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Global Positioning System: Understanding Long Noncoding RNAs through Subcellular Localization

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The localization of long noncoding RNAs (lncRNAs) within the cell is the primary determinant of their molecular functions. LncRNAs are often thought of as chromatin-restricted regulators of gene transcription and chromatin structure. However, a rich population of cytoplasmic lncRNAs has come to light, with diverse roles including translational regulation, signaling, and respiration. RNA maps of increasing resolution and scope are revealing a subcellular world of highly specific localization patterns and hint at sequence-based address codes specifying lncRNA fates. We propose a new framework for analyzing sequencing-based data, which suggests that numbers of cytoplasmic lncRNA molecules rival those in the nucleus. New techniques promise to create high-resolution, transcriptome-wide maps associated with all organelles of the mammalian cell. Given its intimate link to molecular roles, subcellular localization provides a means of unlocking the mystery of lncRNA functions.

Introduction

The most abundant yet least understood class of mammalian genes are the long noncoding RNAs (lncRNAs) (Ulitsky and Bartel, 2013). Their defining feature is a lack of protein-coding sequence (Derrien et al., 2012). Nevertheless, they are transcribed and spliced into long transcripts (>200 nt) that can be post-transcriptionally capped and polyadenylated, similar to protein-coding messenger RNAs (mRNAs) (Guttman et al., 2009; Derrien et al., 2012).

LncRNAs were first discovered in eukaryotes in the early nineties, with the imprinted maternally expressed transcript *H19* (Pachnis et al., 1984; Brannan et al., 1990). First classified as an mRNA, its lack of a long and conserved open reading frame (ORF) coupled with its lack of ribosomal interaction prompted the insight that *H19* was a non-protein-coding RNA (Pachnis et al., 1984; Brannan et al., 1990). Importantly, it was noted at the time that *H19* is localized to the cellular cytoplasm (Brannan et al., 1990).

The lessons of *H19* were somewhat overlooked amid discoveries of subsequent lncRNAs: in 1992, *XIST* (X-inactive specific transcript), responsible for X chromosome inactivation in placental mammals (Brown et al., 1992; Brockdorff et al., 1992); in 2002, *Airn*, responsible for imprinting of the *Igfr2* gene (Sleutels et al., 2002); in 2003, *MALAT1*, a promoter of metastasis (Ji et al., 2003); and from 2006, several lncRNAs encoded within HOX clusters that regulate gene expression either in *cis* (Petruk et al., 2006) or in *trans*, like *HOTAIR* (Rinn et al., 2007). One feature these share is a detected (or presumed) enrichment in chromatin and roles in regulation of histone modifications and gene transcription. These discoveries contributed to the widespread view of lncRNAs as nuclear-restricted epigenetic regulators (Khalil et al., 2009; Chen and Carmichael, 2009; Mercer and Mattick, 2013).

Since then, more than 100,000 lncRNAs have been mapped in the human genome (Cabili et al., 2011; Hangauer et al., 2013; Iyer et al., 2015; Ma et al., 2015; Uszczynska-Ratajczak et al., 2018; Guttman et al., 2009). LncRNAs have been linked to diverse biological roles and diseases, prompting rapid growth in their research (Esteller, 2011). Yet just a tiny fraction of lncRNAs has been functionally characterized in any detail, in the range of ~500–1,500 human genes (<2%) (Ma et al., 2015, 2018; Quek et al., 2015), and their roles continue to be elusive and controversial (Yan et al., 2012). Although some lncRNAs appear to function solely through the act of transcription (Kornienko et al., 2013; Latos et al., 2012; Anderson et al., 2016) or splicing (Engreitz et al., 2016), the majority of characterized examples seem to function as mature RNA molecules (Marchese et al., 2017). The challenge of deciphering the biological and disease roles of the tens of thousands of remaining lncRNAs introduces an acute need for methods to predict their functions or at least to classify them in a meaningful way.

We hypothesize that these questions can be addressed through the insight that lncRNA functions are intimately linked with their subcellular locations. Thus, this review is built around three questions: (1) To what extent are lncRNAs localized, and how does this reflect their cellular roles? (2) How can we develop comprehensive spatial maps of lncRNAs within the eukaryotic cell? (3) What are the factors that determine the localization of lncRNAs? A final aim is to re-evaluate views of lncRNA as solely chromatin-restricted transcripts, in light of growing volumes of evidence pointing to their important and widespread roles in the cytoplasm.

LncRNAs: Location and Function

Allocating RNA to subcellular domains is a fundamental regulatory mechanism of cells (Kuriyan and Eisenberg, 2007). The

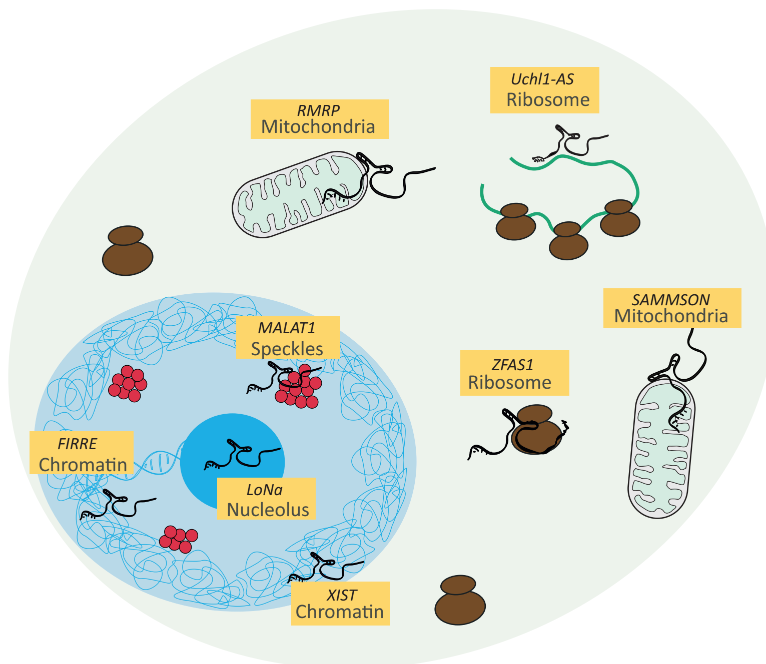

Figure 1. Examples of LncRNAs with Specific Subcellular Localization

original discovery of ''heterogeneous nuclear RNA'' (now known as messenger RNA, mRNA) noted its transport from nucleus to cytoplasm (Cobb, 2015; Georgiev et al., 1963; Sibatani et al., 1962; Georgiev, 1961). Asymmetric distribution of mRNA within the cell was first observed in 1983 in the context of ascidian development (Jeffery et al., 1983). Later, in 1986, Lawrence and Singer observed non-random distributions of mRNAs encoding cytoskeletal proteins in the cytoplasm, suggesting a mechanism for determining local protein concentrations (Lawrence and Singer, 1986). Since then, mRNA localization has been linked to diverse processes, from dampening gene expression noise (Bahar Halpern et al., 2015) to establishing morphogen gradients and contributing to cell fate determination (Driever and Nüsslein-Volhard, 1988; Kloc et al., 2002).

Although it was initially thought that subcellular localization was a property of a minority of RNA species (Bashirullah et al., 1998), it now appears to be the norm (Wilk et al., 2016). With new methodological advances, the estimate of RNA non-uniformly distributed in *Drosophila* models has increased from 10% to 70%–90%, suggesting that control of subcellular localization is a general regulatory principle of RNA (Dubowy and Macdonald, 1998; Tomancak et al., 2002; Benoit Bouvrette et al., 2018; Jambor et al., 2015; Lécuyer et al., 2007).

In contrast to protein-coding genes, lncRNA genes' ultimate product is RNA. Therefore, lncRNA functions depend on RNA physical interactions, which in turn depend on proximity (Voit et al., 2015). Thus, studying lncRNA subcellular localization and its dynamic changes is a crucial step toward elucidating functions and mechanisms of newly discovered lncRNAs (Chen, 2016).

LncRNA Subcellular Localization Is Finely Regulated

Messenger RNAs are generally exported to the cytoplasm, where they will be recognized and bound by ribosomes. In contrast, lncRNAs must find their way to one of many specific points of action and possible molecular interactors, inside or outside the nucleus.

Although the prevailing narrative describes lncRNAs as nuclear-enriched, epigenetic regulators (Khalil et al., 2009; Chen and Carmichael, 2009; Mercer and Mattick, 2013), subsequent research shows that the number and importance of cytoplasmic lncRNAs has been underestimated (Zeng et al., 2018; Mas-Ponte et al., 2017; van Heesch et al., 2014; Cabili et al., 2015; Carlevaro-Fita et al., 2016; Benoit Bouvrette et al., 2018; Ulitsky and Bartel, 2013). Using RNA fluorescence *in situ* hybridization (FISH) for direct visualization of RNA expression (described below), Cabili and colleagues observed complex localization for a set of 34 lncRNAs (Cabili et al., 2015), which could be divided into five categories: large nuclear foci, large nuclear foci with single molecules scattered through the nucleus, predominantly nuclear without foci, cytoplasmic and nuclear, and predominantly cytoplasmic (Cabili et al., 2015). Benoit Bouvrette and colleagues found ~75% of lncRNAs present in cytoplasmic fractions of human and *Drosophila* cells (Benoit Bouvrette et al., 2018). Finally, using RNA FISH and *Drosophila* models, Wilk and colleagues estimated a higher proportion of lncRNAs enriched in the cytoplasm (40%) compared to the nucleus (4%) (Wilk et al., 2016).

LncRNAs are observed in a wide variety of cell compartments and organelles. Within the nucleus, lncRNAs have been detected in nucleoli (non-bounded nuclear organelles in which ribosomal biosynthesis takes place), chromatin speckles (nuclear domains containing proteins associated with transcription and pre-mRNA maturation), and paraspeckles (heterochromatin domains involved in mRNA nuclear retention), where their expression dominates over mRNAs (Zhu et al., 2016; Li et al., 2018; Clemson et al., 2009; Mercer et al., 2008; Guttman et al., 2009; Derrien et al., 2012). In the cytoplasm, lncRNAs can be localized in mitochondria (Dong et al., 2017), ribosomes (Ingolia et al., 2011; van Heesch et al., 2014; Zeng et al., 2018; Carlevaro-Fita et al., 2016), extracellular membranes (Lin et al., 2017), and exosomes (Gezer et al., 2014) (Figure 1).

The primary definition of lncRNAs is their lack of a translated ORF, making their observation in association with ribosomes particularly unexpected (Guttman et al., 2013; Ingolia et al., 2011). These observations have been confirmed and extended by subsequent studies using the same (Ingolia et al., 2014; Zeng et al., 2018) or alternative approaches, like polysome profiling (van Heesch et al., 2014; Carlevaro-Fita et al., 2016). The latter studies quantified lncRNA-ribosome interactions, finding a large proportion of lncRNAs to be engaged by active ribosomes (van Heesch et al., 2014) and reporting lncRNA-ribosome interactions to be very frequent (Carlevaro-Fita et al.,

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Box 1. Global Analysis of Relative and Absolute LncRNA Localization

There are two alternative approaches to estimate RNA localization: absolute and relative (Figure 3A). The absolute localization refers to the ratio of molecules or mass of RNA between two compartments. Although more intuitive, absolute localization is rarely measured by global approaches, exceptions being FISSEQ and other FISH-based methods (see Table 1 for a comprehensive list). Rather, most global subcellular RNA maps are based on relative localization, because sequencing-based approaches evaluate the concentration of RNA. These consistently report that lncRNAs have a nuclear enrichment (Clark et al. 2012; Derrien et al. 2012). We repeat similar analyses here with ENCODE CeFra-seq data from nine cell lines, displaying the median cytoplasmic/nuclear value in Figure 3B (Djebali et al. 2012). The data may be interpreted as the ratio of concentration in cytoplasm and nucleus and clearly show that the average lncRNA has greater nuclear concentration across all cells in contrast to mRNAs.

We attempt here to systematically compare relative and absolute lncRNA localization using the same data. To do this, we have developed a new approach, ALEC (absolute localization estimation from CeFra-seq) for inferring absolute localization from matched CeFra-seq and whole-cell RNA-seq data. ALEC converts relative to absolute localization by estimating the total amounts of cytoplasmic and nuclear polyA+ RNA in a cell. It achieves this by modeling total RNA expression as the sum of nuclear and cytoplasmic concentrations (all in units of fragments per kilobase per million mapped reads, FPKM) (Figure 3C). Resulting coefficients for cytoplasmic (α) and nuclear (β) expression reflect the difference in absolute polyA+ content of the two compartments. These coefficients are then used to estimate the absolute localization of RNAs (Figure 3A, right panel; see Supplemental Information).

We ran ALEC in nine human cell lines for a total of 5,888 GENCODE v19 lncRNA (intergenic) genes and a similarly expressed set of 1,566 mRNAs (maximum FPKM across samples < 10). However, for each cell line, only the subset of expressed lncRNA and mRNA was included. The results are predictions of absolute localization for every lncRNA in each cell and may be interpreted as the ratio of molecules between the two compartments of an average cell. In six cell lines analyzed, the median lncRNA ratio is positive. In other words, it suggests that the average lncRNA in these cells has more copies in the cytoplasm than the nucleus.

It should be noted that (1) this analysis assumes that RNA-seq has no systematic biases in one or other of the compartments used and (2) these conclusions are provisional upon validation by more accurate single-cell/single-molecule approaches.

The full dataset of relative and absolute localization estimates is available at https://www.gold-lab.org/lncrna-characterisation.

2016). Nevertheless, the biological significance of lncRNA-ribosomal interactions remains unresolved. While a minority of lncRNAs probably encode undiscovered ORFs (Ruiz-Orera et al., 2014; Shang et al., 2015; Derrien et al., 2012), it is possible that a large fraction of lncRNA-ribosome interactions are not translationally productive and instead may reflect other processes, including degradation (Kurihara et al., 2009; Carlevaro-Fita et al., 2016; Zeng et al., 2018), regulation of bound mRNAs (Yoon et al., 2012), or simply off-target trafficking of capped and polyadenylated lncRNAs (Guttman et al., 2013).

In summary, lncRNAs have complex spatially regulated distributions within the cell, which point to underlying sequence-encoded localization signals and suggest that their roles go beyond the simple "act-of-transcription" functions.

Linking LncRNA Localization with Function

It follows from fundamental biochemistry that the local concentrations and hence subcellular localization of proteins and RNAs determines their molecular interaction network (Voit et al., 2015). Therefore, an RNA's location can yield valuable insights into its interaction partners and hence its cellular role (Figure 1). It can also have important consequences for experimental studies, as explained in Box 1. The iconic example of this is *XIST* (X-inactive specific transcript), which is localized in the heterochromatic Barr body (now recognized as the inactive X chromosome) (Brown et al., 1992), consistent with its role in dosage compensation (Penny et al., 1996). Similar yet opposite roles are played by the two roX lncRNAs in *Drosophila* (Ilik et al., 2017). Within the nucleus another example is *FIRRE*, which interacts with the nuclear-matrix factor hnRNPU and modulates nuclear architecture and *trans*-chromosomal interactions (Hacisuleyman et al., 2014).

Similarly, lncRNAs regulating gene transcription are detected in the chromatin fraction. Mondal and colleagues sequenced RNA associated with repressive chromatin. They identified *MEG3*, which targets the activity of transforming growth factor-b (TGF-b) pathway genes by RNA-DNA triplex formation (Mondal et al., 2015). Elsewhere in the nucleus, *NEAT1* localization not only marks but is necessary for the formation of paraspeckles (Clemson et al., 2009). Similarly, speckles—domains associated with pre-mRNA maturation—are characterized by localization of *MALAT1*, which is involved in regulation of alternative splicing (Sun et al., 2018; Tripathi et al., 2010). Enrichment in nucleoli, the site of ribosomal genesis, is suggestive of ribosomal-regulatory function (Scheer and Hock, 1999). Using this logic, the nucleolar-specific lncRNA *LoNA* was discovered and found to regulate transcription and methylation of ribosomal RNA (Li et al., 2018).

Cytoplasmic-localized lncRNAs are expected to perform roles appropriate to the cytoplasm. In accordance with this, lncRNAs that interact with microRNAs (miRNAs) tend to be enriched in the cytoplasm. *Linc-MD1* is proposed to modulate muscle differentiation timing by sponging two miRNAs, which in turn regulate muscle-regulatory transcription factors (Cesana et al., 2011; Shukla et al., 2011). Similarly, lncRNAs that regulate stability or translational efficiency of mRNAs are cytoplasmically and ribosomally enriched: *lincRNA-p21* negatively regulates *JUNB* and *CTNNB1* translation in HeLa cells, and *AdipoQ-AS* and *Uchl1-AS1* regulate translation of their antisense mRNAs (Cai et al., 2018; Carrieri et al., 2012; Yoon et al., 2012). LncRNA co-localization with ribosomes or endoplasmic reticulum (ER) can also reflect roles in translational regulation, such as for *ZFAS1*, which regulates mRNAs encoding proteins from the

ribosomal complex (Hansji et al., 2016). Nuclear-encoded lncRNAs that are localized in mitochondria may maintain nuclear-mitochondrial crosstalk or regulate cell respiration, such as *SAMMSON*, involved in ribosomal RNA maturation and mitochondrial translation (Mercer et al., 2011; Rackham et al., 2011; Noh et al., 2018; Vendramin et al., 2018; Dong et al., 2017). Finally, lncRNAs are also found in exosomes, poorly understood extracellular vesicles that are thought to mediate cell-to-cell communication via RNA cargoes (Chakrabortty et al., 2015). These tend to be enriched with miRNA seeds as well as RNA-binding protein (RBP)-binding domains, suggesting a possible role as miRNA sponges that transport miRNA to exosomes (Ahadi et al., 2016).

These localization patterns are not exclusive, and many lncRNAs are detected in more than one fraction or organelle. For instance, *RMRP* is found in the nucleus and the cytoplasm, and it has been shown to be a component of the mitochondrial RNase MRP complex as well as to interact with the telomerase reverse transcriptase (TERT) (Chang and Clayton, 1987; Maida et al., 2009). Another example is *lincRNA-p21*, which is nuclear enriched in mouse and regulates p53-dependent apoptosis. Interestingly, its human ortholog can be both nuclear and cytoplasmic and has gained cytoplasmic functions involving hypoxia-enhanced glycolysis and translation regulation (Huarte et al., 2010; Dimitrova et al., 2014; Bao et al., 2015; Yang et al., 2014; Yoon et al., 2012). However, lncRNAs are transcribed in the chromatin, and naturally they will always display a nuclear expression component, modified by transcription, export, and degradation rates (Chen and van Steensel, 2017).

In summary, there is growing evidence not only that lncRNAs have specific localization patterns, but that these patterns can point to useful hypotheses about their functions.

Dynamic Changes in LncRNA Localization

Similar to gene expression in general, subcellular localization of lncRNAs can be a dynamic process. *NORAD* and *SNHG1* lncRNAs display both nuclear and cytoplasmic distributions in human HCT116 colon cancer cells. However, upon DNA damage stress, they are retained in the nucleus (Munschauer et al., 2018; Shen et al., 2017). Interestingly, while *SNHG1* upregulation under normal conditions promotes cell proliferation in several cell types, upregulation coupled to nuclear retention inhibits proliferation (Shen et al., 2017). Nuclear *SNHG1* competes with p53 for binding to p53-negative regulator hnRNPC, leading to higher p53 levels and apoptosis (You et al., 2014; Shen et al., 2017). Another example is the mouse antisense lncRNA, *Uchl1-AS1*, which under rapamycin treatment shuttles from the nucleus to the cytoplasm. There it interacts with *Uchl1* mRNA and recruits ribosomes, promoting translation (Carrieri et al., 2012). In a similar way, during adipocyte differentiation, *AdipoQ-AS* translocates to the cytoplasm where it forms RNA duplexes with its antisense *AdipoQ* mRNA, repressing translation and inhibiting adipogenesis (Cai et al., 2018). Finally, lncRNAs can also be involved in the dynamic regulation of nucleo-cytoplasmic shuttling of proteins. For example, in resting T cells the cytoplasmic lncRNA *NRON* (noncoding repressor of NFAT) acts as a scaffold for a protein complex that traps the transcription factor NFAT, impeding its localization to the nucleus, after T cell activation (Willingham et al., 2005; Sharma et al., 2011; Tsao et al., 2013). Thus, lncRNA localization may be a mechanism by which regulatory networks respond dynamically to changing cellular states.

Relativity: LncRNAs Are Not Nuclear-Enriched in an Absolute Frame of Reference

An important source of confusion over RNA localization arises from how we define nuclear/cytoplasmic enrichment for polyA+ RNA (Mas-Ponte et al., 2017). First, it must be remembered that the total mass of RNA in the nucleus and cytoplasm of a single cell is not equal. In fact, the cellular cytoplasm tends to contain several-fold more molecules of RNA than the nucleus, and this amount probably varies by cell type (Mayer and Churchman, 2017; Carlevaro-Fita et al., 2016).

In bulk studies (described below), researchers separate cells into nuclear and cytoplasmic fractions and purify RNA, from which equal masses are analyzed in downstream quantification by PCR or RNA sequencing (RNA-seq) (Mayer and Churchman, 2017). Thus, the mass of transcript *X* per unit of mass of polyA+ RNA—in other words, the concentration of transcript *X*—is measured. When concentration levels are compared between cytoplasm and nucleus, one can learn about a cytoplasmic/nuclear (C/N) ratio based on relative concentration estimations (Figure 2). For RNA-seq, this may be expressed in units of relative concentration index (RCI), the $\log_2$-transformed ratio of fragments per kilobase per million mapped reads (FPKM). We term these quantification approaches "relative localization."

More intuitive, perhaps, is the alternative "absolute localization": the ratio of molecules of transcript *X* in the cytoplasm and nucleus (Figure 2). Differences in the overall amount of polyA+ RNA in the nucleus and cytoplasm will give rise to differences in localization estimated by relative or absolute methods, including instances of outright disagreement in whether an RNA is nuclear or cytoplasmic enriched (such as Cell 2 in Figure 2).

Given the difficulty in estimating absolute transcript levels within cellular compartments without information on initial amount of polyA+ RNA per fraction, almost all global studies to date have reported the relative localization (Clark et al., 2012; Derrien et al., 2012). The relative localization of lncRNAs tends to be more nuclear in human cells (median values shown in Figure 3B). However, when using the same data to predict absolute localization values (see Box 2), the median of lncRNAs in the majority of cell lines is actually cytoplasmic (Figure 3D). In other words, if one thinks in terms of absolute numbers of molecules in a single cell, then the average lncRNA has a cytoplasmic majority in most cell lines.

In conclusion, lncRNA localization goes beyond the nucleus, and the compartment preference of lncRNA transcripts can be assessed from different perspectives, leading to distinct results.

Technology for Mapping LncRNA Subcellular Localization

Transcriptome-wide subcellular RNA maps require high-throughput, sensitive, and accurate techniques. Traditionally, these have utilized two distinct strategies: oligonucleotide hybridization coupled to imaging, and biochemical fractionation coupled to RNA quantification. The former reveals the exact location of molecules within intact cells; the latter physically isolates fractions from a cell population and quantifies their RNA. More recent techniques combine the benefits of both

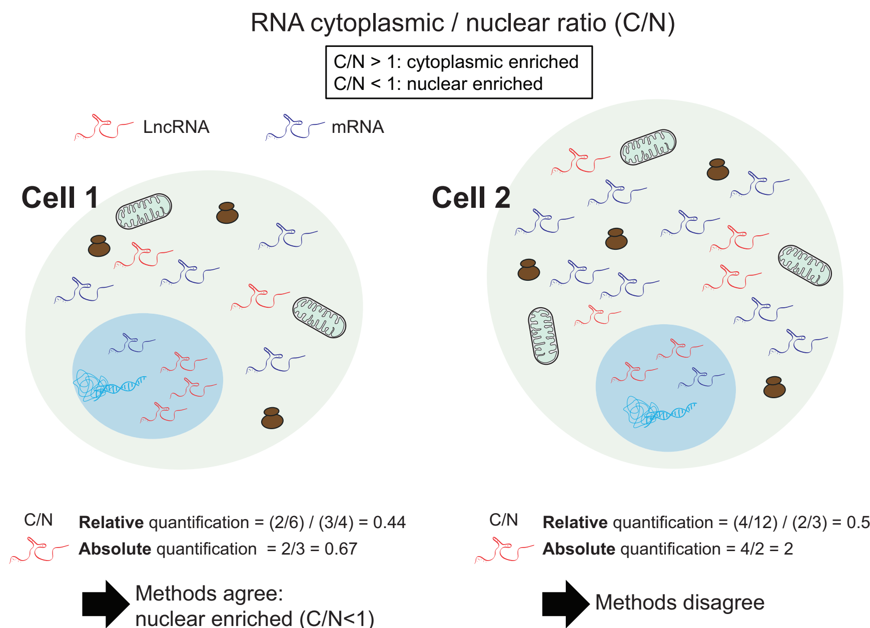

Figure 2. Two Ways of Quantifying RNA Subcellular Localization: Absolute versus Relative

Cells 1 and 2 contain different numbers of molecules of mRNA (blue) and lncRNA (red) in their cytoplasm and nucleus. Note that the mRNA and lncRNA indicate one specific transcript of each, for example, *GAPDH* and *RMRP*. Shown below are two different ways to quantify cytoplasmic/nuclear (C/N) localization of the lncRNA. The relative method calculates the ratio of concentrations in the two compartments. The absolute method calculates the ratio of molecules in the two compartments. For Cell 1, both measures agree that the lncRNA is nuclear enriched (C/N < 1). Conversely, notice how for Cell 2, the relative localization implies that the lncRNA is nuclear enriched (C/N_{rel} = 0.5), while absolute localization indicates cytoplasmic enrichment (C/N_{abs} = 2).

approaches, promising to rapidly improve the volume and quality of subcellular RNA maps.

LncRNAs have special characteristics that make their study particularly difficult. First, they tend to be lowly expressed: in bulk RNA, lncRNAs tend to be present at a steady-state concentration approximately one order of magnitude below mRNAs (Djebali et al., 2012), and many are quantified at less than one copy per cell (Seiler et al., 2017; Djebali et al., 2012). Second, lncRNAs have high coverage by repetitive sequences (Kelley and Rinn 2012), complicating their detection by both hybridization and bulk techniques. A summary of available lncRNA maps is shown in Table 1.

RNA In Situ Hybridization

In situ hybridization (ISH), amongst the oldest and most widely used methods for RNA localization, entails the incubation of intact cells with labeled complementary oligonucleotide probes to enable their visualization by microscopy (Hougaard et al., 1997; Puvion-Dutilleul and Puvion, 1996; Levsky and Singer, 2003; Gall and Pardue, 1969). Single-molecule fluorescence ISH (smFISH) employs multiple probes that hybridize in tandem and thereby amplify the fluorescent signal for detection of low-abundance targets (Raj et al., 2008; Femino et al., 1998; Khatun et al., 2013). RNA is indirectly visualized by fluorescence microscopy, and molecular counts can be inferred informatically from fluorescence images. Thus, smFISH provides absolute quantification of molecules in cellular compartments (Figure 2). Among other findings from smFISH, we have learned that lncRNA localization in the nucleus can either be diffuse, in foci, or part of speckles and paraspeckles, like *MALAT1* and *NEAT1*, respectively (Hutchinson et al., 2007; Tripathi et al., 2010; Cabili et al., 2015; Clemson et al., 2009).

Due to low expression and repetitiveness, lncRNAs are challenging targets for smFISH, resulting in low signal and high off-target detection, respectively (Cabili et al., 2015). In addition, the requirement for customized fluorescent probes makes smFISH time-consuming and expensive, and studies generally cover at best several tens of targets (Cabili et al., 2015; Mercer et al., 2008). Using digoxigenin (DIG)-labeled antisense RNA probes instead, and tyramide signal amplification (TSA)-based protocol, FISH has been scaled up to hundreds and thousands of targets (Lécuyer et al., 2008, 2007; Wilk et al., 2016). Overall, smFISH is considered the gold-standard technique for single-gene studies.

Biochemical Fractionation

Biochemical cell fractionation overcomes many of the drawbacks of ISH by physically isolating subcellular compartments and quantifying their RNA. It can be based on intact organelle purification, protein immunoprecipitation, or partition along sucrose gradients (Lee et al., 2010). RNA is then extracted and quantified using reverse-transcription polymerase chain reaction (RT-PCR) for individual genes or RNA-seq for high-throughputs (CeFra-seq: biochemical cell fractionation combined with RNA-seq) (Taliaferro et al., 2014). By aggregating across many cells, these approaches have high sensitivity for low-abundance transcripts (Djebali et al., 2012). RNA-seq can quantify the entire transcriptome, including unannotated lncRNAs.

With this approach, populations of lncRNAs have been mapped to chromatin fractions (Bhatt et al., 2012; Werner and Ruthenburg, 2015; Derrien et al., 2012), ribosomes (Ingolia et al., 2011; van Heesch et al., 2014; Carlevaro-Fita et al., 2016; Zeng et al., 2018), and exosomes and mitochondria (Noh et al., 2016; Li et al., 2018; Ahadi et al., 2016; Mercer et al., 2011), among other subcellular compartments (Table 1).

Fractionation-based methods are restricted by availability of appropriate isolation protocols and depend on the purity of resulting isolates (Sultan et al., 2014). Contamination across fractions can introduce technical noise and give systematically false locations. For example, cytoplasmic RNAs attached to the exterior of nuclear membranes precipitate with nuclei, becoming false-positive nuclear transcripts (Nathanson et al.,

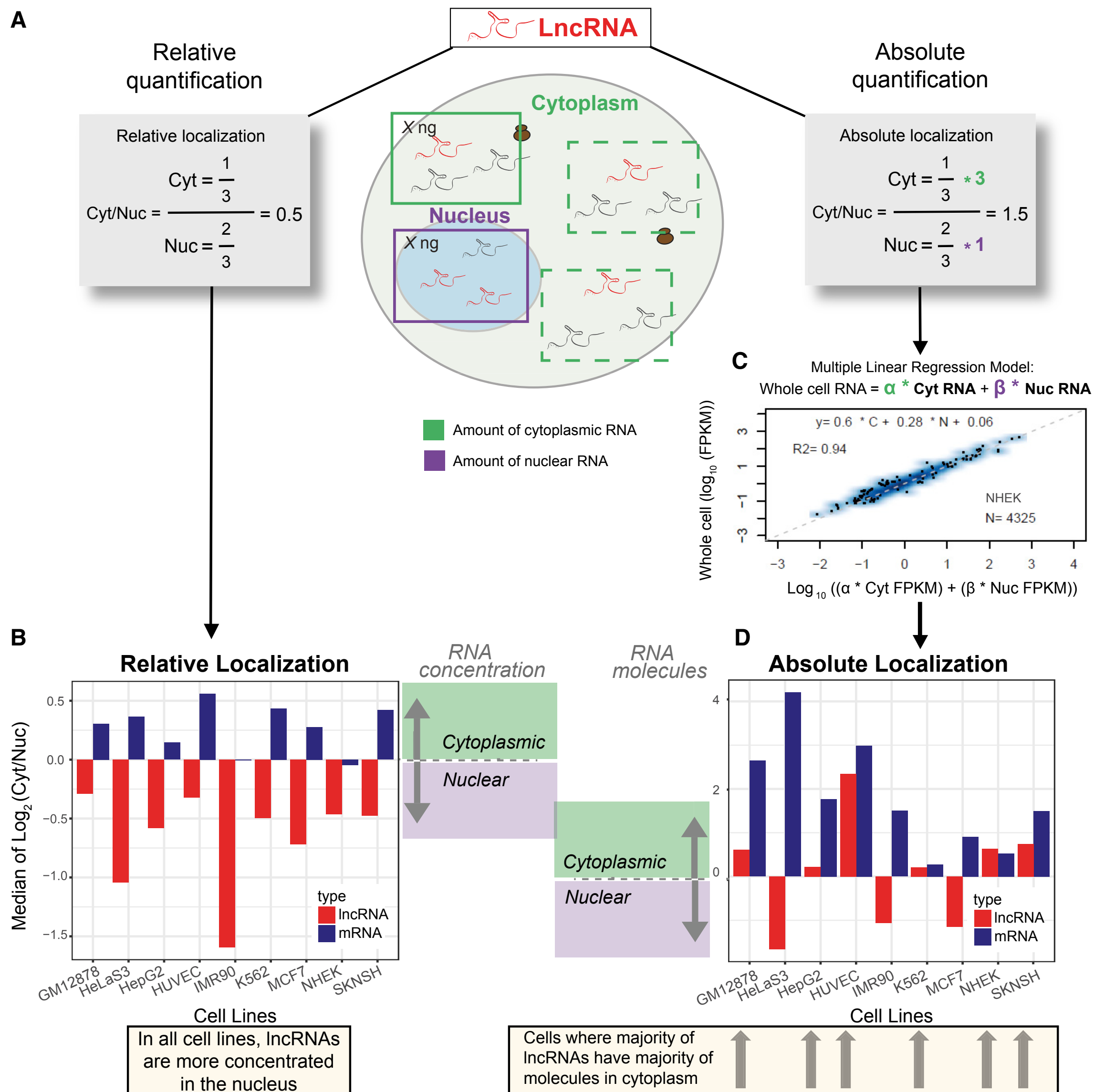

Figure 3. Comparing the Relative and Absolute Cytoplasmic/Nuclear Localization of LncRNAs in CeFra-Seq Data Using a New Approach

(A) A hypothetical cell has RNA molecules in the cytoplasm (cyt) and the nucleus (nuc), including a lncRNA of interest (red) and the remaining polyA+ RNA molecules (gray). Gray boxes contain calculations of cytoplasmic/nuclear localization (C/N) using the relative method (left panel, calculated using RNA concentrations) and absolute method (right panel, calculated using RNA molecule counts). For the absolute quantification, the number of molecules is counted by multiplying the concentration by a correction factor (green, purple), which accounts for the different total mass of polyA+ RNA in those compartments (represented by the colored rectangles in the cell). Specifically, there are three units of mass in the cytoplasm and one in the nucleus.

(B) Using ENCODE RNA-seq data from whole cell, cytoplasm, and nucleus (Djebali et al., 2012; Mas-Ponte et al., 2017), we estimated the relative C/N ratios for all expressed lncRNAs. This is equivalent to the relative concentration index (RCI) (Mas-Ponte et al., 2017). The median of this value, log_2 transformed, is shown, along with the equivalent for expression-matched mRNAs. For all cell lines, the median lncRNA value is negative, indicating that the concentration of lncRNAs is consistently greater in the nucleus.

(C) A multiple linear regression model was trained to estimate α and β coefficients that reflect differences in total polyA+ RNA content in the cytoplasm and nucleus.

(D) Similar data are shown as for (B), but now for absolute quantification. Absolute localization is estimated using the equation from (A) (right side) with the α and β coefficients estimated in (C). These values can be thought of as the ratio of molecules of lncRNA in the cytoplasm and nucleus. Again, the log_2-transformed value for the median lncRNA is shown, alongside equivalent mRNAs. For six indicated cells, the lncRNA value is positive. This indicates that, in these cells, more than half of lncRNAs have more than half of their molecules in the cytoplasm.

2003). Similarly, in unsynchronized populations, cells undergoing mitosis will release their nuclear RNAs as the nuclear membrane breaks down. This may give rise to cytoplasmic and ribosomal detection of lncRNAs such as *XIST*, which appear to be entirely nuclear in smFISH studies (Carlevaro-Fita et al., 2016; Cabili et al., 2015).

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Box 2. Practical Implications of LncRNA Localization

Subcellular localization also has practical implications. Genetic perturbations used to infer lncRNA functions by overexpression or knockdown must be effective in the same cellular compartment where the target lncRNA population is located. This is not always the case; therefore, phenotypes arising from the most widely used perturbation methods may be the result of off-target effects (Jackson et al. 2006). For example, conventional RNA interference (RNAi) approaches of siRNA or shRNA are effective on cytoplasmic RNA populations but often ineffective for nuclear molecules (Lennox and Behlke, 2016; Ntini et al. 2018; Sarshad et al. 2018; Stojic et al. 2018; Maamar et al. 2013). On-target perturbations observed using RNAi experiments may be driven by the cytoplasmic RNA population alone, while the nuclear population is unaffected (Lennox and Behlke, 2016; Stojic et al. 2018). Similarly, overexpression studies may often fail to rescue functions of nuclear lncRNAs when transcripts from transfected plasmids localize to the cytoplasm (Dimitrova et al. 2014; Shen et al. 2017). This may be avoided by the use of special nuclear-retained constructs (Shen et al. 2017). Such considerations will become particularly important as lncRNAs are studied as drug targets (Bonetti and Carninci, 2017; Leucci, 2018; Gutschner et al. 2013). Fortunately, the new generation of perturbation methods, such as antisense oligonucleotides (ASOs) and various CRISPR-Cas9 modalities, are effective in the nucleus (Ideue et al. 2009; Stojic et al. 2018).

Importantly, fractionation methods yield quantitative measurements. However, in most cases these quantifications provide only a measure of transcripts' relative concentration in cellular fractions (Box 1). This may explain the relatively low correlation between RNA smFISH and RNA-seq results, while RNA smFISH and qPCR correlate better (Cabili et al., 2015; Wilk et al., 2016).

In conclusion, fractionation provides quantitative and large-scale data.

APEX-RIP

Recently, innovative methods have been developed to overcome the deficiencies of conventional techniques. A recent elaboration of the fractionation approach, APEX-RIP (Kaewsapsak et al., 2017), combines APEX (engineered ascorbate peroxidase)-catalyzed proximity biotinylation (Rhee et al., 2013) and RNA immunoprecipitation (RIP) (Gilbert et al., 2004) to map RNAs at vastly improved spatial resolution (Kaewsapsak et al., 2017). Using gene fusion, this technique applies APEX-catalyzed proximity biotinylation to proteins from the cellular compartment under study. This is followed by protein-RNA crosslinking and RIP, pulling down biotinylated species (Kaewsapsak et al., 2017). In this way thousands of lncRNAs are mapped to specific compartments without the need for organelle-specific purification.

Kaewsapsak et al. recently mapped cytoplasmic and nuclear localization of hundreds of lncRNAs (Kaewsapsak et al., 2017). An extended series of compartment maps are described in a subsequent preprint article (Fazal et al., 2018). Apart from the advantages and disadvantages associated with RNA-seq discussed above, APEX-RIP offers good specificity and sensitivity in targeting the transcriptome of membrane-bound organelles. Moreover, it is possible to target several subcellular compartments with the same protocol, including fractions that may be difficult to isolate with traditional approaches. A particular drawback of APEX-RIP is the fact that it requires prior knowledge of protein markers of organelles of interest and genetically modified cell models.

Fluorescent In Situ *RNA Sequencing*

Other techniques seek to reconcile the dichotomy of *in situ* imaging and fractionation methods. The remarkable fluorescent *in situ* RNA-sequencing (FISSEQ) (Lee et al., 2014) harnesses a next-generation sequencing (NGS) flow cell to perform FISH, thereby achieving high-throughput absolute RNA localization. In this technique, RNA is reverse-transcribed into *in situ*-amplified, stably crosslinked cDNA amplicons, which are then sequenced using sequencing by ligation. Fluorescent probes are hybridized to the adaptor sequence and imaged using confocal microscopy (Lee et al., 2014).

In contrast to RNA smFISH, FISSEQ offers *in situ* information at high-throughput levels. However, this comes at the expense of lower read coverage compared to standard RNA-seq experiments, reducing sensitivity for lowly expressed RNAs. For example, Lee and colleagues sequenced 14,960 amplicons representing 4,171 genes in human fibroblasts, but only 6.9% mapped to noncoding RNAs (Lee et al., 2014). Furthermore, it is not clear whether FISSEQ can equally access all sites of the cell. Finally, another caveat is the fact that it requires customized instrumentation, which can be a limitation in several cases.

STARmap (spatially resolved transcript amplicon readout mapping) is a related technique that extends the principal to intact tissues, providing 3D positional information of RNA expression (Wang et al., 2018b).

Multiplexed Error-Robust Fluorescence In Situ *Hybridization*

To overcome the smFISH limitation of low-throughput coverage, Chen and colleagues developed MERFISH (multiplexed error-robust fluorescence *in situ* hybridization) (Chen et al., 2015; Moffitt and Zhuang, 2016). MERFISH uses combinatorial FISH-labeling fluorophores and sequential imaging, allowing single-cell FISH for hundreds of RNA species at the same time (Wang et al., 2018a; Moffitt et al., 2016a, 2016b; Chen et al., 2015). Co-localization of fluorophores across imaging rounds is used to identify the unique combination of signals of each transcript (Chen et al., 2015).

Although numerically it would be possible to address localization transcriptome wide, the resulting accuracy may not be high enough, as discussed by the authors. Moreover, it would represent large economic costs and hands-on time. Another limitation of the method is the optical diffraction limit. Authors reported low resolution in nuclear compartments given the high and dense fluorescence signal there (Chen et al., 2015). This has not been a problem for mRNAs, given their principally cytoplasmic nature. However, this is a bigger issue for mapping RNAs across intracellular compartments, as well as for studying nuclear lncRNAs.

Table 1. LncRNA Localization Resources

Method	Quantification Method	Quantification Type	Organelles	Single-Cell	Species	Reference
Fractionation	RNA-seq	Relative	Nucleus; cytoplasm; cytoplasmic membrane; insoluble cytoplasmic fraction	No	*Drosophila* (D17); human (HepG2)	Benoit Bouvrette et al., 2018
Fractionation	RNA-seq	Relative	Nucleus; cytoplasm	No	Human (HEK293T)	Sultan et al., 2014
Fractionation	RNA-seq	Relative	Chromatin; nucleoplasm; cytoplasm	No	Mouse (macrophages)	Bhatt et al., 2012
Fractionation	RNA-seq	Relative	Nucleus; cytoplasm; cytoplasmic membrane; insoluble cytoplasmic fraction; chromatin; nucleoplasm	No	Human cell lines	ENCODE
Fractionation	Microarray	Absolute	Cytosol; polysomes	No	Human (K562)	Carlevaro-Fita et al., 2016
Fractionation	RNA-seq	Relative	Nucleus; cytosol; mono- and polysomes	No	Human (LS-174T-pTER-β-catenin)	van Heesch et al., 2014
Fractionation	RNA-seq	Relative	Mitochondria	No	Human (143B cells)	Mercer et al., 2011
APEX-RIP	RNA-seq	Relative	Mitochondrial matrix; nucleus; cytosol; endoplasmic reticulum (ER)	No	Human (HEK293T)	Kaewsapsak et al., 2017
APEX-seq	RNA-seq	Relative	Nucleus; nucleolus; nuclear lamina; nuclear pore; cytosol; ER membrane; outer mitochondrial membrane; mitochondrial matrix; ER lumen	No	Human (HEK293T)	Fazal et al., 2018
MERFISH	Image analysis	Absolute	Cytoplasm	Yes	Human (IMR90)	Chen et al., 2015
FISSEQ	Solid sequencing by ligation	Absolute	Nucleus; cytoplasm	Yes	Human primary fibroblasts	Lee et al., 2014
Single-cell RNA FISH	Image analysis	Absolute	N/A	Yes	Human (hFF, hLFs and HeLa)	Cabili et al., 2015
FISH	Image analysis	Absolute	N/A	Yes	*Drosophila*	Khatun et al., 2013
Fractionation + FISH	RNA-seq + image analysis	Relative / absolute	Nucleus; cytoplasm	Both	Mouse (liver and MIN6 cell line)	Bahar Halpern et al., 2015

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Therefore, although promising, to date this approach has not provided high-throughput subcellular maps for lncRNAs.

RNA Zipcodes Specifying Subcellular Destinations

In recent years, the creation of transcriptome-wide subcellular RNA maps has become possible thanks to the techniques described above (Taliaferro et al., 2014; Kaewsapsak et al., 2017; Ji et al., 2003; Chen et al., 2015; Lee et al., 2014), and these maps have begun to reveal lncRNAs enriched in specific cellular locations (Table 1). The next logical question is how this localization is orchestrated genome-wide. To this aim, researchers have begun to systematically search for domains that trigger lncRNA subcellular localization (Lubelsky and Ulitsky, 2018; Shukla et al., 2018; Carlevaro-Fita et al., 2018).

Mechanisms Regulating the Major Membrane-Bound Separation: Nucleus versus Cytoplasmic Localization

While the mechanisms governing the cellular distribution of RNA species like mRNAs or transfer RNA (tRNA) have been the subject of study for ~30 years (Nakielny et al., 1997), research on lncRNAs has only recently begun (Zhang et al., 2014a; Lubelsky and Ulitsky, 2018; Shukla et al., 2018). Given that lncRNAs share post-transcriptional modifications with mRNAs that are critical for mRNA trafficking and nuclear export (Hautbergue, 2017), two fundamental questions are: (1) To what extent do the regulatory mechanisms discovered for mRNAs also apply to lncRNAs? (2) What mechanisms explain the distinctive aspects of lncRNA localization (including chromatin, nucleolus, speckles, and other specific patterns)?

During mRNA trafficking, transcripts are transported out of the nucleus via the nuclear pore complex (NPC), into the cytoplasm, and thence to the ribosome. This is mediated by *cis*-acting RNA elements termed "zipcodes" (Singer, 1993), which regulate RNA stability and localization. Zipcodes are composed of sequence motifs ranging from ~5 to hundreds of nucleotides, generally at the 3′ UTR of transcripts, that alone or in synergy recruit *trans*-acting proteins, creating ribonucleoprotein (RNP) transport particles (Kloc et al., 2002). For example, hnRNPA1 and hnRNPL proteins recognize UAGGG(A/N) and CA repeat motifs, respectively, and contribute to RNP formation and mRNA nuclear export (Chaudhury et al., 2010).

mRNA export requires mature transcripts that are spliced, capped, and polyadenylated (Valencia et al., 2008; Hamm et al., 1990; Köhler and Hurt, 2007). In the nucleus, proteins from the transcription-export complex (TREX) and the nuclear RNA export factor 1 (NXF1) facilitate the export of RNPs to the cytoplasm through NPCs (Hautbergue, 2017). Protein complexes such as the TREX complex specifically bind the CAP-binding complex, exon-junction complex, and other processing factors and thereby promote the transport of mRNAs (Hautbergue, 2017).

Many lncRNAs share these features, making plausible their export along similar pathways (Guttman et al., 2009; Derrien et al., 2012). Recent work from Viphakone and colleagues found lncRNAs interacting with NFX1 and TREX complexes (Viphakone et al., 2018). Interestingly, nuclear lncRNAs showed less NXF1 binding compared to cytoplasmic ones, as previously reported for the nuclear lncRNA *XIST* (Cohen and Panning, 2007; Viphakone et al., 2018). Both studies suggest that the lack of binding to the nuclear export adaptor of those lncRNAs may lead to nuclear retention, possibly explaining the elevated nuclear/cytoplasmic ratios of lncRNAs. The features of these lncRNAs that prevent their recognition by canonical transport machinery remain unknown.

Other evidence supports a model where lncRNAs and mRNAs share nuclear export pathways. The lncRNA *RMRP* and other noncoding RNAs use the same alternative protein-export receptor (CRM1) as some mRNAs (Williams et al., 2018; Noh et al., 2018). *RMRP* binds the HuR protein, which shuttles from nucleus to cytoplasm in a CRM1-dependent manner, mobilizing RNA molecules in the process (Brennan et al., 2000; Noh et al., 2016; Williams et al., 2018). Once in the cytoplasm, RNP particles can be further transported to specific locations, ensuring mRNA localization to its site of translation. For example, the chicken ZBP1 *trans*-acting protein binds *β-actin* mRNA in the nucleus through a 54-nt-long zipcode containing a tandem repeat of an ACACCC motif and localizes the RNA to the cell periphery (Chabanon et al., 2004; Kislauskis et al., 1994).

LncRNA-Specific Localization Mechanisms

For those lncRNAs in the cytoplasm and nucleus that display localization patterns distinct from mRNAs, alternative localization mechanisms must exist. RNA:protein, DNA:RNA, and RNA:RNA interactions may actively anchor lncRNAs to nuclear regions, impede the binding of export factors, or differentially target lncRNA stability.

Evidence for lncRNA sequence motifs driving nuclear localization is emerging from single-gene studies. Zhang and colleagues identified an AGCCC motif responsible for nuclear localization of *BORG* lncRNA (Zhang et al., 2014a). Intriguingly, the motif also correlates with nuclear localization of other lncRNAs and mRNAs. Miyagawa at al. described several large fragments (~1 kb) of *MALAT1* that promote nuclear enrichment and two of them (named E and M) that confer localization to speckles (Miyagawa et al., 2012). A 156-bp local repeating RNA domain (RDD), present in 8 exonic copies in human *FIRRE,* localizes the transcript to the nucleus (Hacisuleyman et al., 2014). This function is conserved by the 16 copies of the RRD in the mouse *Firre* ortholog. Furthermore, recent studies showed examples of nuclear domains that dynamically respond to cell states. Human *SNHG1* and *lincRNA-p21*, both nuclear and cytoplasmic transcripts involved in p53 mediated apoptosis, are retained in the nucleus following DNA-damage treatment by doxorubicin (Zhao et al., 2018; Hall et al., 2015; Shen et al., 2017; Huarte et al., 2010). *SNHG1* binds to NCL protein through a G-rich motif (Shen et al., 2017) while *lincRNA-p21* retention is linked to two primate-conserved inverted repeat Alu elements (IRAlus) (Chillón and Pyle, 2016).

More generally, the frequency of short nucleotide sequences (k-mers) can be used to predict RNA localization with good accuracy, underlining the fact that *cis*-encoded localization signals contribute to localization (Su et al., 2018; Cao et al., 2018). Given the known differences in lncRNA and mRNA sequence composition, this may help explain lncRNA-specific localization (Haerty and Ponting, 2015). In summary, distribution of lncRNAs to different cellular compartments can be dynamically regulated through novel localization elements.

"Negative regulation" is likely to be another cause of observed relative nuclear enrichment of lncRNAs. The apparent

nuclear/cytoplasmic localization at steady state is simply proportional to cytoplasmic degradation, δ, divided by export rate, λ (Bahar Halpern et al., 2015). Greater transcript degradation in the cytoplasm can therefore give rise to apparent nuclear enrichment (Clark et al., 2012). Given that lncRNAs tend to have shorter half-lives than mRNAs (Mukherjee et al., 2017; Clark et al., 2012), this may help explain their distinct C/N concentrations. Therefore, both active localization and passive degradation processes are capable of causing observed patterns of RNA localization.

Most recently, systematic studies have opened up the question of lncRNA-specific localization mechanisms, with the surprising discovery of a role for repetitive or transposable elements. This is detailed in the following section.

Repetitive Surprises from High-Throughput Screens for LncRNA Localization Elements

Global approaches using either experimental or analytical strategies have recently been developed to comprehensively identify localization zipcodes. Lubelsky and Ulitsky screened libraries of 109-mer fragments of 37 human lncRNAs fused to a green fluorescent protein mRNA reporter to identify regions that regulate nuclear/cytoplasmic localization (Lubelsky and Ulitsky, 2018). Unexpectedly, this identified C-rich motifs derived from antisense Alu repeats and defined a core 42-nt motif called SIRLOIN (SINE-derived nuclear RNA localization). This sequence recruits hnRNPK protein and promotes nuclear localization of host transcripts for both lncRNAs and mRNAs. C-rich motifs within mouse B1 repeats exhibit a similar effect, suggesting a conserved regulatory mechanism (Lubelsky and Ulitsky, 2018).

Shukla and colleagues presented a similar approach with 153-mers from 38 human lncRNAs with previously defined localization patterns (Cabili et al., 2015; Shukla et al., 2018). They found conserved long sequences (>300 nt) responsible for nuclear localization, with a common 15-nt C-rich pattern, similar to the SIRLOIN motif. Surprisingly, the 15-nt motif alone was not sufficient to re-localize the cytoplasmic reporter (Shukla et al., 2018), highlighting the importance of sequence context. The power of these approaches is obvious in their unbiased sensitivity for discovering localization zipcodes; however, they are limited to studying a relatively small number of lncRNAs.

These findings chime with recent interest in the relationship between transposable elements (TEs), lncRNAs, and localization (Johnson and Guigó, 2014; Chen et al., 2008; Carlevaro-Fita et al., 2018). TEs are mobile DNA elements capable of copying and inserting themselves into genic and intergenic regions (Fedoroff, 2012), which represent approximately half of the human genome (Lander et al., 2001; Kelley and Rinn, 2012). In contrast to mRNAs (~40%), the majority of lncRNAs (~83%) carry at least one exonic TE element (Kelley and Rinn, 2012).

It has been proposed that exonic TEs may be repurposed as functional domains that interact with DNA, RNA, or proteins (Kapusta et al., 2013; Johnson and Guigó, 2014). This "copy-paste" model may explain how lncRNA genes are capable of acquiring new functions over short evolutionary timescales (Johnson and Guigó, 2014; Kapusta et al., 2013; Kelley and Rinn, 2012). In support of this, *trans*-acting proteins interact extensively with exonic TEs to affect stability and splicing of the host transcript (Kelley et al., 2014).

Unbiased bioinformatic approaches are also capable of identifying novel *cis*-regulatory sequences. In a recently published study to map functional TEs, correlation was used to discover a link between certain TE types and the nuclear localization of their host transcript (Carlevaro-Fita et al., 2018). Intriguingly, the three identified TEs, L2b, MIRb, and MIRc, are all ancient, implying that recently born lncRNAs repurpose pre-existing repetitive sequences. The benefit of this bioinformatic motif-discovery approach is its genome-wide scale, although this also reduces its statistical power, and it is restricted to defined classes of TEs. Together these findings support a zipcode model similar to mRNAs, where elements, both short (5–10 nt) and long (hundreds of nucleotides), contribute to specifying localization through *trans*-acting proteins.

Discussion

Here we have argued that understanding lncRNAs' subcellular localization is a powerful and indeed necessary step toward understanding the nature and mechanisms of their functions in the cell. Localization may be added to a small but growing list of features for classifying and predicting functions of lncRNAs. These include genomic organization (antisense, intergenic, etc.) (Derrien et al., 2012), TE content (Kelley and Rinn, 2012), processing (Mukherjee et al., 2017), chromatin modifications (enhancer- or promoter-type) (Marques et al., 2013), sequence motifs (Kirk et al., 2018), post-transcriptional processing (Mukherjee et al., 2017), and transcript length (St. Laurent et al., 2015). We would argue that cell localization determines molecular contacts and hence biological functions and is therefore a fundamental lncRNA feature (Kuriyan and Eisenberg, 2007).

Subcellular mapping and analysis methods are advancing rapidly. They still involve trade-offs of sensitivity and scale and of quantitative ensemble versus semiquantitative single-molecule measurements, which particularly affect lowly expressed and repetitive lncRNAs. Future methods involving single-cell analysis coupled to single-molecule RNA quantification would be a logical endpoint in solving these issues.

RNA localization is driven by the combination of distinct "zipcodes" encoded in primary sequence (Singer, 1993), which we have begun to systematically discover by bioinformatic predictions and cell-based screens. These zipcodes range from sequence domains of hundreds of nucleotides to shorter motifs such as hexamers, structural elements, and transposable element fragments (Zhang et al., 2014b; Werner et al., 2015; Chen and Carmichael, 2009; Lubelsky and Ulitsky, 2018; Shukla et al., 2018). However, it is likely that transcript localization is also determined by other factors. Among them, a potentially important mechanism that has not been covered here is chemical modification of RNA. Sparse evidence is available at present linking RNA modifications and localization (Kaneko et al., 2003). Moreover, work from mRNA suggests that it plays an important and unappreciated role in trafficking and stability and will no doubt be an important avenue of future research (Roundtree et al., 2017).

Finally, we have argued here for a more nuanced perception of lncRNA localization and to dispel the lingering view that all or even a majority of lncRNAs are nuclear-specific epigenetic regulators. There is now a convincing body of evidence for

important cytoplasmic lncRNA populations, with a growing roster of clearly elucidated mechanisms. Indeed, using a new approach to estimate absolute localization of RNAs with RNA-seq data (Figure 3 and Box 1), we have shown here that cytoplasmic-enriched lncRNAs are the majority in many cell types. The one-size-fits-all chromatin-enriched model for lncRNAs should be rejected in favor of a more balanced view, that lncRNAs are found throughout the cell.

In conclusion, subcellular localization is the window through which one of the greatest contemporary mysteries in biology may be understood: what are the cellular functions of lncRNAs?

SUPPLEMENTAL INFORMATION

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