoblastoma protein (pRBF) function and cell-cycle arrest by inhibiting cyclin-dependent kinases (CDK4/6), and p14 increases the functionality of p53 signaling (20). Coordinated regulation of this locus is governed by polycomb repressive complex–2 (PRC-2), which trimethylates histone H3 on lysine 27 (H3K27) in transcript-
As a result, HEIH recruitment of PRC-2 to tumor suppressors may facilitate HCC tumorigenesis.

ANRIL genomic region (24). Many of these SNPs are found within through regulation of this locus. A screen for lncRNAs upregulated STAT1 binding and leads to upregulation of ANRIL, this SNP may SNPs for type 2 diabetes, heart disease, and cancers overlap with the PRC-2 to silence p16 and other genes involved in cell cycle control. er, suggesting a easier (CAD) — disrupts the binding site for STAT1, a transcription factor that represses ANRIL expression (25). Because it abrogates transcription can affect the transcriptional state of the locus, which is frequently deleted or silenced in cancers (23). Specifically, ANRIL is upregulated in prostate cancer tissues (22), and risk-associated SNPs for type 2 diabetes, heart disease, and cancers overlap with the ANRIL genomic region (24). Many of these SNPs are found within enhancer elements, and one — associated with coronary artery disease (CAD) — disrupts the binding site for STAT1, a transcription factor that represses ANRIL expression (25). Because it abrogates STAT1 binding and leads to upregulation of ANRIL, this SNP may contribute to CAD through ANRIL-mediated silencing of p15.

trans-acting IncRNAs also play a role in tumor suppression through regulation of this locus. A screen for IncRNAs upregulated in hepatocellular carcinoma (HCC) identified high expression in hepatocellular carcinoma (HEIH), a lncRNA that binds EZH2, the methyltransferase subunit of PRC-2 (26). HEIH knockdown resulted in derepression of PRC-2 target genes, including p16, and HEIH overexpression increased H3K27 levels at the p16 promoter, suggesting a trans model of regulation in which HEIH recruits PRC-2 to silence p16 and other genes involved in cell cycle control. As a result, HEIH recruitment of PRC-2 to tumor suppressors may facilitate HCC tumorigenesis.

**Development.** Paralleling their role in regulation of the localized genes. (27) Like HEIH, HOTAIR overexpression leads to genome-wide retargeting of the PRC-2 complex, resulting in a PRC-2 occupancy and gene expression pattern that promotes metastasis in breast (28) and colorectal cancers (29).

Countering the transcriptional repression of PRC-1/2 complexes, the thrihorax group (TrxG) proteins trimethylate lysine 4 of histone H3 (H3K4) in order to establish and maintain HOX gene transcriptional activation. LncRNAs recruit TrxG proteins, such as the MLL-1 complex, to chromatin for activation of specific HOX genes. The lncRNA HOTTIP binds WDR5, an adapter protein for MLL-1, and recruits the MLL-1/WDR5 complex to 5′ HOX genes (30). HOTTIP-mediated recruitment of MLL-1 occurs in cis and is facilitated by chromosomal looping, which brings the nascent HOTTIP transcript into close spatial proximity with its 5′ HOX target genes. This interaction is disrupted by HOTTIP knockdown, which results in a loss of H3K4me3 across the HOXA locus and a notable shortening and bending of distal bony elements, including the radius, ulna, and third digit. Whereas HOTTIP knockdown most strongly reduced expression of 5′ HOX genes (HOXA10–HOXA13), knockdown of Mira, another lncRNA transcribed within the HOXA locus, specifically reduced expression of HOXA6/7 (31). Unlike HOTTIP, Mira binds directly to MLL-1 and recruits the complex to the HOXA6/7 promoters. Through the activation of HOXA6/7, Mira indirectly triggers expression of 15 germ-layer marker genes during early mouse ES cell differentiation, further underscoring the importance of lncRNAs in the HOX developmental program.

Although the HOX cluster is a good model for understanding lncRNA-mediated repression and activation (Figure 1A), the full scope of epigenetic regulation by IncRNAs in development appears to be much broader. A recent report identified 137 lncRNAs that temporospatial limb and organ development along the anatomical anterior-posterior axis. Expressed from the HOXC locus, the lncRNA HOTAIR drives transcriptional repression of HOXD in trans through recruitment of PRC-2 and LSD1, a complex that removes a chromatin modification (H3K4me2) associated with transcriptional activation (27).

**Figure 1**

Models for lncRNA-mediated epigenetic, transcriptional, and posttranscriptional regulation. (A) IncRNAs can recruit chromatin-remodeling components to specific genomic loci, reprogramming the state of chromatin in order to silence or activate transcription. (B) IncRNAs housed within nuclear subcompartments can bind promoter-associated proteins, recruiting specific genomic loci to the subcompartment and driving transcription of the localized genes. (C) IncRNAs can act as ceRNAs, which harbor miRNA binding sites and therefore reduce miRNA levels available for targeting mRNAs. In all cases, IncRNAs are shown in blue.
globally affect gene expression levels in mouse ES cells, demonstrating that IncRNAs play roles in early development and stem cell biology. Many of these IncRNAs (approximately 30%) interact with chromatin-modifying proteins and affect gene expression patterns that maintain ES cell pluripotency or repress lineage-specific differentiation (32). Beyond early development, IncRNA scaffolds for chromatin-remodeling components maintain developmental programs in specific tissues, such as the retina (33). Despite the apparent diversity of target genes regulated by these IncRNAs, several questions underpin all cases of epigenetic control: it will be important to determine the RNA structural motifs that interact with chromatin-remodeling components, identify cases in which multiple complexes are recruited to a single IncRNA, and discover how IncRNAs target these complexes to specific regions of chromatin. In addition, a recent study in fission yeast showed that transcription of protein-coding mRNAs, along with their degradation factors, nucleates heterochromatin formation at specific loci in response to developmental signals (34). Moreover, it will be interesting to explore whether IncRNAs and protein-coding transcripts share mechanisms for exerting epigenetic control over genomic loci.

Transcriptional and cotranscriptional control While the examples of epigenetic control involve recruitment of chromatin-remodeling components, transcriptional control often governs recruitment of RNA polymerase-II (PolII), transcription factors, and coregulators to the promoter regions of specific genes. Just as they recruit chromatin-modifying proteins, IncRNAs also nucleate assembly of transcription factors in diverse signaling pathways, including IncRNA-mediated repression of target genes in the p53 pathway (reviewed in ref. 35) and IncRNA coactivation of nuclear receptor signaling (reviewed in ref. 36). Yet the mechanisms employed by IncRNAs in transcription control extend well beyond scaffolds, as IncRNAs can also serve as decoys that bind and repress the activity of transcription factors or repress target genes by forming a triple helix with promoter DNA, blocking PolII binding and transcription initiation (reviewed in ref. 37). Although mechanisms and disease associations (38) for IncRNA transcriptional regulators have been reviewed, recent reports extend both the mechanistic scope of IncRNA-mediated transcriptional control and the clinical relevance of these pathways.

Cell cycle control. Cell cycle progression is intimately associated with expression of specific sets of IncRNAs (39). It is also well-established that some IncRNAs abundantly localize to nuclear bodies, which can house protein factors involved in transcriptional activation (e.g., interchromatin granules [IGCs]) or repression (e.g., Pc group [PcG] bodies) (reviewed in ref. 40). A recent report shows that these IncRNAs play a role in cell cycle control by recruiting specific genomic loci to each nuclear compartment, resulting in transcriptional activation or repression. The IncRNAs TUG1 and NEAT2 associate with PcG bodies and ICGs, respectively, and serve as scaffolds for protein factors involved in transcriptional repression and activation within their respective compartments (Figure 2).
science in medicine

1B). These two IncRNAs also bind polycomb 2 protein (Pc2), which is associated with the promoters of cell cycle regulator genes and also contains a chromodomain that binds histone modifications. TUG1 binds the methylated form of Pc2, recruits the protein to PcG bodies, and increases its affinity for repressive heterochromatin marks, reinforcing localization within the PcG-repressed compartment. In contrast, NEAT2 binds the demethylated form of the protein, recruiting it to ICGs and increasing its affinity for active chromatin modifications. As a result, Pc2 methylation and demethylation govern the physical relocation and transcription of the cell cycle regulator genes to which it is bound (41). It will be interesting to learn whether other IncRNA scaffolds can recognize covalent modifications on promoter-associated signaling proteins and explore whether this is a common mechanism for conditional gene localization and transcriptional control.

Stress response. Direct repression of PolIII is another form of transcriptional control exerted by Alu IncRNAs, which are approximately 300-600 products of PolIII transcription from prevalent genomic repeats known as Alu elements (42). These IncRNAs are often upregulated by stress induction, such as heat shock, and in cancers, including hepatocellular carcinoma (43). Alu knockdown in heat shock–induced kidney cells lead to derepression of four genes, prompting biochemical studies identifying specific Alu regions that bind RNA PolII (44) and demonstrating that Alu IncRNAs exert transcriptional repression by blocking contacts between PolIII and promoter DNA (45). Identifying the regulatory factors that restrict Alu repression to specific target genes remains an important area of research and may complement efforts focused on the clinical implications of this regulatory pathway.

A recent report provides an intriguing link between Alu expression and geographic atrophy (GE), a form of macular degeneration that is a common cause of blindness. Retinal tissues affected by GE exhibit reduced levels of Dicer1, an enzyme that cleaves long double-stranded RNA and is a core component of small RNA gene processing. Interestingly, knockdown of other enzymes required for miRNA processing did not lead to the GE phenotype, and Dicer1 knockdown caused accumulation of Alu IncRNAs (46). Overexpression and direct injection of Alu IncRNAs into the retina resulted in GE, and the condition could be ameliorated by increased Dicer1 expression. Although it appears that Dicer1 is needed to cleave toxic long double-stranded RNA Alu transcripts, failure to produce the smaller Alu products may also contribute to GE. Moreover, it will be important to learn whether the cleaved Alu products are functional in the retina and whether downregulation of Dicer1 in other diseased tissues, such as cancers (47), alters Alu expression levels. Furthermore, using antisense oligonucleotides to reduce toxic Alu IncRNA levels might be a useful therapeutic strategy to combat GE.

Posttranscriptional control

Epigenetic and transcriptional control by IncRNAs share common themes: IncRNA–protein interactions and IncRNA-DNA/chromatin interactions establish a considerable diversity of linkages between transcriptional activator or repressor proteins (e.g., transcription factors or chromatin-remodeling components) and the genomic loci to which these proteins are recruited. Although effort continues to focus on these two areas, IncRNAs have an increasingly appreciated role in posttranscriptional regulatory networks. Many IncRNAs that exert posttranscriptional control fall within the broader class of competitive endogenous RNAs (ceRNAs), which harbor miRNA binding sites (miRNA response elements; MREs) and reduce miRNA levels available for targeting mRNAs (Figure 1C and refs. 48, 49). As a result, ceRNA upregulation is typically followed by symmetric upregulation of transcripts that the sequestered miRNA normally targets. Because miRNAs regulate thousands of human genes and each miRNA may have many targets, the scope of ceRNA regulation may be significant, and recent reports highlight its importance in development as well as tumor suppression.

Cell cycle control and tumor suppression. Evidence for IncRNAs acting as ceRNAs emerged from a study on phosphatase and tensin homolog (PTEN), a tumor suppressor for which gene dosage changes are linked to cancer susceptibility (50). PTEN was in part chosen for investigation because it is subject to miRNA regulation and has a noncoding pseudogene, PTEN-P1, that harbors a 3′ untranslated region (UTR) with the same MRE. Overexpression of the PTEN-P1 3′ UTR regulated PTEN levels in trans, but only in the presence of Dicer1, which suggests that miRNAs must be present in order to establish the regulatory link (51). Because it exerts trans regulation by sequestering PTEN-targeting miRNAs miR-19b and miR-20a, PTEN-P1 is a tumor suppressor, and copy number losses at PTEN-P1 are observed in colon cancer cells. The same study showed that a similar relationship exists between v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), which is amplified in numerous human tumors, and its pseudogene, KRAS1P, suggestive of a broad role for IncRNA ceRNAs in tumorigenesis. Two recent reports have extended this initial study by showing that PTEN is also affected by coding ceRNAs, including the Zeb2 transcript in melanoma (52) and the RB1 transcript in glioblastoma (53). Collectively, these pioneering efforts on ceRNAs establish a framework for understanding the role of IncRNAs as posttranscriptional regulators in tumor suppressor or oncogenic miRNA networks. It will be interesting to understand the sensitivity of these networks to changes in ceRNA abundance relative to the total miRNA pool.

Development. Further support for the importance of IncRNAs acting as ceRNA is provided by a recent study on muscle cell differentiation, a process modulated by miRNAs and subject to miRNA dysregulation in diseases such as myocardial infarction and Duchenne muscular dystrophy (DMD). Expressed in skeletal muscles, the IncRNA linc-MD1 (a long intergenic noncoding RNA) contains MREs for miR-133 and miR-135, which regulate transcription factors involved in myogenic differentiation and muscle cell integrity, including MEF2C and MAML1 (54). Knockdown of linc-MD1 resulted in a symmetric reduction of these two myogenic markers, whereas overexpression led to the expected increase in protein levels. Although linc-MD1 levels, as well as those of MEF2C and MAML1, were strongly reduced in DMD myoblasts, linc-MD1 overexpression restored MEF2C and MAML1 synthesis and partially rescued timing of the differentiation program. Importantly, these results suggest that ceRNAs may be dysregulated in tissues that exhibit aberrant differentiation, such as DMD. They also highlight the potential of therapeutic strategies that restore dysregulated ceRNA networks (Figure 2B).

Future perspectives

Beyond the examples discussed herein, recent advances in RNA sequencing technology have led to the discovery of thousands of IncRNAs that are upregulated in diseases, such as prostate (55), liver (56), and hepatocellular (26) cancers. A subset of these disease-
associated lncRNAs as well as other clinically relevant lncRNAs is shown in Table 1. Although the ability to identify lncRNAs that correlate with disease far outpaces the elucidation of mechanistic links, sequencing technologies also provide ways to help close this gap. Because lncRNAs exert regulatory function through molecular interactions, sequencing technologies have been adapted to support high-throughput mapping of the lncRNA interactome. RNA immunoprecipitation followed by sequencing (RIP-seq) identified thousands of cis- and trans-acting lncRNAs that associate with PRC-2 (57), and direct cross-linking of RNA-protein (CLIP-seq) interactions in vivo is a promising strategy to improve stringency of such protein-RNA interactome measurements (58). Just as these methods take a protein-centric view, chromatin isolation by RNA purification (ChIRP) takes an RNA-centric view, as a lncRNA can be isolated from a cross-linked pool of chromatin to retrieve and enumerate associated DNA sequences and protein (59). Emerging technologies for transcriptome-wide determination of RNA structure (e.g., parallel analysis of RNA structure [PARS]) provide complimentary information (60), allowing researchers to associate interaction domains with the underlying RNA structures. Collectively, these methods should help identify structural domains that allow lncRNAs to associate specifically with other proteins and chromatin (Figure 2A). It may soon be possible to test phenotypic consequences of these interactions in model organisms, such as zebrafish, using morpholino oligonucleotides that specifically target and suppress lncRNA domains (61). Such studies may help inform whether molecular interactions or cryptic small peptide coding capacity (15) give rise to lncRNA function.

Further characterization of the lncRNA interactome and follow-up functional studies may have at least two clinical implications. Initially, lncRNAs may serve as targets for clinical intervention. Although they may now be used as biomarkers for breast (28), hepatocellular (26, 62), liver (63), prostate (55), and lung (64) cancers, lncRNAs may also be targeted when dysregulated expression gives rise to disease or aberrant development. Antisense oligo therapy may be used to reduce toxic lncRNA overexpression, as in the case of Alu toxicity in the retina (Figure 2B). In addition, siRNA knockdown of Xist, a well-studied lncRNA that leads to epigenetic silencing of one female X-chromosome for gene dosage compensation (reviewed in ref. 65), can improve the development-

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<td>ANRIL</td>
<td>Binds PRC-1/2; represses INK4b-ARF-INK4a locus</td>
<td>Oncogenic: upregulated in prostate cancer; SNPs linked to heart disease, diabetes, cancers</td>
<td>21–25</td>
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<tr>
<td>H19</td>
<td>Binds PRC-2; plays a role in cell-cycle arrest</td>
<td>Oncogenic: overexpressed in hepatocellular carcinoma</td>
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<td>HOTAIR</td>
<td>Binds PRC-2 and LSD1; represses HOXD and other PRC-2 target genes</td>
<td>Oncogenic: overexpression promotes breast and colon cancer metastases</td>
<td>27–29</td>
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<tr>
<td>Kcnq1ot1</td>
<td>Binds G9a and PRC-2; represses imprinted genes at 11p15 locus</td>
<td>Hypomethylation observed in Beckwith-Wiedemann syndrome and cancers</td>
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<tr>
<td>Alu</td>
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<td>Gas5</td>
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<tr>
<td>MEG3</td>
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<td>Tumor suppressor: downregulated in numerous cancers</td>
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<td>Tumor suppressor: mediates repression of p53 target genes</td>
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<td>SRA</td>
<td>Binds NRs; NR coregulator</td>
<td>Oncogenic: upregulated and drives NR signaling in cancers</td>
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<td>BACE1-AS</td>
<td>Binds BACE1 mRNA; increases mRNA stability and BACE1 protein levels</td>
<td>AD: overexpressed in AD, leading to toxic overabundance of BACE1</td>
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<tr>
<td>HULC</td>
<td>Binds miR-372; results in CREB phosphorylation</td>
<td>Oncogenic: overexpressed in liver cancer</td>
<td>56</td>
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<tr>
<td>linc-MD1</td>
<td>Binds miR-133 and miR-135; regulates muscle-specific transcription factors</td>
<td>DMD: downregulated in DMD myoblasts</td>
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<tr>
<td>PTEN-P1</td>
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<td>Tumor suppressor: copy number losses in cancers, altering PTEN gene dosage</td>
<td>51–53</td>
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AD, Alzheimer's disease; GR, glucocortioid receptor; linc-, long intergenic noncoding RNA; NR, nuclear receptor.
tial competence of embryos cloned via somatic cell nuclear transfer (66). Beyond its potential benefits for human-assisted reproductive technologies (67), this study suggests that mRNA knockdown may be effective in cases where their overexpression leads to pathogenic epigenetic reprogramming.

lncRNAs may also find clinical application as tools for biotechnology. Characterization of the lncRNA interactome will reveal a wealth of linkages that may be required in order to obtain synthet- ical control over gene regulatory networks. Applying lncRNAs for control over chromatin modifications is a particularly interesting possibility, considering the importance of epigenetic memory in tumorigenesis, stem cell maintenance, and aging. lncRNAs that target chromatin-remodeling components to specific loci (Figure 2C) may be useful tools for controlling the state of cultured stem cells, as lncRNAs have already been shown to play important roles in maintaining pluripotency and driving differentiation programs. Furthermore, a challenge in regen- erative medicine is to restore aged tissues to a young-adult state without resetting the differentiation program to embryonic or postnatal developmental stages. As current efforts for tissue rejuvenation may be affected by the epigenetic signature of aged cells (68), synthetic lncRNAs may compliment existing strategies by reprogramming specific regions of the epigenome. The potential benefits for disease diagnosis and treatment as well as stem cell therapy and regenerative medicine certainly jus- tify ongoing efforts aimed at a deeper mechanistic understand- ing of lncRNA function.

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