Long noncoding RNA and protein abundance in lncRNPs

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ABSTRACT

Although long noncoding RNAs (lncRNAs) are generally expressed at low levels, emerging evidence has revealed that many play important roles in gene regulation by a variety of mechanisms as they engage with proteins. Given that the abundance of proteins often greatly exceeds that of their interacting lncRNAs, quantification of the relative abundance, or even the exact stoichiometry in some cases, within lncRNA–protein complexes is helpful for understanding of the mechanism(s) of action of lncRNAs. We discuss methods used to examine lncRNA and protein expression at the single cell, subcellular, and suborganelle levels, the average and local lncRNA concentration in cells, as well as how lncRNAs can modulate the functions of their interacting proteins even at a low stoichiometric concentration.

Keywords: lncRNA; lncRNP; concentration; abundance; stoichiometry; single cell; subcellular; suborganelle

INTRODUCTION

The vast majority of the human transcriptome consists of non–protein coding RNAs (ncRNAs), among which those longer than 200 nt are defined as long noncoding RNAs (lncRNAs) (Derrien et al. 2012). The latest annotation suggests that human lncRNAs exceed 170,000 (Zhao et al. 2021), although in a cell-/tissue-specific expression manner (Kim et al. 2015; Seifuddin et al. 2020). Currently annotated lncRNAs comprise species that are derived from different genomic origins and are processed by distinct mechanisms (for reviews, see St Laurent et al. 2015; Wu et al. 2017; Kopp and Mendell 2018). Over the past decade, well-documented studies focused on lncRNA modes of action have shown that these large and single-stranded molecules are often functionally engaged with distinct sets of proteins (Statello et al. 2021). They can act as guides, recruiters, scaffolds and decoys to modulate the structure and function of chromosome architecture and nuclear condensates, to regulate transcriptional and post-transcriptional processes, or to directly interfere with mRNA translation, post-translational modifications and signaling

pathways. Thus, lncRNAs can trigger significant cellular responses in many biological contexts.

Given their large size and flexible conformation, it remains a challenge to fully understand how lncRNAs exert their effects. In particular, the abundance of RNA binding proteins (RBPs) often greatly exceeds that of interacting lncRNAs, except in a few cases, such as the human telomerase reverse transcriptase (hTERT) and the telomerase RNA (*hTR*) that form an lncRNP at about a 1:1 ratio (Xi and Cech 2014; Ghanim et al. 2021). Quantitative comparison of lncRNA and protein levels shows that the copy number of the majority of proteins per cell varies from 10,000 to 80,000,000 (Hein et al. 2015), remarkably exceeding that of lncRNAs, with expression of 0.3–1000 molecules per cell (Table 1). Further, most proteins associate with many other factors, not just lncRNAs to which they can bind. Thus, distinct mechanisms have been adapted by lncRNAs to overcome their low abundance compared to their interacting proteins for achieving measurable effects. Understanding the subcellular localization of lncRNAs and the local concentrations of both proteins and RNAs is

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TABLE 1. lncRNP complexes with copy number quantification in cells

lncRNA	Length (nt)	Localization	Copies of lncRNA (per cell)	Interacting protein(s)	Copies of interacting proteins per cell	Function and mode of action	Key references
<i>hTR</i>	451	Nucleoplasm and telomeres	1150 (HeLa)	hTERT	500 (per cell)	An essential telomerase component required for telomere homeostasis	(Xi and Cech 2014)
<i>HOTTIP</i>	3764	Nucleus (chromatin)	~0.3 (foreskin fibroblast)	WDR5/MLL	N/A	Recruits WDR5/MLL for H3K4me3 across <i>HOXA</i> to enhance transcription with an AML-like phenotype in <i>Hottip</i> transgenic mice	(Wang et al. 2011; Cheng et al. 2015; Luo et al. 2019)
<i>lincRNA-EPS</i>	2531	Nucleus (chromatin)	11 (BMDM)	hnRNP L	N/A	Suppresses immune gene transcription by controlling nucleosome positioning	(Atianand et al. 2016; Mumbach et al. 2019)
<i>Xist</i>	17,000	Nucleus (inactivated X chromosome)	~100 (MEF)	RBM15, PCGF5, SPEN, CELF1, and others	N/A	Essential for X-chromosome inactivation in female mammals	(Brown et al. 1992; Hartshorn et al. 2003; McHugh et al. 2015; Sunwoo et al. 2015; Markaki et al. 2020)
<i>PNCTR</i>	>10,000	Nucleus (perinuclear compartment, PNC)	~36 (HeLa)	PTBP1	~286,000 (per cell)	Modulates cancer cell proliferation by sequestering the splicing factor PTBP1	(Yap et al. 2018)
<i>NEAT1_2</i>	22,700	Nucleus (paraspeckles)	53 (per paraspeckle in HeLa)	NONO, SFPO, FUS, RBM14	~2000 NONO per paraspeckle	Nucleates paraspeckle formation	(Chujo et al. 2017; Yamazaki et al. 2018)
<i>LETN</i>	4500	Nucleolus (granular component, GC)	~200 (HUH7 and HCC827)	NPM1	8,800,000 (per cell)	Maintains cancer cell proliferation by modulating nucleolar function	(Wang et al. 2021)
<i>SLERT</i>	694	Nucleolus (dense fibrillar component, DFC)	826 (PA1)	DDX21	13,000 (per DFC), 78,000 (per nucleolus), and 1,040,000 (per cell)	Controls FC/DFC phase separation and promotes Pol I transcription	(Xing et al. 2017; Wu et al. 2021)
<i>circFAM120A</i>	556	Cytoplasm (monoribosomes)	22-25 (HT29, 293FT and HT29)	IGF2BP2	~136,000 (per cytoplasm) and ~200,000 (per cell)	Promotes cell proliferation by outcompeting <i>FAM120A</i> mRNA binding to IGF2BP2	(Li et al. 2021)
<i>FAST</i>	547	Cytoplasm	140 (H9)	β-TrCP	3269 (per cytoplasm) and 8173 (per cell)	Maintains hESC pluripotency by activating WNT pathway	(Guo et al. 2020)
<i>lncRNA-ACOD1</i>	Variants: 2330 and 2259	Cytoplasm	~100 (BMDM)	GOT2	N/A	Facilitates viral replication by enhancing glutamic-oxaloacetic transaminase GOT2 activity	(Wang et al. 2017)
<i>NORAD</i>	5300	Cytoplasm (NORAD-PUM bodies) Cytoplasm	400 (HCT116) 70 (U2OS)	PUMILLO (PUM1/PUM2) PUMILLO (PUM1/PUM2)	15,000 PUM1/2,000 PUM2 (per cell) 200 PUM1/550 PUM2 (per cell)	Sequesters PUM to maintain genomic stability	(Lee et al. 2016; Elguindy and Mendell 2021) (Tichon et al. 2016)

therefore more informative mechanistically than only knowing average cellular concentrations.

As one example, for *cis*-acting lncRNAs, an abundance of one to 10 molecules per cell is likely sufficient for functioning at a single locus or at several loci by directly base-pairing with the genomic DNA or by recruiting chromatin modifiers to impact transcriptional outputs. This would explain how a number of low-abundance lncRNAs have been shown to exert pronounced biological effects (Table 1). For *trans*-acting lncRNAs, a higher abundance (10–1000) has been reported for their potential roles as recruiters, scaffolds, or decoys of proteins. Of note, such a higher abundance is still far below that of their interacting proteins; thus, the exact working model of a particular lncRNA depends not only on how many RBP binding motifs or structural modules exist within the lncRNA but also on its subcellular distribution (Table 1). In addition to relative abundance, it is worthwhile noting that the exact stoichiometry of lncRNA and protein within a functional lncRNP can provide clues into detailed lncRNA working mechanisms, as illustrated by well-studied lncRNPs, such as *NORAD* interacting with *PUMILIO* (*PUM*) (Elguindy and Mendell 2021), and *SLERT* interacting with *DDX21* (Wu et al. 2021). In this review, we summarize methods used to quantify lncRNA and interacting RBPs at the cellular, subcellular and suborganelle levels. We also discuss how cells deal with the generally low lncRNA–protein concentration ratios through a variety of regulatory modes of action.

STOICHIOMETRY OF CLASSICAL lncRNPs

Several classical cellular ribonucleoprotein complexes (RNPs) have now been well studied such as the catalytic ribonucleoprotein complex RNase MRP involved in precursor ribosomal RNA (Lan et al. 2020), the RNase P complex that selectively cleaves transfer RNA-like substrates (Guerrier-Takada et al. 1983; Bartkiewicz et al. 1989; Evans et al. 2006) and the telomerase complex that acts in lengthening telomeres during DNA replication (Kim et al. 1994). For each of these functional RNPs a defined stoichiometry of RNAs and proteins is required for their assembly (Gray and Gopalan 2020). A useful example is the human telomerase that is minimally composed of a catalytic hTERT and an RNA component, *hTR*. *hTR* is a 451 nt lncRNA acting as the template for telomere elongation by associating 1:1 with hTERT that is capable of *de novo* DNA synthesis (Schmidt and Cech 2015). Stoichiometric analyses by spike-in qRT-PCR and northern blotting (NB) experiments showed ~1150 *hTR* per HeLa cell, and by a two-step immunoprecipitation (IP)–western blotting (WB) method showed ~500 hTERT per HeLa cell (Xi and Cech 2014). Interestingly, quantification of endogenous levels of telomerase revealed ~240 active telomerase monomers or ~120 dimers, a number comparable to that

of telomeres in late S phase per HeLa cell (Cohen et al. 2007; Xi and Cech 2014). These analyses suggested that telomerase was not an abundant RNP and that unassembled nontelomerase *hTR* or hTERT existed in cells. Overexpression of either *hTR* or hTERT led to increased telomerase activity, supporting the notion of a quick assembly of the subpopulations of nontelomeric hTERT and *hTR* into functional telomerases in cells (Xi and Cech 2014). Methodologically, the nonequivalence of copy number between telomerase and its components indicates that only measuring the total cellular copy number of a lncRNA of interest and its corresponding protein may be inadequate to indicate the relevant abundance of functional lncRNPs.

METHODS TO DETECT THE ABUNDANCE OF lncRNAs AND THEIR ASSOCIATED RBPs

Different from *hTR* and hTERT in telomerase, most lncRNA-binding proteins also bind to many other RNAs in cells; thus, it is important to detect the relative abundance of lncRNAs and their associated RBPs within the particular lncRNPs for functional insights. Also, unlike telomerase, which can be quantified by telomerase activity, we lack biochemical assays to detect most lncRNP complexes. Methods to quantify lncRNA expression have therefore moved from classic biochemical quantification (Xi and Cech 2014; Lee et al. 2016; Xing et al. 2017) to combining subcellular fractionation and quantitative imaging (Guo et al. 2020; Li et al. 2021), and more recently, high resolution microscopy at the suborganelle level to dissect the relative abundance of lncRNA and protein in lncRNPs (Wu et al. 2016; Yao et al. 2019; Elguindy and Mendell 2021; Wu et al. 2021).

BIOCHEMICAL QUANTIFICATION OF lncRNAs AND RBPs IN CELLS

Biochemical quantification is the most well-established method to calculate the average lncRNA copy number per cell. To do so, one needs to construct an *in vitro* benchmark system, termed a “standard curve,” which can be generated by using NB or qRT-PCR (Xi and Cech 2014; Xing et al. 2017; Li et al. 2021; Wu et al. 2021) analyses with a series of dilutions of purified *in vitro* transcribed (IVT) lncRNAs of interest (Fig. 1A). Then, the total RNA extracted from a defined number of cells (i.e., 1 million cells) is collected and subjected to NB followed by band quantification or by qRT-PCR to obtain the respective C_t values. Based on the standard curve (Fig. 1A), the copy number of a particular lncRNA per cell can be determined by dividing the total number of cells used to extract the total RNA.

One must take two matters into consideration during this biochemical quantification protocol. First, for RNAs that are strongly encapsulated with proteins, chromatin,

FIGURE 1. Quantification of cellular and subcellular lncRNA expression. (A) A workflow to illustrate the biochemical quantification of the copy number of a lncRNA per cell. The standard curve is generated by northern blotting or qRT-PCR of in vitro transcribed and purified RNA "X" at different concentrations. Total RNA is extracted from a certain number of cells by means of classical TRIzol or an improved heat/shearing TRIzol method. See text for detail. (B) A pipeline of isolating cytoplasmic, nuclear soluble, and insoluble fractions to quantify lncRNA at the subcellular level. See text for detail. (C,D) Imaging quantification of lncRNP components at subcellular and suborganelle levels (Yao et al. 2019; Wu et al. 2021). (C) Image acquisition. Firstly, the gene locus of the marker protein in the region of interest (ROI) is tagged with fluorescence protein (FP) via CRISPR/Cas9-mediated knock-in (KI) to circle the subdomain. smFISH of the target lncRNA is performed to gain signals of the lncRNA of interest in the whole cell. (D) Imaging processing. 3D stacks of the protein and the lncRNA of interest are imported into Imaris. 3D rendering of the marker protein and the lncRNA was produced, respectively, with surface building function modules to obtain their integrated densities within corresponding surfaces. The percentage of the protein and the lncRNA in ROI is calculated by the formula shown in the panel, where N is the copy number of proteins/RNAs per granule, I_{granule} is the intensity of proteins/RNAs in total granules, I_{total} is the intensity of proteins/RNAs in the whole cell, N_{total} is the copy number of proteins/RNAs per cell, and N_{granule} is the number of granules per cell.

nuclear matrix or in dense condensates in cells, it is compulsory to utilize an improved RNA extraction method by shearing or heating to disrupt such dense complexes, thereby leading to enhanced extractability of RNA by TRIzol (Chujo et al. 2017; Wu et al. 2021). Second, we highly recommend the use of IVT RNA rather than plasmid

DNA as the template to generate the standard curve. Using IVT RNAs as the template for standard curves involves a cDNA synthesis step and thus takes the efficiency of the reverse transcription reaction into account. Furthermore, knowing a copy number of a particular RNA per cell, by combining the RNA-seq transcriptomic data, one can roughly get an idea of the copy number of additional RNAs by comparing their expression in samples with the same sequencing depth (Liu et al. 2019).

In principle, in these biochemical quantifications, specificities of NB probes and qRT-PCR primers are of importance to be considered to avoid potential bias in calculating concentrations (Li et al. 2021). For instance, using different qRT-PCR primer sets targeting *hTR* gave noteworthy differences in *hTR* levels, even as much as 10-fold variation. To solve this concern, gradient dilutions of in vitro purified *hTR* as spike-in were incorporated into the endogenous *hTR* to obtain qRT-PCR signals for abundance (Xi and Cech 2014).

Similarly, the copy number of a protein of interest per cell can be calculated, by first generating the standard curve as a reference concentration with a series dilution of purified proteins by WB and then calculating the protein copy number from a defined number of cells (Li et al. 2021). Of note, the accuracy of antibodies for WB should be considered to avoid potential misdetection. On the other hand, though, mass spectrometry (MS)-based quantitative proteomics provides a sensitive way to analyze proteins on a global scale across cell and tissue type (Aebersold and Mann 2003; Schubert et al. 2017). Generally, there are two quantitative MS strategies, isotopic labeling (Gygi et al. 1999; Ong et al. 2002;

Ross et al. 2004) and label-free approaches (Chelius and Bondarenko 2002; Ahrne et al. 2013). Among isotopic labeling methods, SILAC (stable isotope labeling with amino acids in cell cultures) (Ong et al. 2002; Wang et al. 2018a) is the most popular strategy in which specific amino acids with a nonradioactive, isotopically labeled form are

incorporated into all proteins in given samples. For label-free absolute quantification, all proteins are digested into peptides and subjected to liquid chromatography to measure the summarized ion counts; then these counts are converted into signal values compared to the total amount of proteins injected into the mass spectrometer, or correlated to a set of spike-in reference peptides with known concentrations (Chelius and Bondarenko 2002; Ludwig et al. 2012). Although large scale quantification is allowed in these measurements, a huge number of cells is required to gain confidence in quantitative MS, because the readout is limited as a proxy for protein levels. Consequently, single-cell proteomics, termed scMS, has major limitations on throughput and proteomic depth (Budnik et al. 2018; Zhu et al. 2018). Nevertheless, in principle, the copy number of a protein of interest can be extracted for particular types of cells that have been analyzed by quantitative MS (Castello et al. 2012, 2016). This can then be used to determine the levels of RBPs containing intrinsically disordered regions (IDR) in RNA interactomes that have emerging roles in phase separation (Das et al. 2014; Zhang et al. 2015; Maharana et al. 2018; Zhou et al. 2018; Guillen-Boixet et al. 2020).

BIOCHEMICAL QUANTIFICATION OF lncRNAs AND RBPs AT THE SUBCELLULAR LEVEL

lncRNAs often possess subcellular preferences for function instead of being evenly distributed throughout the cell (Cabili et al. 2015; Wu et al. 2016; Sun et al. 2018). More than the copy number of a lncRNA per cell, its specialized localization indeed more profoundly impacts the function of its interacting proteins. One simple way used to determine subcellular localization involves separating cytoplasmic and nuclear RNAs followed by NB and qRT-PCR quantification (Fig. 1B). In brief, cells are treated with nonionic detergent, such as Igepal, to solubilize membrane lipids and release cytoplasmic contents. After centrifugation at low speed (i.e., 1000 g), the soluble and cytoplasmic RNAs in the supernatant are extracted by TRIzol or other RNA extraction reagents (Chen et al. 2008; Lo Piccolo et al. 2015; DeCaprio and Kohl 2020). The pelleted nuclear fractions are further incubated with prolonged nonionic detergent or gentle ionic detergent, such as deoxycholate, to destroy the nuclear membrane and release nuclear soluble and insoluble fractions. The latter insoluble fractions mainly consist of chromatin-associated RNAs (Ramsby and Makowski 2011) and nuclear condensate-enriched RNAs (Chujo et al. 2017). In theory, the nuclear insoluble fraction is resistant to high salt, nonionic detergent and nuclease treatment; thus, special attention should be paid to isolate lncRNAs from the insoluble fraction using appropriate buffers (i.e., by including high salt, 2 M NaCl) (Takata et al. 2009).

In the cytoplasm, some lncRNAs are specifically localized to certain organelles for function. For example, the lncRNA SAMMON is mitochondrial localized and plays a role in promoting melanoma growth and survival via binding mitochondrial p32 protein (Leucci et al. 2016). Some lncRNAs are enriched in ribosomal fractions and affect mRNA translation (Essers et al. 2015; Hansji et al. 2016). For instance, ZFAS1 binds to the small 40S ribosomal subunit and regulates ribosome production and assembly (Hansji et al. 2016). A recent study showed that *circFAM120A* bound the translational inhibitor IGF2BF2 to prevent this protein from engaging with *FAM120A* mRNA on monoribosomes, resulting in enhanced *FAM120A* mRNA translation (Li et al. 2021). It should be noted that *FAM120A* mRNA is expressed at 250–600 copies per cell, while *circFAM120A* is expressed at only 22 copies per cell. Interestingly, to be competitively competent for IGF2BF2 binding, the majority of *circFAM120A*, but just a small fraction (<10%) of *FAM120A* mRNA, is specifically present on monoribosomes, resulting in a 3:1 to 5:1 of *FAM120A* mRNA and *circFAM120A* stoichiometry on the monoribosomes, as shown by polyribosome profiling assays that segregate single ribosomes and polyribosomes via sucrose gradients (Li et al. 2021). Such specific localization of *circFAM120A* provides a sufficient level to trap IGF2BP2 on monoribosomes, thereby facilitating *FAM120A* mRNA translation and ultimately cell proliferation (Li et al. 2021).

Inside the nucleus, isolating soluble and insoluble nuclear fractions can roughly indicate a subnuclear localization of lncRNAs, but it remains a challenge to delineate their precise subnuclear localization patterns using biochemical assays. Different types of membrane-less nuclear bodies are difficult to isolate biochemically. The largest nuclear structures, nucleoli (1–5 μ m [Lafontaine et al. 2021]), are more amenable to be isolated from a nuclear fraction while other smaller nuclear bodies are far more difficult to purify using sucrose cushions. Isolation of nucleoli could be achieved by treating sonicated nuclear fractions with various concentrations of sucrose and magnesium chloride (Andersen et al. 2005), which allowed investigators to uncover the role of *Alu*-containing RNAs in the maintenance of nucleolar structure and function (Caudron-Herger et al. 2015). Paraspeckles (0.2–0.5 μ m [Sasaki and Hirose 2009; Mao et al. 2011b]) are difficult to isolate biochemically and were discovered by immunofluorescence (IF) imaging validation of proteomic results of the sucrose density—isolated nucleolar components including PSP1 and NONO (Fox et al. 2002). IF with anti-PSP1 and anti-NONO revealed that they did not localize to nucleoli but rather to previously undefined regions that are localized adjacent to nuclear speckles (Fox et al. 2002, 2005).

Beyond the above-mentioned traditional biochemical analyses, recently developed proximity labeling-based methods including BioID (biotin identification) (Choi-Rhee et al. 2004; Roux et al. 2012), APEX (engineered

ascorbate peroxidase) (Martell et al. 2012; Hung et al. 2016), HRP (horseradish peroxidase) (Hopkins et al. 2000; Li et al. 2014), and PUP-IT (pupylation-based interaction tagging) (Liu et al. 2018), coupled with quantitative MS offer a high-throughput means for systematic analysis of spatially restricted proteomes (Bosch et al. 2021). In addition, RAP-MS (RNA antisense purification coupled with mass spectrometry) revealed Xist interacting proteins (McHugh et al. 2015), IP-MS identified thousands of proteins across almost all protein classes in HeLa cells (Hein et al. 2015) and RNA CHART (capture hybridization analysis of RNA targets) -seq using antisense oligonucleotides for *NEAT1_2* revealed novel RNA components of paraspeckles (West et al. 2016) as well as the crosstalk between paraspeckles and mitochondria (Wang et al. 2018b). Moreover, DNA CHART-seq and CHART-MS of *NEAT1* and *MALAT1* have also been used to show the shared DNA and protein components between paraspeckles and nuclear speckles (West et al. 2014).

IMAGING AND SUPER RESOLUTION IMAGING QUANTIFICATION OF lncRNPs AT THE SUBCELLULAR AND SUBORGANELLE LEVEL

Compared to biochemical separation that is performed using bulk cells, imaging and super resolution imaging provide an in situ measurement of cellular components. Based on the biochemical quantification of lncRNA copy number per cell (Fig. 1A,B), the exact molecule number in designated localized regions can be examined by calculating fluorescent intensities of the regions of interest (ROI) and whole-cell regions through single molecule RNA FISH (smFISH) experiments of the target lncRNAs (Fig. 1C,D; Wu et al. 2016; Yao et al. 2019; Wu et al. 2021). Of note, ROI is represented by the known marker protein in the targeted subdomain (Fig. 1C,D). It is worthwhile mentioning that quantitative imaging demands high-resolution and high-quality images, devoid of unwanted contamination by background signal intensity.

The subregion quantification of a particular protein is similar to that of lncRNA by circling the interest region through protein IF imaging (Wu et al. 2016; Guo et al. 2020; Wu et al. 2021). Since the expression level of RNA and protein per cell is based on biochemical quantification from the standard curve generated from bulk cells (Fig. 1A, B), the averaged quantification per cell does not account for cell-to-cell variability, and accordingly, the calculated copy number at a particular region can vary.

A more precise way for quantification of proteins in living cells is to fuse proteins of interest to a fluorescent protein such as monomeric enhanced green fluorescent protein (mEGFP) using CRISPR/Cas9 technology. This can be used to indicate both protein expression and localization. By generating a fluorescence intensity standard curve of purified mEGFP in vitro at different concentrations, the ex-

act copy of protein in the whole cell and the specific subcellular region can be retrieved (Fig. 1C; Yao et al. 2019). However, one potential disadvantage of this method is that the fused mEGFP domain (~27 kDa) may change the localization, stability or function of the protein of interest.

Recently developed super-resolution microscopy has enabled the observation and quantification of subcellular structures at the nanometer level (West et al. 2016; Wu et al. 2016, 2021; Xing et al. 2017; Colognori et al. 2019; Elguindy and Mendell 2021). By consolidating super-resolution imaging and specific cellular organelles of interest, the meaningful local concentration of RNA and protein in one lncRNP could be accurately calculated. For instance, structural illumination microscopy (SIM) allied with CHART RNA-seq using antisense oligonucleotides that target different *NEAT1_2* regions revealed protein and RNA components arranged as core-shell structures in paraspeckles (West et al. 2016; Wang et al. 2018b). SIM, together with iCLIP RNA-seq, revealed the distinct binding preferences of lncRNA and RBPs in terms of *sno-lncRNAs*, *SPA lncRNAs* and splicing factors RBFOX2, hnRNP M and TDP43 in a particular nuclear region that is absent from patients with Prader-Willi syndrome (PWS) (Wu et al. 2016).

THE SUBCELLULAR LOCALIZATION AND ABUNDANCE OF lncRNAs PROVIDE CLUES ABOUT MODES OF ACTION

Knowing the subcellular localization gives clues about whether a lncRNA acts in *cis* or in *trans*; combining its intrinsic sequence, conformational features and the lncRNA local concentration can further illustrate how a lncRNA exerts transcriptional or post-transcriptional effects by interacting with proteins that are expressed at different levels in cells (Fig. 2).

LOW ABUNDANCE CHROMATIN-ASSOCIATED lncRNAs CAN BE INVOLVED IN THE IN CIS AND IN TRANS REGULATION OF TRANSCRIPTIONAL OUTPUTS

Nuclear soluble and insoluble fractionation followed by qRT-PCR or RNA-seq analyses could first give clues about chromatin-associated lncRNAs (Fig. 1B) and their copy number per cell can be retrieved by biochemical quantification (Fig. 1A). Chromatin-associated lncRNAs are found to be expressed at low copy number, at a range of 0.3–100 copies per cell (Table 1). Their modes of action can be classified into two groups: base-pairing with DNA through complementarity to form RNA:DNA hybrids (R-loops) or RNA:DNA triplexes and recruiting or sequestering chromatin-associated RBPs at one locus or several loci (Fig. 2A). It is worthwhile noting that these modes are not mutually exclusive, because base-pairing with DNA very often recruits specific proteins for functions.

FIGURE 2. The subcellular localization and relative abundance of lncRNA determine its mode of action. (A) Examples of working modes of chromatin-associated lncRNAs. (a) Base-pairing with the complementary genomic DNA directs lncRNA localization and mode of action. (Top) lncRNA *TARID* (*TCF21* antisense RNA inducing demethylation) forms an R-loop in the *TCF21* (transcription factor 21, a tumor suppressor gene) CpG island promoter to recruit a proposed R-loop reader, GADD45A (growth arrest and DNA-damage-inducible, alpha), and cooperates with TET1 (10–11 translocation 1) and TDG (thymine-DNA glycosylase) to drive demethylation of the *TCF21* promoter, resulting in *TCF21* transcription activation (Arab et al. 2014, 2019). (Bottom) The lncRNA *Khps1* (antisense orientation to *SPHK1*) forms RNA–DNA–DNA triplexes upstream of the transcription start site of proto-oncogene *SPHK1-B* (Sphingosine kinase 1, isoform B) with a purine-rich sequence. *Khps1* prompts an open chromatin structure by recruiting the histone acetyltransferase p300/CBP to the *SPHK1* promoter to facilitate *SPHK1-B* transcription (Postepska-Igielska et al. 2015). (b) Chromatin-associated lncRNA *Xist* (X inactive specific transcript) serves as a recruiter paradigm along the female inactive X chromosome (Xi), which contains ~1000 genes (Tukiainen et al. 2017). *Xist* recruits different chromatin-modifying factors to form super-molecular complex (SMC) to silence adjacent genes on Xi (Brockdorff 2018). (B) Examples of working models of trans-acting lncRNAs. (a) One lncRNA possesses multiple binding motifs (top) (Yap et al. 2018) or forms multiple structural modules (bottom) (Guo et al. 2020) to decoy RBP function. (b) Locally enriched lncRNA interacts with multiple RBPs to form nuclear condensates. *NEAT1_2* (nuclear paraspeckle assembly transcript 1, the long isoform) is the scaffold RNA of paraspeckles. It folds in half to keep the core region in the center and 5'/3' termini in the shell within paraspeckles. The radially arranged *NEAT1_2* provides an interacting network for paraspeckle proteins (West et al. 2016), among which SFPQ (splicing factor proline and glutamine rich) and NONO (non-POU domain containing octamer binding protein) play critical roles in initiating paraspeckle assembly (Yamazaki et al. 2018). (c) Multiple lncRNAs are enriched as a group to interact with RBPs. Five *sno-lncRNAs* (small nucleolar RNA-ended lncRNA) and two SPAs (5' small nucleolar RNA capped and 3' polyadenylated) are transcribed from the Ch15q11–13, and are absent in patients with PWS (Prader–Willi syndrome). These lncRNAs interact with multiple RBPs to form microscopically visible foci that sequester RBPs to affect pre-mRNA alternative splicing (Wu et al. 2016). (d) Many circRNAs possess 16- to 26-bp duplexes and function as a group to inhibit PKR activation (Liu et al. 2019).

In the first scenario, a few copies per cell of lncRNAs that are associated with chromatin are likely adequate for their proposed modes of action. Formation of complex structures at specific genomic regions may in turn recruit additional chromatin modifiers and transcriptional factors to achieve a measurable gene expression change (Li et al.

2016; Arab et al. 2019; Feretzaki et al. 2020). This can be exemplified by the lncRNA *TARID* (*TCF21* antisense RNA inducing demethylation) that forms an R-loop at the *TCF21* promoter in *cis* to trigger the sequential process of GADD45A recruitment, TET1 association, DNA demethylation and *TCF21* transcription activation (Fig. 2A;

Arab et al. 2014, 2019). In terms of the formation of triplex structures, *Khps1* (antisense transcript of *SPHK1*) is tethered to the *SPHK1-B* promoter in *cis* via DNA–RNA triplexes and recruits p300/CBP to acetylate histones, promoting an active chromatin conformation to enhance *SPHK1-B* transcription (Fig. 2A; Postepska-Igielska et al. 2015).

In the second scenario, lncRNAs are located in the proximity of chromatin to scaffold or recruit protein binding (Fig. 2A). P53 induced intergenic noncoding RNA p21 (*lincRNA-p21*), is expressed at 20–80 copies per cell (Yang et al. 2014), interacts with hnRNP K and prevents reprogramming by maintaining the heterochromatic state of pluripotency gene promoters (Bao et al. 2015). Chromatin modifier complex deposition mediated by lncRNAs can also extend over a long range. The most prominent example is the lncRNA *Xist*, which is 17,000 nt in mouse and is transcribed exclusively from the inactive X chromosome with ~100 copies per cell (Brown et al. 1992; Hartshorn et al. 2003; McHugh et al. 2015; Sunwoo et al. 2015; Markaki et al. 2020). *Xist* spreads directly from its transcription locus to distal sites across the X chromosome (Lee and Bartolomei 2013; Markaki et al. 2020) in differentiated female cells to silence an X chromosome by recruiting SPEN and a range of other proteins sequentially to form a transcriptionally silent nuclear compartment (Fig. 2A; Chu et al. 2015; Dossin et al. 2020; Markaki et al. 2020).

LOCALIZED ENRICHED lncRNAs CAN CONTAIN MULTIPLE BINDING MOTIFS TO ACHIEVE HIGH EFFICIENCY AS DECOYS OR SCAFFOLDS FOR PROTEINS

lncRNAs acting in *trans* as decoys or scaffolds in principle require higher abundance. However, even lncRNAs expressed at copy numbers ranging from dozens to hundreds of copies per cell are still outnumbered by their interacting proteins (Table 1). To overcome this issue, lncRNAs often harbor many binding motifs (Yap et al. 2018) or form multiple binding modules (Guo et al. 2020) to interact with proteins. Further, their localized accumulation offers a sufficiently high concentration to achieve measurable effects on interacting proteins, especially in that cellular neighborhood.

PNCTR is a >10,000 nt transcript produced by RNA polymerase I (Pol I). It is expressed only at ~36 copies per cell, is exclusively localized to the perinucleolar region (PNC) and is capable of recruiting a substantial amount of PTBP1 (Yap et al. 2018). PTBP1 is an apoptosis activator and prefers to bind YUCUYY and YYUCUY motifs (Fig. 2B), which are highly abundant in *PNCTR* sequence (2178 and 565, respectively). These motifs serve as PTBP1 concentration “sensors” and enable *PNCTR* to sequester 7%–27% cellular PTBP1 in theory, which indeed was confirmed as 11%–31% by quantifying IF-smFISH experiments. *PNCTR* thus provides ample binding sites for

PTBP1 to antagonize PTBP1 splicing regulation in a pro-survival function for cells (Fig. 2B; Yap et al. 2018). In another example, *FAST* is a 547 nt, cytoplasmic transcript that is expressed at 140 copies per human embryonic stem cell (hESC). It forms five independent stem-loops that allow binding of ~700 molecules of β -TrCP (an E3 ubiquitin ligase that targets phosphorylated β -catenin in the WNT destruction complex) (Fig. 2B), accounting for >20% of the total ~3269 copies of β -TrCP in the cytoplasm (Guo et al. 2020). This has enabled a suppressed β -catenin degradation and activated WNT signaling that contributes to the maintenance of hESC pluripotency (Guo et al. 2020).

Except for being targeted by the same proteins, some lncRNAs can interact with a variety of proteins, exemplified by one of the most well-studied lncRNAs, *NEAT1_2* (Fig. 2B). *NEAT1_2* is a 22,700 nt transcript, expressed at ~1000 copies per HeLa cell (Chujo et al. 2017) and serves as the key scaffold lncRNA of paraspeckles (Sasaki et al. 2009; West et al. 2016), a type of compact and membrane-less nuclear body exhibiting liquid-like properties (Yamazaki et al. 2018). In HeLa cells, each paraspeckle contains ~50 *NEAT1_2* molecules (Chujo et al. 2017), providing many binding sites for paraspeckle proteins (PSPs) via distinct subdomains (Hennig et al. 2015; Yamazaki et al. 2018). In the core-shell spheroidal structure of a paraspeckle, the 5' and 3' termini of *NEAT1_2* display a shell localized pattern and the middle domain of *NEAT1_2* contains several functional subdomains. The middle region of *NEAT1_2* binds to NONO, SFPQ, and FUS, which is sufficient for paraspeckle assembly (West et al. 2016; Yamazaki et al. 2018). Among the over 40 identified paraspeckle proteins, NONO and SFPQ have strong affinity with *NEAT1_2* subdomain 12–13k, lower but substantial affinity with the adjacent 9.8–12k and 15.4–16.6k, to initiate paraspeckle assembly (Yamazaki et al. 2018). In addition, FUS provides an interaction network through its PLD (prion-like domain), leading to paraspeckle assembly via phase separation (Fox et al. 2018). This was also illustrated by RNA pull-down assays combining SILAC-MS analysis where quadruplex-forming G-rich sequences in *NEAT1_2* were important for NONO, SFPQ, PSPC1 binding (Simko et al. 2020).

DIFFERENT lncRNAs CAN ACT AS A GROUP TO ENSURE MEASURABLE EFFECTS ON PROTEIN INTERACTIONS

In addition to the single type of lncRNA that acts as a decoy or scaffold, multiple different lncRNAs may localize proximately and act as a group by containing the same binding motifs for proteins; alternatively, different lncRNAs can form similar structural modules that allow the same protein to bind these RNAs to achieve a measurable effect.

Five *sno-lncRNAs* and two *SPA lncRNAs* are paternally transcribed from Ch15q11–13, a region that is not transcribed in patients with PWS due to parental chromosome deletion, uni-chromosome disomy or epigenetic defects (Yin et al. 2012; Zhang et al. 2014; Wu et al. 2016). While PWS region *sno-lncRNAs* range from 1100 nt to 2900 nt in size, *SPA lncRNAs* are larger transcripts, 34,000 nt for *SPA1* and 16,000 nt for *SPA2*. These *snoRNA*-biogenesis-facilitated *lncRNAs* are processed from the same polycistronic transcript driven by a strong paternal Pol II promoter (Wu et al. 2016), ultimately yielding relatively abundant *SPAs* and *sno-lncRNAs* (approximately one-third of the level of *lncRNA MALAT1* in H9 cells). Once produced, these *lncRNAs* are all localized to their site of production and form microscopically visible *lncRNP* accumulations, referred to here as PWS bodies (Fig. 2B; Wu et al. 2016). Super-resolution microscopy together with iCLIP RNA-seq revealed that these *lncRNAs* are colocalized with splicing factors RBFOX2, hnRNP M, and TDP43 and provide specific binding motifs with certain preferences (Wu et al. 2016). For example, *sno-lncRNAs* prefer to bind RBFOX2 (UGCAU and GCAUG motifs), whereas *SPA1* prefers to bind TDP43 (tandem UG-repeat sequences) as revealed by iCLIP analysis (Wu et al. 2016). Quantitative imaging of each RBP and these PWS-region *lncRNAs* has shown that greater than 1% of each RBP could be sequestered by these *lncRNAs*, suggesting that the PWS body takes up 0.02%–0.1% of the nuclear volume but sequesters greater than 1% of each associated RBP. As a consequence, in a PWS hESC cellular model lacking all these seven *lncRNAs*, altered splicing events that are associated with the mislocalization of these RBPs on pre-mRNAs were identified (Wu et al. 2016). Some of the misregulated pre-mRNA splicing events were confirmed in hiPSCs derived from a PWS patient (Wu et al. 2016).

Another example can be illustrated by *circRNAs*, which are known to be expressed at a low level in general (Chen 2020). Using an optimized circSHAPE-MaP approach to measure the folding conformation of *circRNAs* (Liu et al. 2019), it was found that 26 out of 34 examined *circRNAs* contain one to four intramolecular 16- to 26-bp RNA duplexes (*intra-dsRNAs*), which are seen less frequently in their linear mRNA cognates. This has allowed the short *intra-dsRNA*-containing *circRNAs* to work as a group to bind PKR and prevent it from dimerization for activation (Fig. 2B; Liu et al. 2019). PKR is a double-stranded RNA-dependent protein kinase associated with direct antiviral activity and immune responses, and in this process, PKR activation requires *dsRNAs* longer than 33 bp, while short *dsRNAs* of 16 to 33 bp in length can block its activation (Zheng and Bevilacqua 2004; Bou-Nader et al. 2019). Given this structure-dependent, but sequence-independent, manner for PKR activation, and that each cell has ~10,000 copies of *circRNAs* many of which have one to

four *intra-dsRNA* regions, *circRNAs* as a group can modulate PKR activation (Liu et al. 2019).

lncRNA-INDUCED PROTEIN PHASE SEPARATION PROMOTES EFFICIENT lncRNP ASSEMBLY TO OUTCOMPETE THE SAME BINDING MOTIFS ON OTHER EXPRESSED RNAs

Even for *lncRNAs* that are abundantly expressed at hundreds of copies per cell, they may still display a low stoichiometric ratio compared to total cellular expressed RNAs containing the same binding motifs for a particular protein. For example, *NORAD* plays a prominent role in genome stability by sequestering and antagonizing PUM1 and PUM2, which have inhibitory effects on mitotic, DNA repair, and DNA replication factors related to aberrant mitosis (Lee et al. 2016; Tichon et al. 2016). Depletion of *Norad* in mice causes premature aging due to augmented activity of PUM proteins (Kopp et al. 2019). *NORAD* is expressed at ~400 copies per HCT116 cell, and each transcript contains 18 PREs (PUMILIO response elements) for PUM binding (Lee et al. 2016). Given that there are ~15,000 PUM1 plus ~2000 PUM2 proteins per cell, *NORAD* provides sufficient PUM1 and PUM2 binding sites to restrain functional PUM proteins (Lee et al. 2016). However, in terms of element number, these 7200 PREs (400×18) are unlikely to compete well against overall 130,000–325,000 PREs within the whole mRNA transcriptome (Fig. 3A; Elguindy and Mendell 2021).

PUM proteins contain large amino-terminally located IDRs. Super-resolution microscopy has shown that *NORAD* and PUM are colocalized in the cytosol to form microscopically visible foci named NP (*NORAD* and PUM) bodies. NP bodies have a liquid–liquid phase separation property, in which PUM protein phase separation is driven by *NORAD* in a PRE-number-dependent manner. *NORAD* thus efficiently sequesters PUM beyond simple competitive titration based on RNA valency and PRE abundance by creating a micro-environment to effectively recruit a large number of PUM to these sites. In such a model, once *NORAD*-PUM clustering via multivalent RNA-binding interactions is established, additional PUM will be recruited and concentrated by IDR–IDR interactions of PUM proteins and finally enlarged into a NP body.

Given the fact that many RBPs, for example, those enriched in paraspeckles, undergo phase separation (Hennig et al. 2015; Fox et al. 2018) and that the *lncRNA NEAT1_2* mid-region itself contains a phase separation property (Yamazaki et al. 2018), it remains possible that paraspeckles, and likely other *lncRNP* complexes such as *Xist* RNP (Markaki et al. 2020), may nucleate their formation via an efficient multivalent mechanism, in addition to coupling with Pol II transcription (Mao et al. 2011a; Hnisz et al. 2017; Shrinivas et al. 2019; Rippe and Papantonis 2021).

FIGURE 3. Mechanisms of super-stoichiometry of lncRNA-RBP interaction. (A) NORAD (non-coding RNA activated by DNA damage) drives PUMILLO (PUM) phase separation by multivalent NORAD-PUM and IDR-mediated PUM-PUM interactions to sequester outnumbered PUM proteins (Elguindy and Mendell 2021). NORAD outcompetes other cellular mRNAs by means of PRE (PUMILLO response element) valency and abundance, preventing aberrant mitosis by restraining PUM proteins within a narrow range (Lee et al. 2016; Tichon et al. 2016). (B) SLERT (snoRNA-ended lncRNA enhances preribosomal RNA transcription) is exclusively localized to the nucleolus (Xing et al. 2017). It interacts with the DEAD-box RNA helicase DDX21 with one loop region and transforms a large amount of DDX21 proteins from open to closed conformation by a molecular chaperone-like mechanism (Wu et al. 2021). Closed DDX21 molecules form loosened clusters surrounding FC/DFC to maintain the proper liquidity required for RNA polymerase I (Pol I) transcription (Wu et al. 2021).

lncRNAs CAN PROMOTE CONFORMATIONAL CHANGE OF PROTEIN CLUSTERS BY A MOLECULAR CHAPERONE-LIKE MECHANISM AT A SUBSTOICHIOMETRIC RATIO

Super resolution microscopy lncRNP in situ studies have shown an extremely low stoichiometric ratio of the lncRNA *SLERT* to its binding protein DDX21, which together play important roles in human nucleolar organization and Pol I transcription (Fig. 3B; Xing et al. 2017; Wu et al. 2021). In the nucleolus, Pol I transcription of rDNA occurs at the border of fibrillar centers (FC) and dense fibrillar components (DFC) (Fig. 3B), making FC/DFC organization as a

unit for Pol I transcription and nascent pre-rRNA processing (Yao et al. 2019). *SLERT* is a H/ACA-type snoRNA-ended lncRNA, transcribed and processed from the *TBRG4* locus, and then translocated to the nucleolus for function (Xing et al. 2017). Depletion of *SLERT* led to reduced cell proliferation, consistent with its role in promoting Pol I transcription (Xing et al. 2017).

SLERT is exclusively localized at FC/DFC units, where it binds to DDX21 (Xing et al. 2017), a DEAD-box RNA helicase that can adopt an open or closed conformation upon substrate binding (Chen et al. 2020). DDX21 is largely enriched outside of each FC/DFC, where it forms a discontinuous shell-like structure coating each FC/DFC by switching its intermolecular and intramolecular interactions mediated by unstructured amino- and carboxy-termini. Increased intermolecular interaction led to DDX21 multimerization, resulting in decreased size of the shell and FC/DFC structure, reduced mobility of FC/DFC components, and suppressed Pol I transcription (Fig. 3B; Wu et al. 2021). Intriguingly, there are about 13 *SLERT* and 13,000 DDX21 molecules per FC/DFC (Wu et al. 2021), and yet these 13 *SLERT* are capable of promoting the significantly more abundant DDX21 proteins in FC/DFC to adopt a closed conformation, ultimately leading to the loosened environment of an FC/DFC unit and proper Pol I transcription (Fig. 3B; Wu et al. 2021). *SLERT* prefers to bind DDX21 with open conformation. It was proposed

that under this super-stoichiometric ratio, once *SLERT* induces a DDX21 protein to adopt the closed conformation, this DDX21 would be released, allowing the recruitment of another one with an open conformation to initiate conformational transition, resulting in the formation of hypomultimerized DDX21 molecules in FC/DFC, which is required for FC/DFC liquidity and Pol I processivity. Moreover, without *SLERT*, DDX21 clusters are strongly engaged with rDNAs to prevent Pol I binding to rDNAs for transcription (Fig. 3B).

How exactly *SLERT* acts as a molecular chaperone to coordinate DDX21 conformational change in clusters warrants future studies that will require the development of

new methods for the analysis of phase-separated lncRNPs. Nevertheless, both studies (Fig. 3A,B) have raised the intriguing possibility that lncRNPs may work as clusters in certain condensates, rather than independently in solution as previously thought.

PERSPECTIVES

Functional studies on lncRNAs have been burgeoning in the past decade. Numerous efforts have been made to explore their biogenesis, expression, localization, turnover, conformation, and function. These efforts have led to our current understanding of how lncRNAs use different mechanisms to overcome their naturally low expression to effectively interact with protein partners. More efforts are certainly needed to understand particular lncRNP complexes by developing new approaches to decode their molecular basis for interactions and to visualize their dynamics at the single molecule and suborganelle levels.

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