

Multimodal Long Noncoding RNA Interaction Networks: Control Panels for Cell Fate Specification

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ABSTRACT Lineage specification in early development is the basis for the exquisitely precise body plan of multicellular organisms. It is therefore critical to understand cell fate decisions in early development. Moreover, for regenerative medicine, the accurate specification of cell types to replace damaged/diseased tissue is strongly dependent on identifying determinants of cell identity. Long noncoding RNAs (lncRNAs) have been shown to regulate cellular plasticity, including pluripotency establishment and maintenance, differentiation and development, yet broad phenotypic analysis and the mechanistic basis of their function remains lacking. As components of molecular condensates, lncRNAs interact with almost all classes of cellular biomolecules, including proteins, DNA, mRNAs, and microRNAs. With functions ranging from controlling alternative splicing of mRNAs, to providing scaffolding upon which chromatin modifiers are assembled, it is clear that at least a subset of lncRNAs are far from the transcriptional noise they were once deemed. This review highlights the diversity of lncRNA interactions in the context of cell fate specification, and provides examples of each type of interaction in relevant developmental contexts. Also highlighted are experimental and computational approaches to study lncRNAs.

KEYWORDS long noncoding RNAs; miRNAs; competing endogenous RNAs; k-mers; cell fate specification

LINEAGE specification decisions in early development provide a blueprint of the body plan in multicellular organisms. Model systems such as embryonic stem (ES) cells are often employed in the study of early cell fate decisions. Understanding cell fate is also critical for regenerative medicine, as cell-based approaches pose significant therapeutic promise. Toward this end, induced pluripotent stem (iPS) cells, which display characteristics of ES cells, and can be patient-derived, have the potential to be differentiated into a myriad of different cell types.

Understanding determinants of cell fate is critical both for understanding early development, and to guide lineage commitment of pluripotent stem cells to enable the replacement

of diseased cell types in patients. While central transcriptional regulators of pluripotency including OCT4, SOX2, and NANOG, which maintain the pluripotent state, and specification factors such as SOX1, MEOX1, and SOX17 (Kan *et al.* 2004; Shimoda *et al.* 2007; Wang *et al.* 2013) are relatively well understood, many key cell-fate determinants remain functionally undefined. Importantly, recent developments in transcriptomics have demonstrated that, although the majority of the mammalian genome is transcribed, protein coding sequences amount to <2% of transcribed genomic sequence (Dinger *et al.* 2008; Alexander *et al.* 2010; Harrow *et al.* 2012), with the number of noncoding RNA (ncRNA) genes equaling, or possibly even outnumbering, protein-coding genes based on estimates from GENCODE and FANTOM (Hon *et al.* 2017; Frankish *et al.* 2019). Already, certain noncoding transcripts, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs; designated as transcripts >200 nt) have been implicated in cell fate decisions for unspecialized cells, including pluripotent stem cells; however, the vast majority of ncRNAs remain understudied.

Using pluripotent cells and their derivatives for illustrations, this review centers on lncRNAs, with a focus on the

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multimodal interactions through which they regulate cell fate specification. These interactions identify lncRNAs as important factors in early developmental processes and suggest that they should be considered in the design of regenerative medicine strategies.

lncRNAs: Interactions as Functional Determinants

The presence and number of lncRNAs appear to correlate with organismal complexity, and their expression patterns show subcellular, cellular, and tissue specificity, which suggests context-dependent roles, particularly in the determination of cell fate (Mattick 2001). Different classes of lncRNAs are defined based on transcription direction and location (for example: sense, bidirectional, antisense, intronic, intergenic; Figure 1) relative to other genes (Mattick and Rinn 2015). While this location classification might be suggestive of mechanism, lncRNA genomic location does not always strictly dictate function (Mattick and Rinn 2015; Quinn and Chang 2016). On the other hand, intermolecular interactions with other RNAs, proteins and chromatin have revealed emerging functional themes, and demonstrated the far-reaching regulatory potential of lncRNAs. Here, we examine the implication of these interactions in cell fate determination and early developmental processes.

Much of the function of lncRNAs depend on their ability to base pair to other RNAs or DNA through conventional or Hoogsteen base pairing, to form complex intramolecular and intermolecular secondary and higher order structures (Mercer and Mattick 2013). The structures formed by lncRNAs regulate and direct interaction with RNA-binding proteins (RBPs) to regulate, negatively or positively, their cellular targets. These proteins are central to lncRNA mechanisms of action and regulation of their function (Rinn and Ule 2014).

Localization of a lncRNA transcript can be suggestive of its functional role and contribution to gene regulation. While cytoplasmic lncRNAs tend to function post-transcriptionally, many nuclear lncRNAs regulate gene expression at the transcriptional level (Rinn and Chang 2012; Mercer and Mattick 2013). Within the nucleus, expression of a gene requires chromatin decompaction, particularly in heterochromatic regions. The compaction state is determined by chemical modifications of nucleosomal histone proteins, controlled by histone-modifying enzymes. lncRNAs have been shown to interact with chromatin modifiers (readers, writers, erasers) and remodelers to facilitate changes in the chromatin's biochemical and accessibility landscape at specific gene loci through both *cis*- and *trans*-acting mechanisms (Figure 2) (Rinn and Chang 2012).

Additionally, lncRNAs have also been shown to have a number of post-transcriptional and cytoplasmic functions in many developmental processes. These include functioning in mRNA stability and translation regulation through protein, miRNA, and mRNA interactions (Figure 2) (Batista and Chang 2013; Yoon *et al.* 2013; Quinn and Chang 2016).

These interactions provide a basis for lncRNA functional classification, but can also be targeted to direct cell fate.

Interactions with proteins: chromatin regulation

The plasticity of pluripotent stem cells is related to the high ratio of euchromatin to heterochromatin (Gaspar-Maia *et al.* 2011), making more chromatin accessible to transcription factors, RNA polymerase, and other proteins necessary for transcription. Pluripotent stem cells also have a high proportion of poised chromatin (Fisher and Fisher 2011), which facilitates the rapid gene derepression required for lineage commitment. Extensive binding of lncRNAs to epigenetic regulators that control chromatin accessibility defines one category of lncRNA function (Mercer and Mattick 2013). Studies using ES cells and other cell types have shown that ~30% of intergenic lncRNAs were bound by at least one epigenetic regulator (Khalil *et al.* 2009), indicating widespread impact of lncRNAs on cell identity at the transcriptional level.

lncRNAs can act in *cis* by binding to neighboring genes and facilitating recruitment of chromatin modifier/remodelers to the target locus (Bassett *et al.* 2014). The act of lncRNA transcription can also have a *cis*-regulatory function in gene expression, and influence genome organization (Bassett *et al.* 2014; Engreitz *et al.* 2016; Melé and Rinn 2016). lncRNAs can function in *trans* as well, either by serving as a recruitment or scaffolding factor on which chromatin modifying proteins assemble, or by modulating the stability of the chromatin regulatory protein complex (Rinn and Chang 2012; Bassett *et al.* 2014).

Cis-regulatory lncRNAs control expression of neighboring genes. In the context of cell fate specification, these lncRNA genes are often located adjacent to key developmental regulators that determine cell fate and organismal development (Bassett *et al.* 2014; Engreitz *et al.* 2016; Melé and Rinn 2016). This mechanism is commonly used for antisense and divergent lncRNAs that are typically <5 kb from, and transcribed in the opposite direction relative to, a transcribed gene. For example, *Evx1as* and its neighboring protein-coding gene, *Evx1*, demonstrate highly correlated expression in murine ES cells (Luo *et al.* 2016). Depletion of *Evx1as* indicated unidirectional regulation of the protein-coding neighbor by the lncRNA where *Evx1as* bound to its own promoter and facilitated binding of Mediator to activate transcription at the locus (Luo *et al.* 2016). This example highlights how lncRNAs can act in *cis*, tethered to their promoter, to modify gene expression near their transcription site.

Another illustration of *cis* influence of a lncRNA is exemplified by *Chaserr*'s regulation of *Chd2*—a chromatin remodeler with roles in cell differentiation in mice (Rom *et al.* 2019). *Chaserr*'s transcript is produced upstream of the transcription start site of *Chd2*, where it collaborates with CHD2 protein to repress *Chd2*'s expression in a negative feedback loop to maintain cellular levels of CHD2 (Rom *et al.* 2019).

*yy*lnc*T*—a member of the divergent subclass of lncRNAs known as yin yang (*yy*) lncRNAs—supports expression of its gene neighbor, *Brachyury (T)* by localizing to its locus during

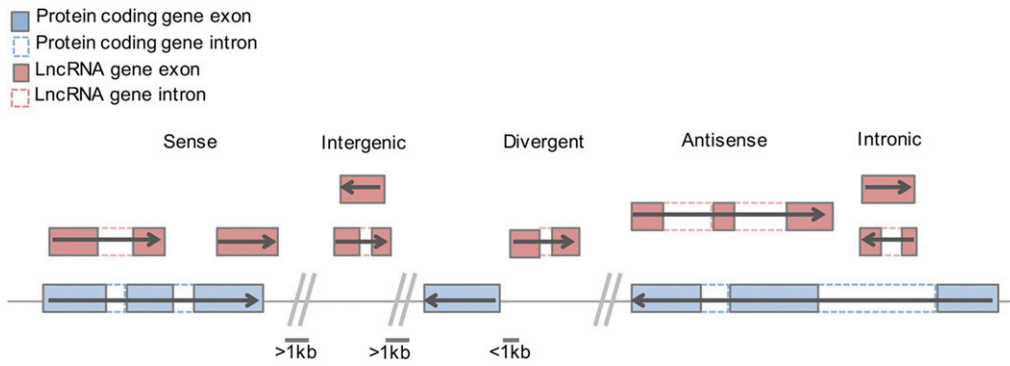


Figure 1 General principles illustrating lncRNA subtypes and genomic origin. lncRNAs may originate from various regions in the genome, including proximal, distal, and overlapping, with respect to protein coding genes. Sense and antisense lncRNAs may, or may not, fully overlap with protein coding genes. Divergent and intergenic lncRNAs are arbitrarily distinguished based on distance from the nearest protein coding gene.

mesoderm commitment in human ES cells (Frank *et al.* 2019). *yy*lncRNAs are primarily encoded from genomic loci of key cell-fate regulators, thus mirroring their developmental expression patterns, and, as a class, they illustrate a broad mechanism through which lncRNAs safeguard cell-fate decisions (Frank *et al.* 2019).

A handful of *cis*-acting lncRNAs are also known to repress gene expression over long genomic distances. In the most extreme example, the lncRNA *Xist* silences gene expression over the entire 165 million base pair X chromosome early during the development of female mammals, as part of the dosage compensation process called X-chromosome Inactivation (XCI) (recently reviewed by Sahakyan *et al.* 2018).

Xist induces stable gene silencing through two parallel pathways. In the first, *Xist* silences actively transcribed genes through an incompletely defined mechanism that involves the protein SPEN and the RNA element Repeat A, which is a tandem repeat located at the 5' end of *Xist*. In parallel, and subsequent to Repeat-A-mediated silencing, *Xist* induces the spread of Polycomb Repressive Complexes (PRCs) over transcriptionally inactive chromatin (Nesterova *et al.* 2019; Żylicz *et al.* 2019). In a mechanistic sense, this spread of PRCs over the X is likely a major means by which *Xist* orchestrates stable silencing that is inherited through subsequent cell divisions (Wang *et al.* 2001; Kalantry *et al.* 2006; Sahakyan *et al.* 2018). Other *cis*-repressive lncRNAs that depend on PRCs for their silencing functions, such as *Kcnq1ot1*, *Airn*, *Morrbid*, and *Haunt*, may utilize similar mechanisms to bring PRCs to chromatin (Regha *et al.* 2007; Terranova *et al.* 2008; Yin *et al.* 2015; Kotzin *et al.* 2016; Schertzer *et al.* 2019). Indeed, both *Kcnq1ot1* and *Airn* were recently shown to require the *Xist* cofactor HNRNPK to induce the spread of PRCs in mouse trophoblast stem cells (Schertzer *et al.* 2019).

In addition, the PRCs, particularly PRC2, have been shown to interact with many RNAs, and the functional consequence of this interaction has not always been clear. For example, the PRC2 component SUZ12 has been shown to interact with a lncRNA to repress a differentiation-inducing transcriptional program in human ES cells. Here, the lncRNA *tsRMST* uses multiple mechanisms, including coregulation with SUZ12 and NANOG, to block expression of lineage specification genes and impede WNT5A-induced epithelial-mesenchymal

transition (Yu and Kuo 2016). SUZ12 and the central pluripotency regulator SOX2 also interacts with *lncRNA_ES1* and *lncRNA_ES2* to contribute to pluripotency maintenance (Ng *et al.* 2012) through unclear mechanisms.

Other lncRNAs, at least partially through their interaction with PRC2, have been implicated in processes supporting lineage commitment. For example, the lncRNA *Braveheart* interacts with PRC2, and, perhaps in part due to a consequence of this interaction, *Braveheart* directs murine pluripotent cells to a cardiac fate by moderating a mesoderm and cardiac-specific transcription factor network (Klattenhoff *et al.* 2013). Nevertheless, through a specific structured element, *Braveheart* interacts with the CNBP/ZNF9 nucleic acid binding protein, and at least a portion of *Braveheart* function can be ascribed to the CNBP/ZNF9 interaction (Xue *et al.* 2016). Moreover, and surprisingly, even though depletion of *Braveheart* results in myogenic defects through its control of central cardiomyogenic regulators including *Mesp1*, *Hand1*, *Nkx2.5*, and *Tbx20*, and general loss of sarcomere gene expression, *Braveheart* null mice were grossly phenotypically normal (Han *et al.* 2018). Conversely, genetic ablation of the lncRNA *Fendrr*, which has also been shown to interact with PRC2, results in mouse embryonic lethality at around E13.75 due to myocardial defects (Grote *et al.* 2013). Here, it has been proposed that *Fendrr*'s interaction with both PRC2 and Trithorax Group/MLL complexes modulates chromatin signatures in control of lateral mesoderm differentiation (Grote *et al.* 2013). PRC2 function has also been reported to be regulated in *trans*, in mouse ES cells and human iPS cells, by the relatively abundant lncRNAs *Rian*, *Mirg*, and *Meg3/Gtl2*, which are produced from an imprinted cluster (Kaneko *et al.* 2014). Examples of these lncRNAs with clear roles for repressing transcription in *cis* also exist (Sanli *et al.* 2018). Additionally, many lncRNAs produced from the developmentally important *Hox* gene clusters also bind PRC2, the most notable of which may be the lncRNA *HOTAIR*. The extent to which lncRNA/PRC2 interactions in the *Hox* clusters contribute to gene regulation in mammalian development, whether the regulation occurs in *cis* or in *trans*, and what the mechanisms are, however, remain unclear (Li *et al.* 2013, 2016; Tsai *et al.* 2010; Amândio *et al.* 2016; Selleri *et al.* 2016; Portoso *et al.* 2017).

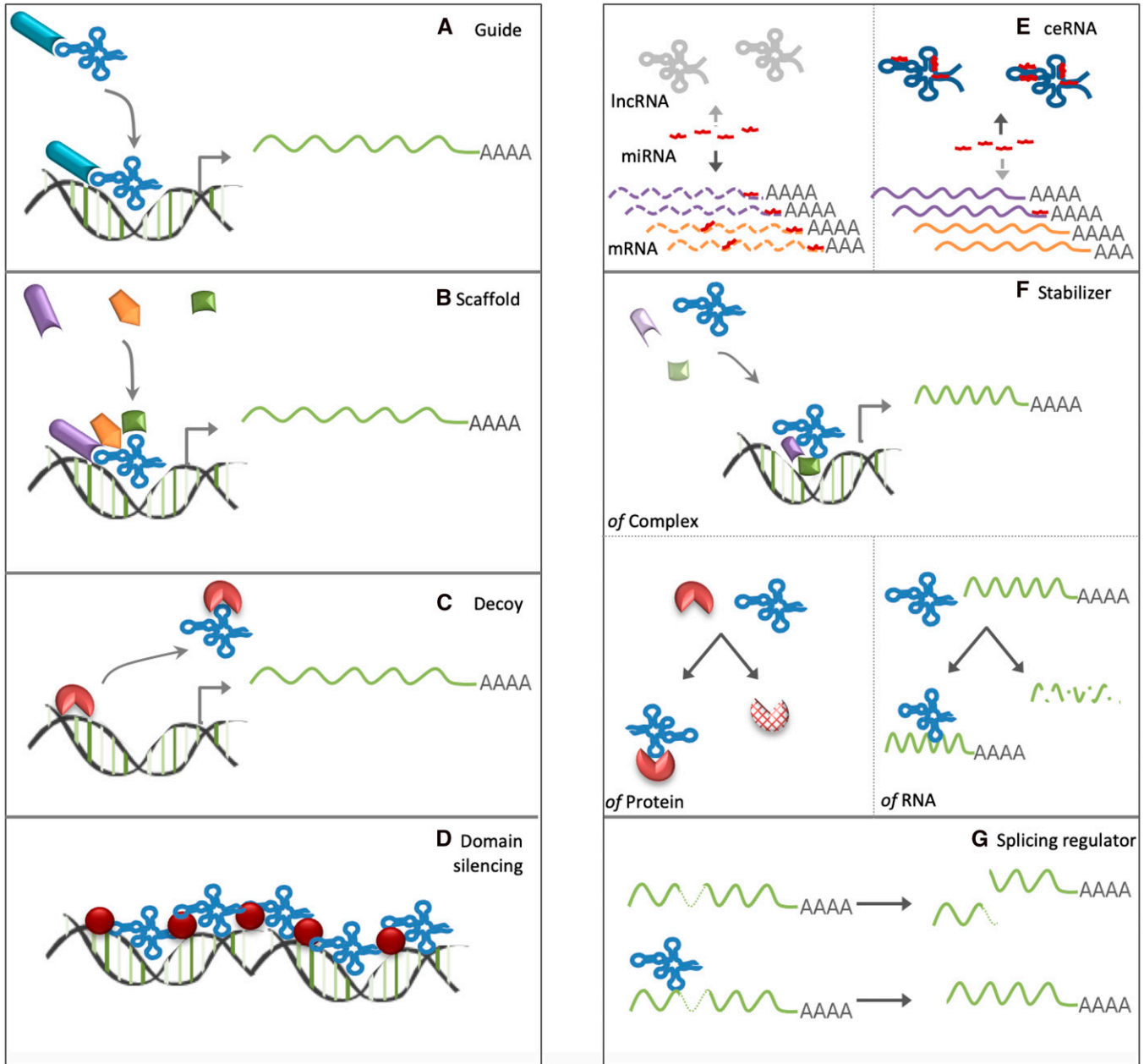


Figure 2 Schematic illustration of the different modes of action for lncRNAs. Localization of lncRNAs to the nucleus or cytoplasm can dictate different mechanisms of action. Based on their ability to bind to DNA and interact with proteins, lncRNAs can guide transcription regulators and epigenetic modulators (A); act as scaffolds to assemble chromatin regulatory factors (B); titrate away regulators of transcription by acting as decoys (C); regulate domain- or chromosome-wide chromatin state to regulate transcriptional output (D); act as ceRNAs to capture regulatory factors such as miRNAs away from target genes (E); contribute to the stabilization of protein complexes, proteins, and mRNAs (F); and influence alternative splicing (G).

Importantly, both crosslinking immunoprecipitation (CLIP) studies of PRC2 as well as those that have studied the RNA-binding properties of PRC2 *in vitro* have found that PRC2 binds RNA with little sequence specificity and nanomolar affinity (Kaneko *et al.* 2013, 2014; Davidovich *et al.* 2015; Wang *et al.* 2017). Collectively, these studies suggest that one function of lncRNAs, and perhaps chromatin-bound RNAs in general, is to tether PRC2 to transcriptionally active regions of chromatin. This tethering may keep PRC2 in a poised state, in close proximity to future target genes, where it can initiate

stable gene silencing upon receipt of the appropriate cues (Kaneko *et al.* 2013, 2014; Davidovich *et al.* 2015; Wang *et al.* 2017). High-affinity and nonspecific interactions with RNA may also govern PRC1 function in an analogous fashion (Bernstein *et al.* 2006; Bonasio *et al.* 2014).

lncRNAs have been shown to interact with a wide variety of chromatin modulatory factors. In addition to PRCs, these include histone methylases (Hendrickson *et al.* 2016). The H3K4 methylase MLL family is necessary for activating the expression of certain genes (Yang *et al.* 2014). WDR5 is a

protein-subunit of MLL, recruiting the complex to target sites for activation (Yang *et al.* 2014). WDR5 engages with several lncRNAs that have been implicated in the self-renewal of ES cells (Yang *et al.* 2014). When the RNA-binding site of WDR5 was mutated in mouse ES cells, rendering it unable to bind lncRNAs, WDR5 was significantly less stable (Yang *et al.* 2014). This loss of stability resulted in a severe decrease in H3K4me3 marks on the promoters of pluripotency-related genes, and a loss of the ES cell state in 50% of colonies (Yang *et al.* 2014).

Interactions between lncRNAs and chromatin readers are exemplified by the interaction between *DIGIT* and BRD3 in the regulation of endoderm differentiation (Daneshvar *et al.* 2016, 2019). Here, *DIGIT* supports BRD3 recruitment to H3K18ac at regions in the genome enriched during endoderm differentiation in human ES cells (Daneshvar *et al.* 2019).

In addition to chromatin remodelers and modifiers, analysis of lncRNA interactomes has also identified transcription factors as key players in lncRNA function. *Panct1*—a nuclear-functioning sense lncRNA transcribed from an intron of the gene coding for its protein interacting partner, TOBF1—exemplifies this type of *trans* interaction in mouse ES cells (Chakraborty *et al.* 2017). *Panct1* was shown to facilitate the binding of TOBF1 to pluripotency marker promoters by way of sequence-directed binding to Oct-Sox motifs, thus recruiting transcription factors such as Oct4 to promote target expression (Chakraborty *et al.* 2017). This interaction illustrates the role of a lncRNA in efficient binding of pluripotency-associated transcription factors to their target sites without direct interaction (Chakraborty *et al.* 2017).

The aforementioned examples demonstrate the breadth of lncRNA–protein interactions that influence transcription in developmental processes, and on which cell specification is at least partly dependent. Many of the described lncRNA interactions involve key transcription factors and epigenetic regulators that affect developmental progression. Modulation of specific lncRNAs could therefore be a viable avenue for specific regulation of target expression in developmental contexts.

Interactions with proteins: stability and sequestration

lncRNAs enhance or repress protein function through a variety of mechanisms including sequestration, binding support, and degradation, as in the aforementioned case of WDR5 and its reliance on lncRNAs for stability (Yang *et al.* 2014). Conversely, in other contexts, lncRNAs have been shown to be dependent on their protein partners for stability to carry out their functions, and regulate their half-life. This is the case for *lncR492*—a noncoding transcript that inhibits neural differentiation in mouse ES cells (Winzi *et al.* 2018). Knockdown of *lncR492* resulted in increased expression of neural markers such as *Pax6* and *Nestin* during differentiation. Proteomic analysis indicated that *lncR492* directly interacts with HuR—a mRNA binding protein with functions in the (de)stabilization of mRNA transcripts. Overexpression and knockdown of HuR

moderated the expression of *lncR492*, increasing and reducing the prevalence of the lncRNA, respectively—a pattern that suggests HuR supports the stability of the *lncR492* transcript. Finally, both *lncR492* and HuR positively influence WNT signaling, which has a known inhibitory effect on neural differentiation (Haegele *et al.* 2003), outlining the axis by which *lncR492* and HUR function (Winzi *et al.* 2018).

lncRNAs can impede protein function through binding and sequestration. In the context of pluripotency, the chromatin mark H3K56 acetylation activates core pluripotency-related genes and is required for the maintenance of the undifferentiated ES cell state. SIRT6 is a chromatin-binding protein that removes this chromatin modification and functionally represses pluripotency-related genes to promote exit from the stem cell state (Etchegaray *et al.* 2015). *LncPRESS1* functions as a molecular decoy for SIRT6, sequestering the protein, which binds to the 3'-end of the lncRNA in human ES cells. This interaction in the nucleus prevents SIRT6 from binding promoters of pluripotency-related genes and repressing transcription via deacetylation (Jain *et al.* 2016). Another regulatory interaction in this network is illustrated by P53, which antagonizes *lncPRESS1*, freeing SIRT6 to further repress pluripotency markers in human ES cells (Jain *et al.* 2016).

Emerging data indicate that regulatory lncRNA–protein interactions occur in specialized microenvironments that display characteristics of phase-separated particles (Hniz *et al.* 2017; Daneshvar *et al.* 2019). These liquid-like condensates are able to exchange molecules dynamically with their surroundings (Bergeron-Sandoval *et al.* 2016; Boeynaems *et al.* 2018; Lu *et al.* 2018). The preceding interactions demonstrate the role lncRNA–protein interactions play in integral processes throughout differentiation and development. Such interactions can be investigated as points of manipulation for control of differentiation processes, especially when they localize to distinct microdroplets, and when the mechanisms for the interactions have been clearly defined.

Interactions with RNA

Similar to the prevalence of lncRNA–protein interactions, lncRNA–RNA interactions are prolific and have widespread effects on cell identity. Interestingly, lncRNAs interplay with multiple other RNA types, from mRNA to other ncRNA, including miRNAs and circular RNAs. Through different mechanisms, these lncRNA–RNA interactions can affect lncRNA function through repressing or supporting downstream targets.

Canonical functions of mRNAs are determined by their availability and potential to be translated. In addition to long-characterized protein factors, the half-life of a mRNA is determined by various co- and post-transcriptional regulatory factors, including lncRNAs. lncRNA interaction with mRNAs or mRNA-regulatory factors can stabilize or facilitate the degradation of the mRNA molecules, increasing or decreasing translational output (Faghihi *et al.* 2008, 2010; Gong and Maquat 2011). For example, *Sirt1-AS* interacts with *Sirt1* mRNA to promote its stability, thereby inhibiting myogenic

differentiation in favor of myoblast proliferation in mice (Wang *et al.* 2016).

lncRNAs have also been shown to alter the translation output of mRNAs by affecting alternative splicing. A prime example of this mechanism of action is the interaction of *Zeb2-NAT*—an antisense lncRNA—with its sense transcript, protein-coding *Zeb2*. *Zeb2-NAT* was demonstrated to bind *Zeb2*'s 5' UTR, which contains an intron where the internal ribosome entry site for the ZEB2 protein resides (Beltran *et al.* 2008). Without protection of the first intron by lncRNA binding, ZEB2 protein levels are significantly diminished. In mice, *Zeb2-NAT* and ZEB2 prevent fibroblasts from being effectively reprogrammed to the pluripotent state, possibly by supporting senescence due to E-cadherin downregulation (Beltran *et al.* 2008; Bernardes de Jesus *et al.* 2018). Conversely, under conditions of decreased *Zeb2-NAT* expression, the mouse fibroblasts readily transitioned to ES cell-like cells in media conditions that support the pluripotent state (Bernardes de Jesus *et al.* 2018). Further, mouse ES cells in *Zeb2-NAT* knockdown conditions were able to maintain the pluripotent state in differentiation-inducing contexts (Bernardes de Jesus *et al.* 2018).

Perhaps even more impactful, based on their numerous targets, is the influence of lncRNAs on miRNAs. These small ncRNAs (~22 nt long) are key post-transcriptional regulatory factors that influence target transcript translational repression and/or degradation (Heinrich and Dimmeler 2012). miRNA function has been implicated in the establishment and maintenance of ES cell pluripotency and differentiation (Heinrich and Dimmeler 2012). Generally, miRNAs and associated proteins assemble to form the RNA-induced silencing complex (RISC), in which the miRNA serves as a guide to target specific mRNAs, which are degraded in proportion to the degree of complementarity with the miRNA (Gregory *et al.* 2005). lncRNAs can affect the efficiency of these processes as well, since they can dictate the abundance of individual miRNAs by supporting miRNA stability or by causing their degradation, for example through template-mediated degradation (Fuchs Wightman *et al.* 2018). Additionally, lncRNAs can alter miRNA function by behaving as a sponge or competing endogenous RNA (ceRNA) that sequesters the miRNA, thus preventing degradation of the miRNA target genes to support or promote exit from the stem cell state (Liu *et al.* 2014, see Table 1).

linc-RoR (Regulator of Reprogramming) exemplifies the ceRNA mechanism in the context of reprogramming and the maintenance of pluripotency. Deviation from precise *linc-RoR* levels results in the differentiation of human ES cells to mesoderm and/or endoderm if its levels are depleted, or the inability of cells to properly differentiate if *linc-RoR* levels are elevated. *linc-RoR* was shown to be a ceRNA for *miR-145-5p*, suppressing the miRNA's negative regulation of stem cell regulatory factors such as OCT4 and SOX2 (Loewer *et al.* 2010; Wang *et al.* 2013).

While *linc-RoR* guides reprogramming, *lncRNA-1064* was shown to support neural differentiation (Weng *et al.* 2018).

Knockdown of *lncRNA-1064* led to a decrease in neural lineage markers and reduced neural differentiation of mouse ES cells *in vitro* and *in vivo* in a murine teratoma model (Weng *et al.* 2018). Mechanistic analysis revealed *lncRNA-1064* contains multiple miRNA target sites, with the most favorable being for *miR-200c* (Weng *et al.* 2018). *lncRNA-1064*-mediated sequestration of *miR-200c* transcripts enables ZEB1/2 to reach their gene targets to signal for neural differentiation (Weng *et al.* 2018). This interaction is similar to that of lncRNA *AK048794*, which functions as a ceRNA with *miR-592* (Zhou *et al.* 2016). In mouse ES cells, *miR-592* was found to bind the 3' UTR of *FAM91A1*, reducing its protein levels and those of pluripotency regulators *Oct4*, *Sox2*, and *Nanog*, although the downstream mechanism is less clear. Altogether, these studies describe instances of lncRNAs functioning as ceRNAs, sequestering miRNAs, and preventing degradation of miRNA targets to either support or undermine pluripotency. Often, lncRNAs acting as ceRNAs can be a part of a multi-component network, such as the case of *AK048794* (Zhou *et al.* 2016).

Because of their prevalence and well-delineated mechanism, lncRNA control of miRNA levels and availability could be harnessed to modulate miRNA levels in therapeutic contexts, particularly for cases where a wholesale loss of the target miRNA would be phenotypically disadvantageous (Kleaveland *et al.* 2018). Effective ceRNA activity would require a minimum threshold level for the lncRNA. Therefore, one important consideration pertaining to ceRNA/sponge function may be the relative abundance of the lncRNA, miRNA, and mRNA target. This is because the number of miRNA and target molecules would typically outnumber that of a lncRNA (Palazzo and Lee 2015). Another key consideration is whether, and how, RNA structure changes in response to the binding of proteins, other RNAs, or even small molecule metabolites to regulate the activity of ceRNA, as this could influence access to specific sites/sequences on the RNA. This is an aspect of lncRNA regulation that has escaped sustained attention so far, but is likely to be an important aspect of ceRNA, and, more generally, lncRNA regulation.

Multimodal interactions

Since lncRNA functions can vary widely, it could be expected that their functions are multifaceted (Figure 3) and not necessarily dependent on a single mechanism even in a single cell type. Previously characterized as a lncRNA, *Tug1*, is produced from a highly conserved locus (Young *et al.* 2005), and is involved in many developmental processes including photoreceptor specification, axonal differentiation, and osteogenesis regulation, where some functions have been found to be mediated by protein interactions with key cell-fate regulators such as LIN28 (Young *et al.* 2005; Guo *et al.* 2018; He *et al.* 2018). While knockout mice are viable, they display sterility with complete penetrance due to defects in spermatogenesis (Lewandowski *et al.* 2019). Intriguingly, in addition to having two distinct noncoding functions, one of *Tug1*'s functions is dependent on an encoded micropeptide.

Table 1 Paradigmatic modes of lncRNA interaction and functional output using ES cells, differentiation and developmental processes as models for cell fate specification

lncRNA	Mode of action	Interactor(s)	Result	Reference
Airn	Transcriptional	Igf2r	Silences Igf2r cluster to guide development	Santoro <i>et al.</i> (2013)
AK028326	Transcriptional	Oct4	Positively regulates Oct-4 to promote self-renewal	Sheik Mohamed <i>et al.</i> (2010)
Apela RNA	Transcriptional	p53, hnRNPL	Interacts with hnRNPL to repress p53-induced apoptosis	M. Li <i>et al.</i> (2015)
Braveheart	Transcriptional	PRC2 (Suz12), MesP1	Regulates cardiac lineage commitment in ES cells	Klattenhoff <i>et al.</i> (2013)
Chaserr	Transcriptional	CHD2	Influences cell differentiation through regulation of Chd2	Rom <i>et al.</i> (2019)
Deanr1	Transcriptional	SMAD2/3, FoxA2	Recruits SMAD2/3 to the FOXA2 promoter to promote endoderm differentiation	Jiang <i>et al.</i> (2015)
DIGIT	Transcriptional	GSC, BRD3	Supports endoderm differentiation	Daneshvar <i>et al.</i> (2016)
Evx1as	Transcriptional	Evx1	Promotes Evx1 expression to regulate mesendodermal differentiation	Luo <i>et al.</i> (2016)
Fendrr	Transcriptional	PRC2, Trithorax group	Targets promoters for proper heart and body wall formation	Grote <i>et al.</i> (2013)
FIRRE	Transcriptional	CTCF	Preservation of silencing of inactive X chromosome	Yang <i>et al.</i> (2015)
Haunt	Transcriptional	HOXA	Inhibits HOXA expression and ES cell differentiation, whereas the Haunt locus is an enhancer for HOXA	Yin <i>et al.</i> (2015)
HERVH	Transcriptional	Oct4	Recruits Oct4 to maintain pluripotency	Lu <i>et al.</i> (2014)
HOTTIP	Transcriptional	WDR5	Binds WDR5 to activate developmental regulators	Wang <i>et al.</i> (2011)
LncPress1	Transcriptional	SIRT6	Binds SIRT6 to promote expression of pluripotency-related genes	Jain <i>et al.</i> (2016)
lncR492	Transcriptional	HuR	Associates with HuR to promote pluripotency	Winzi <i>et al.</i> (2018)
lncRNA_ES1	Transcriptional	SUZ12, SOX2	Interacts with SUZ12 and SOX2 to prevent differentiation	Ng <i>et al.</i> (2012)
lncRNA_ES2	Transcriptional	SUZ12, SOX2	Interacts with SUZ12 and SOX2 to prevent differentiation	Ng <i>et al.</i> (2012)
Meg3/Gtl2	Transcriptional	PRC2 (JARID2)	Regulates recruitment of PRC2 to chromatin in iPS cells	Kaneko <i>et al.</i> (2014)
Panc1	Transcriptional	TOBF1	Regulates recruitment of TOBF1 to Oct-Sox Motifs to support the pluripotent state	Chakraborty <i>et al.</i> (2017)
pRNA	Transcriptional	TIP5, TTF1	Interaction with TIP5, TTF1 contributes to heterochromatin formation required for differentiation	Savić <i>et al.</i> (2014)
RMST	Transcriptional	SOX2	Associates with SOX2 to regulate neural differentiation	Ng <i>et al.</i> (2013)
tsRMST	Transcriptional	PRC2, NANOG, WNT	Associates with PRC2, NANOG, WNT to repress differentiation	Yu and Kuo (2016)
TUNA (megamind)	Transcriptional	NCL, PTBP1, hnRNP-K	Associates with specified RBPs to activate pluripotency genes and neural differentiation genes	Lin <i>et al.</i> (2014)
yyT	Transcriptional	Brachyury	Regulates Brachyury (T) in mesoderm specification	Frank <i>et al.</i> (2019)
AK048794	ceRNA	miR-592	Sponges miR-592 to support pluripotency	Zhou <i>et al.</i> (2016)
Cyrano	ceRNA	mir-7	Inhibit mir-7 to support self-renewal	Smith <i>et al.</i> (2017)
H19	ceRNA	let-7 microRNAs	Modulates let-7 to impede muscle differentiation	Kallen <i>et al.</i> (2013)
HPAT5	ceRNA	let-7 microRNAs	Modulates let-7 to promote pluripotency	Durruthy-Durruthy <i>et al.</i> (2016)
linc-ROR	ceRNA	miR-145	Inhibits miR-145 suppression of self-renewal genes	Wang <i>et al.</i> (2013)
lncRNA-1064	ceRNA	miR-200c	Inhibits miR-200c to regulate neural differentiation	Weng <i>et al.</i> (2018)
MD1	ceRNA	miR-133, miR-135	Supports differentiation by inhibiting miR-133, miR-135	Cesana <i>et al.</i> (2011)
T-UCstem1	ceRNA	miR-9, PRC2	Maintains self-renewal by modulating miR-9 and PRC2	Fiorenzano <i>et al.</i> (2018)
HOTAIR	Scaffold	PRC2, LSD1	Originally proposed to coordinate PRC2 and LSD1 complexes for proper embryonic development, although contested in the more recent literature	Tsai <i>et al.</i> (2010), Li <i>et al.</i> (2013), Amândio <i>et al.</i> (2016), Li <i>et al.</i> (2016), Selleri <i>et al.</i> (2016), Portoso <i>et al.</i> (2017)
XIST	Scaffold	X chromosome, PRC2	Inactivates X chromosome for dosage control	(Brown <i>et al.</i> 1991)
Cyrano	Multimodal/Other	Stat3, signaling network	Supports ES cell maintenance	Smith <i>et al.</i> (2018)
Tug1	Multimodal/Other	Lin28A, Fragile X mental retardation protein	Regulates various differentiation processes including osteogenesis, neuronal differentiation and spermatogenesis	Young <i>et al.</i> (2005), Guo <i>et al.</i> (2018), 1; He <i>et al.</i> (2018), 1; Lewandowski <i>et al.</i> (2019)
Zeb2-NAT	Other	Zeb2	Facilitates Zeb2 processing to regulate EMT and pluripotency	Beltran <i>et al.</i> (2008); Bernardes de Jesus <i>et al.</i> (2018)

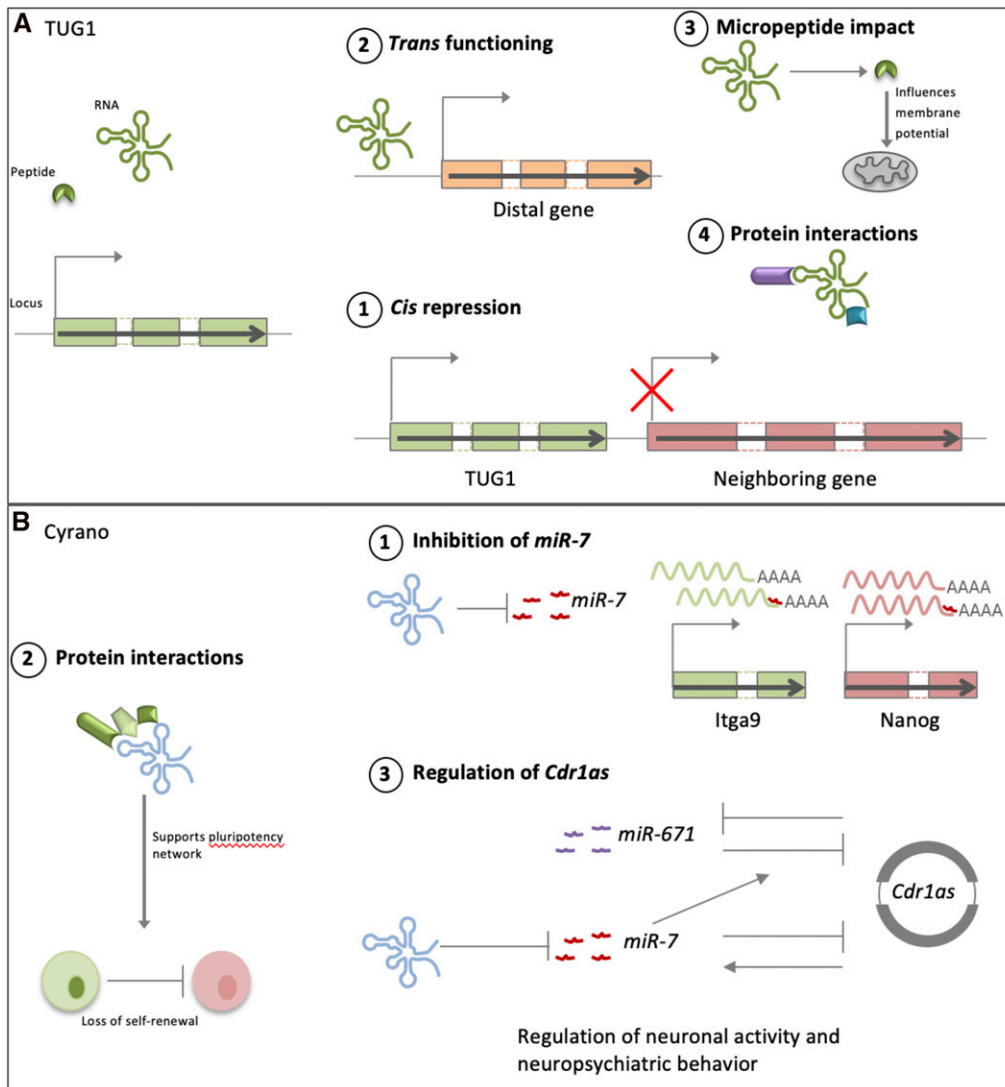


Figure 3 By engaging in diverse interaction patterns, a single lncRNA can impact multiple cellular processes. lncRNAs harness different mechanisms and access multiple networks of interacting partners in a context-specific manner. (A) *TUG1* can (1) regulate neighboring genes in *cis*, (2) function in *trans* to regulate target genes, (3) be translated into a micropeptide that regulates mitochondrial membrane potential, and (4) interact with proteins such as Lin28A to regulate various cell fate decisions. (B) *Cyrano*'s multifaceted functions are illustrated by (1) its ability to inhibit miR-7-mediated repression of *Itga9* and *Nanog*, (2) interact with proteins to support maintenance of the self-renewing pluripotent state, and (3) function in a multi-RNA regulatory network to impact *Cdr1as* expression, and, ultimately, neuronal activity and neuropsychiatric behavior in mice.

Specifically, the *Tug1* locus represses downstream genes in a *cis*-manner, while *Tug1* RNA itself regulates genes that then are dysregulated upon knockout. Additionally, the 5' conserved region of the *Tug1* gene encodes a peptide, TUG1-BOAT that influences mitochondrial membrane potential (Lewandowski *et al.* 2019).

The lncRNA *Cyrano* (*OIP5-AS1*, *1700020I14Rik*, *linc-oip5*, *Oip5os1*) is another illustration of multimodal function. While lncRNAs generally display limited sequence conservation even between closely related species, a 300 nt region shows very high conservation in tetrapods, with ~100 nucleotides conserved between zebrafish and humans (Ulitsky *et al.* 2011). Uniquely, there is a sequence stretch that has nearly perfect complementarity to *miR-7* in all tetrapods examined, which regulates *miR-7* degradation (Kleaveland *et al.* 2018). The conserved sequence folds into a conserved secondary structure within which embeds the *miR-7* binding sequence and partially masks it by base pairing (G. Varani unpublished results). How the RNA structure affects protein

recruitment and degradation remains unclear, however, and it might be that the structure itself is incidental to *miR-7* degradation. Still, it provides tantalizing suggestions that secondary and higher order structure might be essential components of lncRNA regulatory activities.

Cyrano shows rare maternal and zygotic expression during early development (Karlic *et al.* 2017), and in various mammalian cells it is a proliferation regulator (Smith *et al.* 2017; Deng *et al.* 2018; X. Liu *et al.* 2018; Naemura *et al.* 2018). Using proteomic analyses, *Cyrano* was found to interact with a developmental/signaling protein network, through which it partially supports mouse ES cell characteristics (Smith *et al.* 2018). Further, *Cyrano* inhibition of *mir-7*, which targets the pluripotency regulator *Nanog*, as well as *Itga9*, contributes to regulation of stemness and cellular adhesion (Smith *et al.* 2017).

Cyrano has also been shown to exist in a multi-component RNA network with the circular RNA *Cdr1as* and several miRNAs, where it maintains appropriate *Cdr1as* levels in

the brain to control neuronal activity and neuropsychiatric behavior in mice (Piwecka *et al.* 2017; Kleaveland *et al.* 2018). However, despite displaying strong expression and significant molecular and cellular phenotypes, *Cyran* is an example of a lncRNA that does not show an overt developmental phenotype with differing knockout strategies (Han *et al.* 2018; Kleaveland *et al.* 2018), which raises the question of whether there could be compensatory mechanisms for its function.

LncRNA Properties that Underlie Their Intermolecular Interactions

Sequence

A challenge in the lncRNA field is identifying the function of a lncRNA based on analysis of its sequence content alone, which is most easily accessible. This challenge stems from the fact that most proteins interact with RNA through sequence motifs that are degenerate and have hidden structural preferences (Dominguez *et al.* 2018). Compounding the difficulty is that the order of protein-binding modules within a lncRNA is likely to be less important than the mere presence of the binding modules themselves. Thus, two lncRNAs may encode identical functions through different sequence solutions.

An additional obstacle lies in the fact that existing sequence alignment algorithms, which, in large part, have been designed to detect linear sequence relationships between evolutionarily related nucleic acid or protein species, often fail to detect significant homology between lncRNAs (Altschul *et al.* 1990; Rice *et al.* 2000; Edgar and Batzoglou 2006; Wheeler and Eddy 2013). In order to address this problem, Kirk and colleagues recently developed a method called SEEKR (Sequence evaluation through k-mer representation) to quantify nonlinear sequence similarity between lncRNAs (Kirk *et al.* 2018). Rather than evaluating similarity between lncRNAs based on the extent of linear sequence homology, SEEKR functions by counting the abundance of all possible combinations of sequence substrings at a given length, *k*, within a lncRNA, and then scaling these abundances by the extent to which they differ from the mean abundance of each k-mer in the group of lncRNAs being analyzed. The extent of nonlinear similarity between lncRNAs as defined by SEEKR was found to correlate significantly with lncRNA subcellular localization and with protein binding, although the ability to predict either of these two properties from k-mer content alone was minimal. Using a transgenic assay to monitor the ability of a lncRNA to induce *Xist*-like repression, it was found that k-mer content, but not linear sequence homology, strongly correlated with the ability of lncRNAs to induce this type of repression. In a subsequent study, Sprague and colleagues found substantial levels of nonlinear sequence similarity between functional domains in *Xist*, and domains in the lncRNA *Rsx*, a marsupial lncRNA that has been proposed to be a functional analog of *Xist* that arose through convergent evolution (Grant *et al.* 2012; Sprague *et al.* 2019). Collectively,

these data support the notions that different lncRNAs can encode similar function through different spatial arrangements of related, but not necessarily identical, sequence motifs, and that k-mer based classification provides an approach to detect such similarities. The weak-to-modest predictive power of k-mer content in most scenarios hints at ubiquitous and difficult-to-model roles for RNA structure in lncRNA function (Kirk *et al.* 2018). Nevertheless, k-mer based classification schemes, which have been broadly used in other biological contexts (Blaisdell 1989; Burge *et al.* 1992; Kari *et al.* 2015; Lees *et al.* 2016; Pandey *et al.* 2018), represent promising avenues that may ultimately aid in the functional classification of lncRNAs from sequence content alone, much in the way that functional domains can now be routinely identified in proteins (UniProt Consortium 2015).

Structure

LncRNAs function through sequence-specific interactions with proteins that recognize stretches of sequence, as well as with other RNAs or DNAs by base pairing through Watson–Crick or Hoogsteen structures or by forming triple helices. However, intermolecular recognition is often dependent on, or regulated by, specific secondary and tertiary structural features of the lncRNA. Intriguingly, when chemical modification techniques have been used to probe lncRNA structure, it has generally revealed high levels of base pairing, more than in mRNAs and comparable to the ribosome or self-splicing introns—contexts in which secondary structure is complex and essential (Somarowthu *et al.* 2015; Hawkes *et al.* 2016). Conversely, in cells, RBPs such as hnRNPs (Dreyfuss *et al.* 1993) might keep lncRNAs less tightly folded. Furthermore, the observation of secondary structure does not necessarily imply function, especially in the absence of clear evolutionary conservation through covariation, as observed for the ribosome. Similarities shared with the ribosome and RNA enzymes could indicate a lncRNA architecture composed of structured domains, possibly flexibly connected, that establish interactions with other RNAs, chromatin, or specific protein complexes to bring them within functional proximity. While this modular structure hypothesis is appealing because it would provide for intricate functional specificities even in the absence of sequence conservation, it remains to be investigated at the molecular level.

It also remains to be investigated the extent to which secondary structures are functional, and whether they coalesce to form tertiary and higher order structures and interactions. Thus far, relatively few lncRNAs have been characterized at the secondary structure level, including *H19* (Hurst and Smith 1999; Juan 2000), *Xist* (Wutz *et al.* 2002; Fang *et al.* 2015; Lu *et al.* 2016; Smola *et al.* 2016; F. Liu *et al.* 2017), *Braveheart* (Xue *et al.* 2016), *HOTAIR* (Somarowthu *et al.* 2015), *COOLAIR* (Hawkes *et al.* 2016), or *lincRNA-p21* (Chillón and Pyle 2016) and several others.

A few studies indicate nevertheless that lncRNA structure is important for regulation, and the principles learned through these examples might be more broadly applicable to other

lncRNAs. One study showed that regulation of transcription of the *E-cadherin* gene is regulated by a sense lncRNA in epithelial cells that is independently transcribed upstream of the promoter (Pisignano *et al.* 2017). The structure of this RNA is controlled by a SNP that modifies local RNA secondary structure and affects loading of epigenetic enzymes that then regulate the downstream promoter. How common regulation through conformational switching remains unclear, but RNA is structurally malleable and physically well-suited for this mechanism of gene regulation. Riboswitches, for example, are RNA structures that toggle between distinct conformational states upon binding of small molecules and are widespread in bacterial gene regulation (Mandal and Breaker 2004). Riboswitch-like mechanisms of regulation might be present in lncRNAs as well, but their prevalence and function remain to be investigated. A study indicates regulation by conformational switching is provided by the *roX* lncRNA, which targets the MSL complex to the *Drosophila melanogaster* X chromosome as part of the dosage compensation process that occurs in male flies. MSL is recruited to *roX* lncRNA by a conserved stem-loop structure; once bound by the MSL-component MLE, this stem-loop unfolds to form an alternate RNA structure that appears to trap MSL on *roX* (Ilik *et al.* 2013, 2017; Quinn *et al.* 2016). In the case of *Xist*, structured regions, but also regions notable for their absence of structure, likely serve to recruit different subsets of proteins along the length of the lncRNA (Wutz *et al.* 2002; Fang *et al.* 2015; Smola *et al.* 2016; F. Liu *et al.* 2017). Here, toggling between different conformational states might define the subset of proteins that associate with the lncRNA under distinct cellular conditions.

Resource Toolkit for lncRNA Interaction Profiling

RNAs are largely dependent on proteins for their production, processing, transport, and localization. Based on reagent availability and ease-of-study, approaches to investigate RNA-protein interactions have historically been protein-centric. As the diversity and functionality of lncRNAs emerged and expanded, these tools, including RNA-immunoprecipitation (RIP), CLIP and its variations, including HITS-CLIP, PAR-CLIP, iCLIP, and Fast-iCLIP, have begun to reveal the magnitude of protein-lncRNA interactions (Ule *et al.* 2005; Hafner *et al.* 2010; König *et al.* 2010; Flynn *et al.* 2015; J.-H. Li *et al.* 2015; Zarnegar *et al.* 2016). Similarly, efforts are ongoing to define the complete repertoire of RNA-binding proteins using proteomics-based methods such as OOPS, R-DeeP, XRNAX, and DIF-FRAC, which often incorporate RNA dependency in their analysis (Mallam *et al.* 2018; Caudron-Herger *et al.* 2019; Queiroz *et al.* 2019; Trendel *et al.* 2019), and reveal surprisingly widespread RNA-dependent protein functionality, even for well characterized proteins such as CTCF (Caudron-Herger *et al.* 2019).

The expanding RNA functionalities highlight the need for RNA-centered methods to empirically determine binding partners of lncRNAs (Table 2). New computational resources

that enable queries on previously identified interactors, and those that allow for prediction of new interacting candidates have therefore seen remarkable growth in just the last few years.

Experimental methods

Capture hybridization analysis of RNA targets: Capture hybridization analysis of RNA targets (CHART) methodology allows for the identification of chromatin and protein interactors of lncRNAs. First, regions that are accessible for probe-based isolation are mapped using RNase H-dependent digestion. DNA oligonucleotides can then be used to isolate the RNA in an affinity purification step, followed by high-throughput sequencing to determine DNA segments contacted by the lncRNA, or mass spectrometry, to determine the protein interactome of the candidate lncRNA (Simon *et al.* 2011).

Chromatin isolation by RNA purification: Similar to CHART, chromatin isolation by RNA purification (ChIRP) or dChIRP (Chu *et al.* 2011; Quinn *et al.* 2014) uses a probe-based affinity approach built on biotinylated oligonucleotides that tile the lncRNA. Isolated interactors that bind the lncRNA (ChIRP), or bind to a specific domain (dChIRP), can be analyzed by high-throughput sequencing, or by methods that detect proteins, such as mass spectrometry or Western blotting.

RNA antisense purification: The RNA antisense purification (RAP) method (McHugh *et al.* 2015), instead of using short probes (~20 nt) as in CHART or ChIRP, utilizes longer probes of ~60 nt to increase the stability of the interaction in affinity pulldowns.

RNA pulldown: The above-mentioned methods to investigate lncRNA-partner molecules in intact cells were preceded by probe-based isolation of RNAs in cell extracts in pulldown experiments similar to protein coimmunoprecipitation.

Mapping RNA-genome interactions: Mapping RNA-genome interactions (MARGI) uses proximity ligation to connect chromatin-associated RNAs to their genomic targets, thus revealing native RNA-chromatin interactions. Variations in the approach were designed to differentiate between direct interactions (diMARGI), mediated by protein or RNA-tethered interactions, of RNA-DNA chimeras, or passive interactions (pxMARGI) (Sridhar *et al.* 2017).

Global RNA interaction with DNA by deep sequencing: Global RNA interaction with DNA by deep sequencing (GRID-Seq) harnesses *in situ* ligation to identify genome-wide contacts between RNA and chromatin. The developers of this method included mouse ES cells and found distinct *cis*- and *trans*- chromatin interacting RNAs tied to cell-specific gene expression patterns. GRID-Seq particularly enriches for chromatin interactions with nascent RNAs (Li *et al.* 2017).

Table 2 Methods to Investigate lncRNA function in cell fate determination

Approach	Type	Readout	Output
Differential expression analysis	Experimental	Indirect	Gene expression differences between cell types with differentiation, or in a condition of interest
Expression correlation (+/- target or effector)	Experimental	Indirect	Network generation to identify similarly expressed gene clusters, including candidate target molecules or possible upstream regulators
Affinity purification/proximity ligation and deep sequencing (CHART/ChIRP/RAP/MARGI/GRID-Seq/CHAR-Seq/Radicl-Seq/PIRCh-Seq)	Experimental	Direct	Chromatin targets
Affinity purification and mass spectrometry/Western blot (CHART/ChIRP/RAP)	Experimental	Direct	Protein interactions
Single molecule fluorescence <i>in situ</i> hybridization (smFISH) +/- immunofluorescence	Experimental	Direct	Subcellular localization and colocalization with targets or effectors
Conservation analysis	Experimental	Direct	Applicability of function across species
Structural analysis	Experimental	Direct	Secondary structure and functional domain identification; 3D structure
Bioinformatics tools	Computational	Indirect	Predict interactions, assess k-mer content, make structural inferences

Chromatin associated RNA sequencing: Chromatin associated RNA sequencing (CHAR-Seq) is an *in situ* proximity ligation approach coupled with enzymatic chromatin digestion to detect RNA–DNA contacts genome-wide. After sequencing, CHAR-Seq maps the genomic interacting sites of multiple classes of chromatin-associated RNAs including nascent transcripts, ncRNAs involved in regulation of dosage compensation, and *trans*-interacting RNAs involved in RNA processing (Bell *et al.* 2018).

RNA and DNA interacting complexes ligated and sequenced: RNA and DNA interacting complexes ligated and sequenced (RADICL-Seq) identifies genome-wide RNA-chromatin interactions in cross-linked nuclei. Thus far, it has been applied to two cell types—mouse ES cells and mouse oligodendrocyte progenitors—that can differentiate toward multiple cell fates. This approach revealed cell-type specific RNA–chromatin interactions, and was able to identify unique genome occupancy patterns for different classes of transcripts (Bonetti *et al.* 2019).

Profiling interacting RNAs on chromatin: Profiling interacting RNAs on chromatin (PIRCh-Seq) is an antibody-dependent approach that profiles RNA–chromatin interactions, with less enrichment of nascent RNAs cotranscriptionally tethered by RNA polymerases to chromatin. PIRCh-Seq has been used to identify the chromatin-associated transcriptome in both human and mouse ES cells and fibroblasts, as well as mouse neuronal progenitors, where the authors found cell- and allele-specific RNA–chromatin interactions (Fang *et al.* 2019).

Microscopy: As previously discussed, lncRNA localization can provide functional clues. The advent of single molecule imaging approaches facilitates the localization of RNA relative to other interacting molecules. For example, labeled FISH probes used in single molecule fluorescence *in situ* microscopy (smFISH) (Cabali *et al.* 2015; Dunagin *et al.* 2015), followed by immunofluorescence microscopy with three-dimensional

and quantitative fluorescence image analysis allows for the visualization of colocalized lncRNA and protein interactors within subcellular domains (Lino Cardenas *et al.* 2018). Variations, including merFISH (Chen *et al.* 2015) and seqFISH (Shah *et al.* 2016), depend on barcoding in sequential rounds of hybridization to enable the detection of many transcripts simultaneously. The resulting data complexity creates the need for computational tools such as trendsceek (Edsgård *et al.* 2018) to analyze these data. We can expect lncRNA monitoring in spatial transcriptomics to increase as different functionalities continue to emerge.

Bioinformatics

NPInter: Hao *et al.* (2016) is a repository of functional interactions between noncoding RNAs and interacting partners including small and large RNAs, DNA and proteins. At the core of NPInter is a manual curation process based on published literature, with a primary focus on experimentally verified physical interactions, supplemented with *in silico* predictions supported by high-throughput sequence data. The latest version contains >900,000 interactions between noncoding RNAs and other biomolecules from 22 organisms. For RNAs, accession IDs from NONCODE, Ensembl, and RefSeq are supported, and integration with the UCSC Genome Browser facilitates visualization of binding sites for human, mouse, and yeast genomes.

POSTAR: Hu *et al.* (2017), Zhu *et al.* (2018) is a database that enables exploration of post-transcriptional regulatory interactions, based primarily on ~1200 CLIP-Seq data sets. The aim of POSTAR is to contribute a better understanding of how RBPs impact post-transcriptional regulatory processes in six species. Its integration with the UCSC Genome Browser facilitates rapid visualization of RBP binding sites within transcripts.

RAID: The RAID database (Zhang *et al.* 2014; Yi *et al.* 2017), formerly CLIPdb, incorporates experimentally derived and

computationally predicted RNA interactions from the published literature, as well as other databases. RAID includes data for 60 species and >1.2 million individual RNA–protein and 4 million RNA–RNA interactions, respectively. A score that is based on the evidence supporting the interaction indicates the confidence in each interaction.

RNA–protein interaction prediction: Using protein and RNA sequence data, the family of RPISeq machine learning qualifiers (Muppurala *et al.* 2011) provides RNA–protein interaction probabilities. Different versions of the tool provide the probability of interaction between a specific RNA and protein, a specific RNA and up to 100 proteins, or a specific protein and up to 100 RNAs. Additionally, the sequence of a specific protein can be used to query the RPIntDB, which contains >30,000 individual RNA–protein interactions.

RNA–protein interaction predictor: RPI-Pred (Suresh *et al.* 2015) is a Support Vector Machine based prediction tool that uses RNA and protein sequence information and protein structural fragment data. Users can also test multiple candidates including assessing the potential of a single RNA to interact with multiple proteins or multiple RNAs interacting with a single protein.

StarBase: This RNA-centric resource (Yang *et al.* 2011; Li *et al.* 2014) details interactions between various classes of long and short RNAs, as well as between RNAs and proteins as extracted from CLIP-Seq (PAR-CLIP, HITS-CLIP, iCLIP, CLASH), degradome-seq, and RNA–RNA interactome data. Users are also able to impute downstream effects of these interactions based on accompanying gene expression data.

Combined approaches

Structure determination and analysis: The secondary structure of RNA can be predicted from thermodynamic principles, but inaccuracy in the parameters means that experimental input is required to generate a reliable model. This is most often provided in the form of constraints on secondary structure generated from either evolutionary considerations (conservation of base pairs, ideally by covariation) and/or direct experimental mapping of secondary structure using either enzymatic, or, most often, chemical techniques such as SHAPE and dimethyl sulfate (DMS) mapping (Kirk *et al.* 2018), psoralen crosslinking (Lu *et al.* 2016), and high throughput ligation followed by deep-sequencing (Ramani *et al.* 2015). Detection can be achieved efficiently through deep-sequencing, although capillary electrophoresis, and even polyacrylamide gels, can be used at much lower cost when studying single lncRNAs. Because folding *in vitro* and in cells might differ because of kinetic constraints on cotranscriptional RNA folding and the presence of RBPs (Leamy *et al.* 2016), techniques are being developed to probe RNA secondary structures in cellular contexts as well. Here, the primary limitation is sensitivity and the requirement to have sufficient RNA for detection, which could require overexpression since most

lncRNAs are present at relatively low copy number. Higher resolution methods such as SAXS (Small Angle X-ray Scattering) (Rambo and Tainer 2013) or X-ray crystallography and NMR currently have very low throughput and can be used only to investigate a few paradigmatic RNAs or systems of particularly high biological interest.

Conclusion and Perspectives

This review summarizes multiple lines of evidence showing that lncRNAs regulate cellular plasticity and cell fate determination, often through combination of multiple mechanisms. By adding a further layer of complexity to gene regulation, they broadly contribute to gene expression regulation to either (i) maintain a blanket undifferentiated state, (ii) promote exit toward a specified cell type, (iii) reprogram cells to a pluripotent ground state, or (iv) contribute to cell specification control in organismal development. LncRNAs perform these complex functions in integrated networks with a diverse set of cellular players with which they interact physically and/or functionally.

Progress toward the phenotypic assessment of lncRNA depletion occurs through loss-of-function and gain-of-function approaches (Liu and Lim 2018), facilitated by the advent of CRISPR/Cas9 technologies. Indeed, high-throughput screens using CRISPR interference identified >300 lncRNAs that impacted iPS cell growth, with a smaller subset influencing pluripotency maintenance as determined by OCT4 expression (S. J. Liu *et al.* 2017). Functional ablation approaches include poly-A signal insertion proximal to the transcription start site, although a drawback of this insertion is residual background expression, as well as deletion of the lncRNA locus, which results in total loss of lncRNA function, but which may also affect unannotated regulatory elements. Even the genetic manipulation of smaller sequences such as promoters or single exons for well-annotated intergenic lncRNAs should be carried out with caution to avoid modifying regulatory genomic sequences. It should also be noted that DNA-targeting approaches have resulted in differing phenotypes, as exemplified by *Fendrr*. Studies using a reporter gene replacement strategy for *Fendrr* found abnormalities in lung development and lethality at a later time point (Sauvageau *et al.* 2013; Lai *et al.* 2015), compared with the heart and body wall abnormalities resulting in prenatal lethality in earlier investigations (Grote *et al.* 2013). Regardless of the specific technical approach for DNA sequence manipulation, it will be important to study the impact of lncRNA expression ablation on the function and regulation of the interacting proteins, RNAs, and chromatin in development. Specific molecular targeting of the lncRNA itself using CRISPR/Cas13 (Abudayyeh *et al.* 2017; Cox *et al.* 2017) could facilitate such investigations.

Intriguingly, at least several lncRNAs displaying differing levels of conservation, such as *Malat1*, *Neat1*, *Cyranol*, *Braveheart*, *Evx1as*, and *Visc-2*, and found to have profound molecular or cellular functions, had no overt developmental phenotype in knockout animals (Han *et al.* 2018). This

suggests either a primary role for lncRNAs in fine-tuning developmental functions, distinct roles in specific cellular processes requiring situational study, or yet unearthed compensatory functions, potentially by related/familial lncRNAs. Another explanation for the absence of animal phenotypes could be off-target effects of knockdown approaches using RNA interference or antisense oligonucleotide-dependent depletion (Matsui and Corey 2017). Increased use of gene editing approaches such as CRISPR/Cas9 will help to clarify lncRNA functionality in cell and animal models.

Improvements are needed to allow study of lncRNA interactions at the single cell and single molecule level. While technically feasible to a limited extent using imaging technologies, these methods remain specialized and low throughput. These studies would allow the determination of cell fate as single cell expression and epigenetic studies have indicated substantial heterogeneity even within clonal cellular subpopulations. Perhaps dynamic lncRNA interactions contribute to this heterogeneity to dictate differing cell fates as well.

Related to this heterogeneity, it is still unclear whether a classification system that would allow prediction of lncRNA interactions will be found, but, if such a system existed, it is unlikely to be based on broad segments of sequence conservation because these are generally absent in lncRNAs. However, it might be possible to base classification on the identification of shorter sequence stretches (k-mers) or structural features of the lncRNA that facilitate interactions with other biomolecules. Identification of the relevant structural elements would provide insight into lncRNA interactions with noncanonical RNA binding proteins as well, including those without conserved and/or overt RNA binding domains.

There are untapped opportunities for progress in the mechanistic analysis of lncRNA function for better understanding of specific developmental processes and some downstream applications, including personalized therapeutics. For example, cancers typically progress through the acquisition of stemness features (Malta *et al.* 2018) and undifferentiated tumors have poor prognosis because they share immortality and repopulation capacity characteristics with stem cells. lncRNAs have emerged as central oncogenic and tumor suppressive factors involved in misregulated cancer pathways (Berger *et al.* 2018; Chiu *et al.* 2018), and many lncRNAs, such as *Cyrano*, have been shown to support cellular proliferation (Smith *et al.* 2017; Deng *et al.* 2018; X. Liu *et al.* 2018; Naemura *et al.* 2018). Studies of lncRNAs in stem cell contexts will not only enable better understanding of mammalian development and differentiation, but may also eventually facilitate better treatment of cancer and degenerative diseases.

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