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Integrated LncRNA Function Upon Genomic and Epigenomic Regulation

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Summary

Although some long noncoding (lnc)RNAs are known since the 1950s, the past 25 years have uncovered myriad lncRNAs with diverse sequences, structures, and functions. The advent of high-throughput and sensitive technologies has further uncovered the vast heterogeneity of lncRNA-interacting molecules and patterns of expressed lncRNAs. We propose a unifying functional theme for the expansive family of lncRNAs. At an elementary level, the genomic program of gene expression is elicited via canonical transcription and post-transcriptional mRNA assembly, turnover, and translation. Building upon this regulation, an epigenomic program refines the basic genomic control by modifying chromatin architecture as well as DNA and RNA chemistry. Superimposed over the genomic and epigenomic programs, lncRNAs create an additional regulatory dimension: by interacting with the proteins and nucleic acids that regulate gene expression in the nucleus and cytoplasm, lncRNAs help establish robust, nimble, and specific transcriptional and post-transcriptional control. We describe our present understanding of lncRNA-coordinated control of protein programs and cell fate, and discuss challenges and opportunities as we embark on the next 25 years of lncRNA discovery.

eTOC blurb

Herman *et al*. review progress in lncRNA research over the past 25 years. Vast and heterogeneous, lncRNAs interact broadly with gene regulatory machineries. By superimposing a layer of control upon genomic and epigenomic processes, lncRNAs modulate many levels of gene regulation, from transcription to protein modification.

Since the central dogma of molecular biology was proposed by Crick in 1958, we have marveled at the ever-expanding molecular complexity built upon this core process to enable the specific gene expression programs that sustain life. After the discovery of noncoding

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(nc)RNAs, transfer (t)RNAs, and ribosomal (r)RNAs in the 1950s, messenger (m)RNAs, identified in the 1960s, offered templates for protein synthesis (Brenner et al., 1961; Gros et al., 1961). The ncRNA family grew with the discovery of small nuclear (sn)RNAs and small nucleolar (sno)RNAs in the 1980s (Matera et al., 2007), but it was the discovery of small regulatory RNAs from the 1990s [micro (mi)RNAs, piwi-interacting (pi)RNAs, small interfering (si)RNAs] (Wilson and Doudna, 2013), that set the stage for a rapid escalation in the discovery of regulatory long noncoding (lnc)RNAs over the ensuing 25 years. The advent of genomic tiling arrays and especially high-throughput RNA-sequencing technologies in the mid 2000s fueled this explosion, uncovering a vast, versatile, and rich universe of lncRNAs (>200 nucleotides in length), spanning a large range of sizes, sequences, structures, and functions. These technologies soon revealed that a striking ~75% of the human genome is transcribed, and that only 2% of the genes transcribed encode mRNAs with protein-synthesis potential; the vast majority of transcription produces lncRNAs, as reported by the ENCODE Project Consortium (Dunham et al., 2012).

LncRNAs in this huge family are often classified based on their site of transcription relative to protein-coding genes, and thus there are enhancer lncRNAs, promoter lncRNAs, antisense lncRNAs (transcribed in antisense orientation from protein-coding genes), intergenic lncRNAs, and circular lncRNAs (circRNAs) arising from introns and/or exons that are excised and religated (Box 1). In general, lncRNA primary sequences are less conserved across species than mRNAs sequences, their secondary structures are often linked to their functions, and their expression is highly cell type-specific. The past 25 years have uncovered an astonishingly diverse array of mechanisms whereby lncRNAs influence upon gene expression programs and cell function. Despite their heterogeneity, lncRNA-regulated processes share three key features.

First, their complex influence is tied to their wide presence across the entire cellular space (Bridges et al., 2021; Carlevaro-Fita et al., 2019; Fazal et al., 2019). Nuclear lncRNAs associate with specialized domains like paraspeckles, nucleoli, and the lamina, as well as with chromosomes, chromatin domains, and gene regions; accordingly, they modulate nuclear processes like chromatin organization, and RNA transcription and splicing. Cytoplasmic lncRNAs interact with membrane-less cytosolic domains like stress granules, processing bodies, ribosomes, and the cytoskeleton, as well as with membranous cytoplasmic structures like the endoplasmic reticulum and mitochondria; accordingly, they regulate mRNA transport, stability, and translation, as well as protein stability, post-translational modification, and function. Second, lncRNA function is closely linked to their relative abundance. Besides their transcription rates, the relative levels of lncRNAs are influenced by their widely different stability; the presence of 5’ end m^7G caps and 3’ end poly(A) tails, structured 3’ ends, small nucleolar RNA-protein complexes (snoRNPs) at the ends, and covalent circularization of the 5’ and 3’ ends, all modulate their relative stability in the nucleus and the cytoplasm (Yin et al., 2012; Wu et al., 2017). Third, lncRNA function is directly associated to the molecules with which the lncRNAs interact. Although some lncRNAs have intrinsic catalytic function in the absence of proteins (e.g., ribozymes and riboswitches), and some lncRNAs can be translated in certain instances, the function of most lncRNAs is closely associated to their interaction with other nucleic acids and with RNA-binding proteins (RBPs).

Here, we review the progress over the past 25 years in learning about the functions of lncRNAs as regulators of gene expression and cell function. Using a few select examples, mostly from the mammalian world, we present an emerging picture in which there is a basic, foundational ***genomic*** control of gene expression by proteins regulating transcriptional and post-transcriptional processes that affect mRNA production, turnover, and translation. Superimposed upon this level is an ***epigenomic*** program that revises the genomic control by modifying DNA chemistry, RNA chemistry, and chromatin organization. We propose that lncRNAs create an additional dimension of control, superimposed upon genomic and epigenomic layers. In this dimension, lncRNAs form scaffolds to organize DNA regions and modulate transcription, recruit RNAs and cytoplasmic factors to sites of post-transcriptional control, and serve as assembly platforms for multiprotein complexes functionally linked: in effect, they enable a ***supragenomic*** layer of protein expression programs and cell fate (Figure 1).

This proposed supragenomic layer of control globally involves lncRNAs, but it does not occur in isolation. Instead, as we discuss here, it is carried out through the association of lncRNAs with individual proteins and protein complexes, with DNA and chromatin in different states, with RNAs coding and noncoding, and with machineries that control transcription, splicing, translation, phase-separation states, and more. While other ncRNAs like miRNAs, siRNAs, piRNAs, and snoRNAs, are functionally associated to lncRNAs, here we focus primarily on lncRNAs.

Supragenomic control of nuclear functions by lncRNAs

The past 25 years have firmly established that nuclear lncRNAs can influence many processes related to DNA replication, chromatin organization, and gene transcription (Figure 2). As the first functional roles for lncRNAs were in chromatin metabolism, it was generalized early on that lncRNAs had predominantly nuclear functions, although many lncRNA functions were identified in the cytoplasm soon afterwards. Here, we discuss key examples of the supragenomic control of gene expression by nuclear lncRNAs (Figure 3).

LncRNAs implicated in chromatin dynamics

The packaging of DNA and organization into three-dimensional (3D) structures are critical for enabling the carefully orchestrated interactions within and among chromosomes that ensure tight gene expression patterns and genetic transmission during cell division. DNA wraps around histones to form nucleosomes, which then cluster to form loops organized into topologically associated domains (TADs); these domains in turn aggregate into compartments that occupy chromosome territories across the nuclear space. The chromatin must have a stable organization, but it must also be capable of changing to meet the needs of the cell (Wachsmuth et al., 2008). This organization began to be investigated over a century ago and was known to comprise DNA, proteins, and RNA (reviewed by Nickerson et al., 1986; Olins and Olins, 2003). The past 25 years have uncovered many diverse and unexpected ways in which lncRNAs contribute to chromatin regulation.

Chromatin organization.—Many examples have emerged of lncRNAs providing important tiers of control for chromatin assembly. Numerous lncRNAs help to organize

chromatin into active and inactive domains by interacting with major chromatin-modifying proteins like polycomb repressive complex 2 (PRC2), Yin Yang 1 (YY1), and CCCTC-binding factor (CTCF) (Belak and Ovsenek, 2007; Beltran et al., 2016; Cifuentes-Rojas et al., 2014; Hendrickson et al., 2016; Sun et al., 2013; Zhao et al., 2008). One of the first lncRNAs reported, *XIST,* provides scaffolding for chromatin-modifying enzymes like SMCHD1 to drive X-chromosome inactivation (Engreitz et al., 2013; Wang et al., 2018), while telomeric repeat-containing RNAs (*TERRA*) recruit chromatin-modifying proteins TRF2 and PRC2 to support heterochromatin formation at telomeres (Deng et al., 2009; Montero et al., 2018), and lncRNA *ANRIL* regulates neighboring transcription of *CDKN2A* and *CDKN2B* mRNAs by recruiting PRC1 and PRC2 to specific gene promoters in senescent cells (Yap et al., 2010). In other examples, transcription of *Igf2r* non-protein coding RNA (*Aim*) helps to spread polycomb complexes across chromatin, and *HOTAIR* may facilitate chromosome condensation and gene silencing at least in part by interacting with epigenetic regulators PRC2 and LSD1 (Gupta et al., 2010; Latos et al., 2012; Rinn et al., 2007; Tsai et al., 2010). A few circRNAs were also found to modulate transcription in related ways; *circMRPS35* recruited an acetyltransferase to gene promoters, whereas *circFECR1*, *circAFG1*, and *circLRP6* recruited methylating enzymes to inactivate gene promoters (Chen et al., 2018; Jie et al., 2020; Wang and Li, 2020; Zheng et al., 2019).

Chromatin looping.—Following the discovery that transcriptional activity assists with chromatin topology and nuclear compartmentalization, transcription of lncRNAs was likewise found to influence chromatin architecture and looping (Hubner et al., 2013; Mao et al., 2011). A ‘cat’s cradling’ model was postulated in which transcribing lncRNAs successively opened chromatin forming ‘grip holds’ to guide looping interactions (Mele and Rinn, 2016). Enhancer lncRNAs and enhancer-associated lncRNAs (eRNAs, elncRNAs) were also implicated in chromatin topology; for example, transcription of lncRNA *ThymoD* in T-cells triggered local demethylation at CTCF sites, creating a loop that brought together the enhancer and promoter regions of *Bcl11b* during T-cell fate determination (Isoda et al., 2017). In keeping with earlier results that *LINoCR* transcription repositioned nucleosomes and expelled CTCF complexes (Lefevre et al., 2008), genome-wide studies found that RNA polymerase II (pol II) transcription displaced CTCF-anchored chromatin loops and remodeled local architecture (Heinz et al., 2018). Interestingly, CTCF itself interacts with many lncRNAs (Kuang and Wang, 2020) that likely influence its activity. Other lncRNAs involved in transcription-enabled chromatin looping include *Airn* and *Lockd* (Paralkar et al., 2016; Sleutels et al., 2002), and a full class of trait-relevant long-intergenic ncRNAs (TR-lincRNAs) (Tan et al., 2017) and topological anchor point RNAs (tapRNAs) (Amaral et al., 2018). In sum, superimposed on previously known paradigms of chromatin looping, lncRNAs are now found to perform additional regulatory tiers that influence transcription.

R-loops.—Although proteins mediate many interactions between lncRNAs and chromatin, lncRNAs also directly contact DNA, forming hybrid structures that modify chromatin accessibility. Molecular RNA-DNA-DNA hybrids (R-loops), driven by sequence complementarity, are postulated to be widespread and necessary for lncRNA functions, particularly by contributing to gene activation or silencing (Blank-Giwojna et al., 2019; Kuo et al., 2019; Martianov et al., 2007; Schmitz et al., 2010). A recent genome-wide analysis

concluded that lncRNA:DNA triplex-forming structures are enriched in TADs and may predict TAD formation areas (Soibam and Zhamangaraeva, 2021). Many lncRNAs form R-loops in *cis*, with local biological impact; for instance, the *TCF21* antisense lncRNA *TARID* forms a loop at the *TCF21* promoter that induces *TCF21* transcription (Arab et al., 2019). In an interesting example from plants, the lncRNA *APOLO* forms local R-loops in *cis* but also distant R-loops in *trans*, jointly coordinating the expression of a subset of genes responsive to the plant hormone Auxin (Ariel et al., 2020). In addition to controlling the transcription of protein-coding genes, R-loops also play a large role in the transcription of lncRNAs; moreover, R-loops appear to function as intrinsic pol II promoters to drive transcription, and ablating R-loop formation decreased the transcription of antisense lncRNAs (Tan-Wong et al., 2019). CircRNAs may also form R-loop structures to alter transcription elongation, as shown for *circSMARCA5*, which forms an R-loop that pauses transcription at *SMARCA5* exon 15 and reduces SMARCA5 production (Xu et al., 2020).

Transcriptional regulation by lncRNAs

The past two decades have revealed that lncRNAs also influence transcriptional programs by interacting directly with the transcriptional machinery and repressing or activating it. Examples of transcriptional repression include *Airn,* which caused transcriptional pausing at the *Igf2r* promoter (Latos et al., 2012), and antisense lncRNA *GNG12-AS1,* which interfered with the transcription of protein-coding *DIRAS3* mRNA in the sense direction (Stojic et al., 2016). Examples of transcriptional activation include production of the heart development factor HAND2, which was transcriptionally enhanced by two nearby lncRNAs, *Uph* and *Hdn* (Anderson et al., 2016; Han et al., 2019; Ritter et al., 2019). In fact, a global *cis* function for lncRNAs promoting transcription has been proposed, as genes encoding chromatin-remodeling and transcription factors are preferentially located near sites of lncRNA transcription, pointing to a cooperative role for lncRNAs to produce transcription factors (Ponjavic et al., 2009). LncRNAs directly binding transcription factors to influence gene transcription include lncRNA *PANDA*, derived from the *CDKN1A* promoter, which binds nuclear transcription factor Y subunit α (NF-YA) in senescent cells (Hung et al., 2011), lncRNA *PVT1*, whose functions include blocking phosphorylation and degradation of the transcription factor MYC (Tseng et al., 2014), and *LincRNAp21*, induced by p53 and capable of binding HNRNPK in the nucleus to repress transcription (Huarte et al., 2010).

In recent years, nuclear circRNAs modulating gene transcription have also been identified. Numerous circRNAs were found associated with pol II, many of them exon-intron circular RNAs (EIciRNAs) like *circEIF3J* and *circPAIP2*, which interact with snRNA *U1* and promote the transcription of the respective parent genes (Li et al., 2015). Other circRNAs regulate transcription by interacting with transcription factors, as shown for *circHuR* and *circSamd4*, or by inducing promoter DNA demethylation, as shown for *FECR1* circRNA (Chen et al., 2018; Pandey et al., 2020; Yang et al., 2019). Interestingly, intronic circRNAs (ciRNAs) appear to regulate transcription elongation, a step not typically controlled by linear lncRNAs (Faust et al., 2012; Zhang et al., 2013).

Splicing control by lncRNAs

The complex process of splicing is traditionally known to involve short, *cis*-regulatory elements in pre-mRNA and *trans*-acting splicing factors. Over the past two decades, lncRNAs have been found to superimpose key layers of regulation upon splicing. Because both canonical splicing and backsplicing to generate circRNAs largely use the same splicing machinery, it was postulated early on that circRNAs might alter pre-mRNA splicing and mRNA production. In fact, it was proposed that the canonical splicing machinery and the backsplicing machinery compete for shared factors, such that there is a balance between pre-mRNA splicing and circRNA backsplicing (Liang et al., 2017). An example of this balance is the *MBL* locus, which encodes the splicing factor muscleblind (MBL). MBL promotes circularization to yield *circMbl*, and interestingly, *circMbl* binds and sequesters MBL; thus, low MBL levels favors splicing to generate mature *MBL* mRNA, while high MBL levels favors backsplicing to generate instead *circMbl* (Ashwal-Fluss et al., 2014). On the other hand, circRNAs may also promote alternative splicing of the host transcript. As an example, *circSEP3*, arising from exon 6 of *SEP3* DNA, forms an R-loop, in turn slowing down transcription and promoting splicing of mature *SEP3* mRNA (Conn et al., 2017). Instances of circRNAs adding tiers of control on splicing will likely grow, given their innate partnership with the splicing machinery.

The role of linear lncRNAs in splicing is less intuitive, but interesting evidence is emerging. The strong correlation between alternative splicing and the transcription of antisense RNAs has led to the hypothesis that the two processes are connected and evolutionarily conserved (Morrissy et al., 2011). In this scenario, natural antisense transcripts (NATs) transcribed from the opposite strand can form RNA-RNA hybrids with sense pre-mRNAs to modulate the production of splice isoforms (Bardou et al., 2011); for example, lncRNA *asFGFR2* regulates alternative splicing of *FGFR2* mRNA by interacting with the chromatin-modifying proteins PRC2 and KDM2a and thus creating a splicing-specific chromatin signature (Gonzalez et al., 2015). Conversely, transcription of antisense linear lncRNAs can alter pre-mRNA splicing by masking the splice position and inhibiting further processing. An example of such regulation is NAT *Zeb2*, which prevents splicing to maintain a 5'UTR *Zeb2* intron encoding an internal ribosome entry site (IRES) necessary for translation (Beltran et al., 2008). Others function by attenuating pol II transcriptional elongation or by triggering premature termination to affect isoform expression, as is the case for antisense *RNAβ* (Stork et al., 2007). Further regulation of splicing factors by lncRNAs is linked to paraspeckles and nuclear bodies (below). Through these actions, lncRNAs help to establish and refine patterns of alternative splicing and protein isoform production.

LncRNAs in nuclear bodies

Responsible for igniting early interest in the functions of nuclear lncRNAs in the 2000s, membrane-less nuclear bodies such as nucleoli, nuclear speckles, and paraspeckles exist both constitutively and in response to changing cell states (Mao et al., 2011). We highlight distinct roles of linear lncRNAs as architectural (arc)RNAs and backbones of nuclear condensates, and discuss their unique ability to anchor to specific nuclear regions and coordinate the assembly of many RNA- and DNA-binding proteins (Chujo et al., 2016).

NEAT1 is an essential component of paraspeckles, where it forms a scaffold for protein binding (Chen and Carmichael, 2009; Clemson et al., 2009; Hutchinson et al., 2007; Sasaki et al., 2009; Sunwoo et al., 2009). In particular, the 3' end of a long *NEAT1* isoform has subdomains that recruit core paraspeckle proteins NONO and SFPQ to initiate liquid-liquid phase separation (Yamazaki et al., 2018). *NEAT1* can alter splicing by sequestering splicing factors [SR (serine/arginine-rich) proteins] in the condensates to help balance mRNA isoforms (Cooper et al., 2014; Jiang et al., 2009). The pioneering discovery of a lncRNA with various important roles in the subnuclear space set the stage for identifying other types of RNAs that localize to paraspeckles (Fox et al., 2018; Prasanth et al., 2005).

The abundant lncRNA *MALAT1* localizes to nuclear speckles (Hutchinson et al., 2007; Wilusz et al., 2012). Unlike *NEAT1*, *MALAT1* is not essential for nuclear speckle formation, but does orchestrate the complex layering of these nuclear bodies: *MALAT1* is positioned near the periphery while splicing factors SON and SC35 are located centrally (Fei et al., 2017). Like *NEAT1, MALAT1* also regulates pre-mRNA splicing by modulating the availability of splicing factors like SR proteins and transcriptional repressors like PC2 (Engreitz et al., 2014; Tripathi et al., 2012; Yang et al., 2011). Techniques such as RNA *in situ* conformation sequencing (RIC-seq) have provided deep insight supporting a function for *MALAT1* as an "RNA hub" for other nuclear RNAs including like *NEAT1* and *U1* (Cai et al., 2020).

Additional scaffolding of nuclear bodies has been found to be mediated by lncRNAs such as SPAs (5' snoRNA-capped and 3' polyadenylated lncRNAs) (Wu et al., 2016; Yin et al., 2012), whereas nuclear stress bodies were assembled by lncRNAs such as *HSATIII*, through the retention of SR proteins, transcription factors, and scaffold attachment proteins (Jolly et al., 1999). The interactions among these lncRNAs and RBPs were linked to a range of diseases associated with alternative splicing of key transcripts (Yap et al., 2018). Although lncRNAs organizing higher-order nuclear architecture remain to be identified, functional intergenic repeating RNA element (*FIRRE*), expressed from the X chromosome, was capable of bringing together different chromosomes (Hacisuleyman et al., 2014).

Mechanisms of lncRNA nuclear export and retention

We have discussed the functions of many nuclear-enriched lncRNAs, but few lncRNAs are exclusively nuclear or cytoplasmic, and the determinants of their subcellular distribution are not fully known. Early studies suggested that nuclear lncRNAs lacked a default nuclear export pathway (Palazzo and Lee, 2018). However, a recent survey identified NXF1 and TREX as required components for lncRNA nuclear export, with NXF1 being particularly important for the export of long RNAs with high A/U content and few exons, two features often found in lncRNAs (Zuckerman et al., 2020; Zuckerman and Ulitsky, 2019). This discovery linked exon organization and sequence composition as features driving nuclear export for some lncRNAs. Although fewer studies have focused on circRNA export, a recent study found that circRNA length influenced nuclear export, with short and long circRNAs utilizing helicases UAP56 and URH49, respectively, to exit the nucleus (Huang et al., 2018). Soon afterwards, another study found that circRNA N^6-methyladenosine (m^6A)

modification promoted its nuclear export (Chen et al., 2019). The compartmentalization and transport of circRNAs remain areas of active study.

Likewise, elucidating the nuclear retention mechanisms for lncRNAs is critical for understanding their function. Over the years, a number of nuclear retention motifs in lncRNA primary sequences have been identified that drive their nuclear localization (Carlevaro-Fita et al., 2019; Lubelsky and Ulitsky, 2018; Shukla et al., 2018; Zhang et al., 2014). Some nuclear retention signals like repeat insertion domains of lncRNAs (RIDLs) evolved from transposable elements (Carlevaro-Fita et al., 2019; Chillon and Pyle, 2016; Nguyen et al., 2020), while others include repeating RNA domains (RRDs) that direct intracellular localization, as is the case with *FIRRE*, which uses RRDs to interact with hnRNPU to localize to chromatin (Hacisuleyman et al., 2016). These retention elements bind *trans*-acting factors to prevent translocation and maintain nuclear enrichment (Guo et al., 2020b; Schiene-Fischer, 2015). Besides primary sequences, lncRNA secondary structures and post-transcriptional modifications provide another layer of complexity for nuclear retention. A recent study assessing lncRNA features like splicing, architecture, chromatin modifications, and sequence motifs to predict subcellular distribution found that pol II pausing and chromatin marks significantly influenced lncRNA localization (Zuckerman and Ulitsky, 2019). Inefficient splicing of lncRNAs may also contribute to nuclear retention due to poor export efficiency (Guo et al., 2020b). As mentioned earlier, intron-containing circRNAs may remain nuclear to modulate processes like transcriptional elongation, but subsets of exonic circRNAs are also predominantly nuclear (Wang et al., 2019; Yang et al., 2017a; Yang et al., 2017c). For example, exonic *circAmotl1* increased nuclear retention of the proto-oncoprotein MYC and promoted MYC binding to several target promoters (Yang et al., 2017a) and associated with STAT3 to enable its nuclear translocation (Yang et al., 2017c). We are likely just beginning to scratch the surface on the functions of linear and circular lncRNAs in the nucleus.

Supragenomic control of cytoplasmic functions by lncRNAs

Although lncRNA function initially appeared restricted to the nucleus, work over the past 25 years has uncovered many ways in which cytoplasmic lncRNAs superimpose critical regulatory tiers of protein production and function (Figure 2). After export to the cytosol, lncRNAs associate with RBPs and/or nucleic acids, and may be directed to specific cytosolic domains (e.g., processing bodies, stress granules, or polysomes) or organelles (endoplasmic reticulum or mitochondria). As discussed here, cytoplasmic lncRNAs contribute critical layers of refinement, strength, and specificity to canonical cytoplasmic processes such as mRNA turnover and transport, as well as protein translation, stability, and assembly, mitochondrial function, cytoskeletal dynamics, and cell-cell interactions (Figure 4).

LncRNAs affecting mRNA turnover

Cytoplasmic mRNA degradation is driven by deprotecting the 5’ and 3’ ends (5’ decapping and 3’ deadenylation) coupled to exonucleolytic degradation and endonucleolytic cleavage (Schoenberg and Maquat, 2012). These processes are regulated by complex sets of RBPs that recognize labile mRNAs and modulate their recruitment to ribonucleases present in

the cytosol or in degradation centers like the exosome. By modulating mRNA stability, RBPs enable adaptive changes in the transcriptome of cells responding to proliferation, differentiation, activation, and stress. The turnover of mRNAs is further governed by microRNAs, a class of small (~22 nt) ncRNAs that can promote the decay of mRNAs with which they share partial complementarity; microRNAs recruit the RNA-induced silencing complex (RISC), a multiprotein complex that includes the endoribonuclease Argonaute that cleaves the mRNA (Pratt and MacRae, 2009). Together, microRNAs and RBPs tightly regulate the steady-state levels of mRNAs.

Several lncRNAs operate upon processes that modify mRNA turnover. In Staufen 1 (STAU1)-mediated mRNA decay (SMD), lncRNAs were found to either stabilize or destabilize specific target mRNAs. For instance, the 3'UTRs of some mRNAs partially complement lncRNAs, and the resulting double-stranded (ds)RNAs can trigger SMD. In the case of lncRNAs containing repetitive elements like Alu [*½-sbsRNA* (half STAU1-binding site)] or SINEs (short interspersed elements), the resulting dsRNAs trigger mRNA decay through SMD (Gong and Maquat, 2011; Wang et al., 2013). On the other hand, terminal differentiation-induced ncRNA (*TINCR*), a lncRNA highly abundant during epidermal differentiation and capable of binding mRNAs bearing a 25-nt *TINCR* box, also interacted with STAU1 but instead stabilized subsets of mRNAs encoding differentiation proteins (Kretz et al., 2013).

In other examples of lncRNAs forming dsRNAs that affect mRNA outcome, the lncRNA *BACE1-AS* stabilized *BACE1* mRNA, which encodes β-secretase 1 (BACE1), the enzyme that cleaves amyloid precursor protein (APP) to release the neurotoxic Aβ peptide in Alzheimer's disease (Faghihi et al., 2008). The dsRNA region of complementarity shared by *BACE1* mRNA and *BACE1-AS* blocked a miR-485 site, rendering *BACE1* mRNA stable and increasing BACE1 production (Faghihi et al., 2010). In a recent example, lncRNA *OIP5-AS1*, abundant in human skeletal myoblasts, associated through partial complementarity with *MEF2C* mRNA and stabilized it by recruiting HuR to the *MEF2C* 3'UTR, rising MEF2C production and promoting myogenesis (Yang et al., 2020).

Additional lncRNAs have been identified that influence mRNA turnover by sequestering decay-promoting RBPs. The abundant cytoplasmic lncRNA *NORAD* (noncoding RNA activated by DNA damage) contains many binding sites for Pumilio 1/2 (PUM1/2), an RBP that typically reduces the stability and translation of target mRNAs. The efficient sequestration of Pumilio by *NORAD* enabled the production of several proteins involved in maintaining genomic stability. In cells, the genomic instability seen after ablating *NORAD* was rescued by ectopic expression of *NORAD* containing Pumilio binding sites but not by mutant *NORAD* lacking Pumilio binding sites (Lee et al., 2016).

Given their intrinsic stability, circRNAs have been proposed to modulate the decay of mRNAs by binding and 'sponging' microRNAs that would otherwise repress such mRNA. A prominent example of this function was provided by competing endogenous (ce)RNA *CDR1as*/*ciRS-7*, which bears dozens of miR-7 binding sites and can sponge this microRNA (Memczak et al., 2013; Hansen et al., 2013). Some examples of circRNAs sequestering decay-promoting RBPs such as AUF1 (AU-binding factor 1) for *circPCNX* have also

been reported (Tsitsipatis et al., 2021). The low abundance of most circRNAs makes such sponging functions relative rare.

LncRNAs modulating functions of cytoplasmic phase-separation bodies (SGs, PBs)

RBPs and mRNAs assemble into cytoplasmic membrane-less phase-separation bodies, such as stress granules (SGs) and processing bodies (PBs). These particles typically harbor untranslated mRNAs, likely serving as constitutive or stress-induced reservoirs of specific mRNA subsets (Hubstenberger et al., 2017; Kedersha et al., 2005). Although lncRNAs are much less abundant than mRNAs overall in SGs and PBs, they are increasingly recognized as contributing to their assembly and function.

A function was proposed for specific lncRNAs at the interface between PBs and SGs (Pitchiaya et al., 2019). LncRNAs *THOR* and *ARlnc1*, interacting with the PB proteins IGF2BP1 and HuR, respectively (Pitchiaya et al., 2019; Zhang et al., 2018), were found in the outer shell of PBs. The lncRNAs influenced the translation and stability of interacting mRNAs by recruiting them to sites of translational repression (PB cores, SGs), degradation or translation (Pitchiaya et al., 2019).

SGs form dynamically in response to stress stimuli and represent sites of aggregation of untranslating mRNAs (Aulas et al., 2017; Kedersha et al., 2005; Protter and Parker, 2016). SGs assemble via protein-protein interaction networks and recruit subsets of mRNAs during times of stress, but the mechanisms that select these mRNAs are unknown. Specialized transcriptomic analysis recently found that *NORAD* is present in SGs and interacts with other SG RNAs (Khong et al., 2017). Moreover, the RNA helicase eIF4A, which disrupts RNA-RNA associations, prevented the recruitment of RNAs including *NORAD* to SGs (Tauber et al., 2020), although the association of *NORAD* with SG proteins like TIAR and TIA-1 may also contribute to its recruitment to SGs (Namkoong et al., 2018).

While some roles for linear lncRNAs upon the assembly and function of phase-separation bodies are emerging, less is known about circRNAs in these spaces. Oxidative stress was recently found to promote the association of *circ-Hdgfrp3* with neuronal SGs, although in amyotrophic lateral sclerosis (ALS), *circ-Hdgfrp3* associated instead with cytoplasmic aggregates of a mutant form of the RBP FUS linked to ALS pathology (D'Ambra et al., 2021).

The cytoskeleton in lncRNA localization

LncRNAs are beginning to gain recognition in cytoskeletal dynamics. In one study, the lncRNA *TUG1* (taurin upregulated gene 1) promoted the interaction of enhancer of zeste homolog 2 (EZH2) with α-actin (ACTA1). This interaction led to the methylation of ACTA1 and to an acceleration of the polymerization of filamentous F-actin in vascular smooth muscle cells (Chen et al., 2017). Another lncRNA capable of influencing the function of actin filaments, *CRYBG3* bound instead to globular actin (G-actin), blocked the polymerization of actin filaments, and suppressed cytokinesis (Pei et al., 2018). In addition to suppressing the formation of a functional contractile ring needed to complete cell division, *CRYBG3*-bound G-actin sequestered the protein MAL in the cytoplasm, preventing

the formation of the transcriptionally active MAL-SRF (serum response factor) complex, and blocking the transcription of immediate early genes (Pei et al., 2018).

CircRNAs are also believed to interact with the cytoskeleton, as they can be found at sites distant from the nucleus, such as neuronal synapses. The molecular details of their mobilization to synaptic regions are unknown, although they were postulated to recruit RBPs, microRNAs or other nucleic acids to distal sites (You et al., 2015).

LncRNAs influencing translation

Translation is a complex process whereby mRNA molecules associate with ribosomes to serve as templates for protein synthesis. LncRNAs provide a regulatory overlay that influences protein production in different ways: they can base-pair with mRNAs to promote or repress translation, alter the availability of translation regulatory factors, and associate with ribosomes directly, the latter scenario resulting in protein production and altered lncRNA turnover.

Base pairing of *LincRNAp21* with the *JUNB* and *CTNNB1* mRNAs through several regions of complementarity along these two mRNAs led to reduced mRNA association with polysomes and lowered production of JUNB and β-catenin (CTNNB) in cancer cells. This repression was linked to the recruitment of the translational repressor RCK to the *LincRNAp21*-mRNA complexes (Yoon et al., 2012). In an example of the opposite mode of action, base-pairing between the antisense lncRNA *AS-Uchl1* and *Uchl1* mRNA (encoding ubiquitin carboxy-terminal hydrolase L1) promoted translation of UCHL1. Although *AS-Uchl1* is typically nuclear, stress conditions led to its export to the cytoplasm, where the *SINE B2* RNA element in *AS-Uchl1* bound to *Uchl1* mRNA and enhanced its translation (Carrieri et al., 2012). Antisense lncRNAs can also suppress translation, as documented for the interaction of antisense lncRNA *PYCARD-AS1* with *PYCARD* mRNA, which reduced ribosome assembly and PYCARD translation. Interestingly, *PYCARD-AS1* further repressed *PYCARD* mRNA transcription in the nucleus by recruiting transcriptional repressors DNMT1 and G9a to the *PYCARD* promoter (Miao et al., 2019).

Some circRNAs may also influence translation. For example, binding of HuR to the *PABPN1* 3'UTR promoted PABPN1 translation. In this paradigm, high levels of *circPABPN1*, generated from an exon of *PABPN1* pre-mRNA bearing numerous HuR-binding sites, selectively sequestered HuR away from *PABPN1* mRNA and lowered PABPN translation (Abdelmohsen et al., 2017).

LncRNAs with protein-coding potential

In addition to modulating translation of mRNAs through binding the mRNAs directly or by altering the availability of RBPs that modulate translation, many cytoplasmic lncRNAs directly associate with ribosomes (Carlevaro-Fita and Johnson, 2019; Ruiz-Orera and Albà, 2019). Although the consequences of these interactions are not uniform, it is clear that many lncRNAs encode small peptides like myoregulin (MLN), dwarf open reading frame (DWORF), mitoregulin (MTLN), HOXB-AS3 peptide, and many others (Anderson et al., 2015; Huang et al., 2017; Matsumoto et al., 2017; Nelson et al., 2016; Stein et al., 2018). However, not all lncRNAs associated with polysomes are translated (Banfai et al., 2012;

Guttman et al., 2013) and many are instead degraded by nonsense-mediated decay (NMD; Carlevaro-Fita et al., 2016). Moreover, short open reading frames in the 5' segments of lncRNAs led to the ribosomal localization and NMD sensitivity of some lncRNAs (Smith et al., 2014), as shown for lncRNA *GAS5*, which bears premature stop codons (Smith and Steitz, 1998; Tani et al., 2013).

Although circRNAs lack 5' cap structures, a recent report identified IRES elements in thousands of circRNAs that facilitated the translation of encoded proteins in a tissue-specific manner (Chen et al., 2021). The authors followed up on *circFGFR1*, expressing protein circFGFR1p, a protein capable of functioning as a dominant-negative FGF receptor to inhibit proliferation in response to heat stress (Chen et al., 2021). The proteins encoded by linear and circular lncRNAs are intensely studied at present. The apparent paradox embodied by lncRNAs that have coding potential is discussed below (Perspectives section).

LncRNAs influencing post-translational protein modification and stability

With the flow of genetic information typically including post-translational modification, cytoplasmic lncRNAs are increasingly recognized to alter protein functionality after proteins are synthesized. For example, *lnc-DC*, preferentially expressed in dendritic cells, associated with the C-terminus of STAT3 in the cytoplasm. This interaction increased the levels of STAT3 phosphorylation, as *lnc-DC* binding to STAT3 suppressed the phosphatase activity of SHP1/PTPN6 (Wang et al., 2014). In another example, the lncRNA *NKILA* (NF-κB-interacting lncRNA) helped to keep low levels of NF-κB activity by associating with the NF-κB–IκB complex, as *NKILA* binding blocked IκB phosphorylation by the kinase IKK and prevented the activation of NF-κB; accordingly, low levels of *NKILA* led to elevated NF-κB activity in cancer cells (Liu et al., 2015).

Protein expression programs are also controlled via complex and precise protein degradation mechanisms. Here too, lncRNAs can offer a key layer of control to coordinate the degradation of existing proteins. For example, the lncRNA *HOTAIR* promoted ubiquitin-mediated proteolysis in the cytoplasm by binding E3 ubiquitin ligases DZIP3 and MEX3B and their respective ubiquitination substrates, ATXN1 and SNUPN. Through these associations, *HOTAIR* facilitated the ubiquitination of ATXN1 and SNUPN and accelerated their degradation, as shown in senescent cells (Yoon et al., 2013).

In an example of protein stabilization, the lncRNA *FAST* (*FOXD3-AS1*) prevented the degradation of β-catenin. In human embryonic stem cells, *FAST* associated with the WD40 motif (implicated in protein-protein interactions) of the E3 ubiquitin ligase β-TrCP (β-transducin repeats-containing protein). This interaction prevented the association of β-TrCP with phosphorylated β-catenin and blocked β-catenin degradation, in turn activating WNT signaling (Guo et al., 2020a).

Mitochondrial lncRNAs

Mitochondria are membrane-enclosed organelles essential for energy production in mammalian cells. In humans, the mitochondrial DNA genome (mtDNA) is transcribed into 11 mRNAs that encode 13 proteins, 2 rRNAs (*12S* and *16S* rRNA), and 22 transfer tRNAs (Mercer et al., 2011). In addition, mitochondria-encoded linear RNAs (mtlncRNAs) such

as *lncND5*, *lncND6*, and *lncCyt b* were identified by high-throughput RNA-seq analysis (Rackham et al., 2011; Zhang et al., 2021a). The past two decades have begun to identify the supragenomic impact of mitochondrial lncRNAs, including lncRNAs transcribed in the nucleus and imported into mitochondria, on mitochondrial homeostasis and energy metabolism.

In fibroblasts from patients with nonalcoholic steatohepatitis (NASH), three mtcircRNAs were selectively reduced. Ectopic overexpression of one of them, *SCAR* (steatohepatitis-associated circRNA ATP5B regulator), significantly decreased mitochondrial and cytosolic ROS (Zhao et al., 2020a). *SCAR* was found mainly in the mitochondrial matrix and matrix-facing inner membrane and associated with ATP5B; through this interaction, *SCAR* blocked the mitochondrial permeability transition pore (mPTP) and reduced mROS output.

Mitochondria may also harbor lncRNAs transcribed from nuclear DNA (Jeandard et al., 2019). The lncRNA component of the RNA processing endoribonuclease (*RMRP*) was transcribed in the nucleus, exported to the cytosol by HuR, internalized into mitochondria by PNPase, and retained in mitochondria by the RBP GRSF1; these effects were linked to a role for *RMRP* in mtDNA replication (Noh et al., 2016). Another prominent lncRNA transcribed in the nucleus, *SAMMSON*, positively regulated the localization of p32 (C1QBP) in mitochondria, in turn increasing the levels of 1*6S* rRNA, the abundance of mitochondrially encoded proteins COX2 and ATP6, and the activity of the respiratory complexes I and IV (Leucci et al., 2016). In one more example, in response to norepinephrine, *LINC00473* was actively transcribed in the nucleus, translocated to the cytoplasm, and resided at the interphase of mitochondria and lipid droplets. Direct interaction of *LINC00473* with Perilipin 1 (PLIN1) modulated mitochondrial fusion and fission activity, underscoring a role for *LINC00473* as regulator of mitochondrial metabolic signaling (Tran et al., 2020).

Supragenomic impact of lncRNAs in endoplasmic reticulum, Golgi, and plasma membrane

The endoplasmic reticulum (ER) is a complex organelle primarily responsible for the proper folding of proteins. Among the chaperones in the ER, GRP78 facilitates the folding and assembly of nascent polypeptides and senses the accumulation of unfolded proteins, which activate the mammalian unfolded protein response (UPR) and trigger ER stress (Pobre et al., 2019). Many lncRNAs have been implicated in the UPR (Zhao et al., 2020b), but most of them did not directly associate with the ER.

The laminar shear stress-induced *SENCR* lncRNA interacted with the ER-anchoring domain of cytoskeleton-associated protein 4 (CKAP4), retaining CKAP4 at the rough ER. This complex prevented the association of CKAP4 with Cadherin 5 (CDH5), in turn promoting the localization of CDH5 to the plasma membrane. When *SENCR* levels declined, CKAP4 bound CDH5 and this led to the internalization and degradation of CDH5, in turn impairing adherens junction (AJ) function (Lyu et al., 2019). Similarly, another shear stress-induced lncRNA, *LASSIE*, associated with the ER but was required for the barrier function of endothelial cells in the direction of fluid flow. *LASSIE* helped anchor the cytoskeleton with AJs by binding cytoskeletal proteins such as nestin and AJ proteins PECAM-1 (platelet endothelial cell adhesion molecule-1) and VE-cadherin (Stanicek et al., 2020).

Among the few lncRNAs have been found in the Golgi apparatus, the lncRNA *Snhg1* (small nucleolar RNA host gene 1) was found to interact with VPS13D (vesicle trafficking protein vacuolar protein sorting 13 homolog D), a protein implicated in trafficking between the Golgi apparatus and the vascular system. The complex *Snhg1*-VPS13D was required for VPS13D to mobilize IL-7Rα (CD127) to the membrane, in turn affecting the differentiation of memory CD8 T cells (Zhang et al., 2021b). As our understanding of the noncoding transcriptome grows, other prominent lncRNAs with distinct cytoplasmic localization and function will certainly emerge.

Perspectives

The past 25 years have brought into view a legion of lncRNAs that enable critical layers of precision and complexity in gene expression programs. Highly versatile and diverse, lncRNAs create a supragenomic tier of gene regulation that integrates fundamental genomic and epigenomic tiers of gene control. Their rich influence is linked to the wide heterogeneity in lncRNA sequences, structures, interaction partners, and subcellular spaces of residence. In the nucleus, lncRNAs help organize chromatin, direct transcription, and modify nascent RNAs. In the cytoplasm, they modulate mRNA turnover, storage, and translation, as well as orchestrate protein processing events. Given the impact of lncRNAs on these essential cellular processes, their influence in development, physiology, and disease continues to be intensely studied.

A provocative contradiction in lncRNA nomenclature has surfaced in recent years. With the rapid improvement of proteomic methods to detect ever smaller and rarer peptides in the cell, the numbers of lncRNAs found to be translated is also increasing. Thus, the very name ‘long noncoding RNA’ appears inadequate to describe this growing group of lncRNAs. However, given that our knowledge of ‘potentially coding’ lncRNAs (Ruiz-Orera and Albà, 2019) is still evolving, it is not possible to categorically classify them according to translational status. Perhaps we are progressing towards a time in which we will not distinguish lncRNA from mRNA, and instead we consider RNAs on a ‘continuum of engagement in translation’. On one end of this continuum are the RNAs that are largely associated with ribosomes, primarily cytoplasmic, and actively translated; somewhere down the continuum are RNAs that are translated when needed, but may have other structural or catalytic activities; further down the continuum are those RNAs that mainly function outside of translation, perhaps are mostly nuclear, but could occasionally be translated to meet specific needs of the cell; and at the far end of this continuum, we would find RNAs that never appear to leave the nucleus, have no recognizable reading frames, and are not found associated with ribosomes. It is unclear at present if or when such view may be adopted.

Besides the information gained from progress in proteomic detection, current and future advances in lncRNA biology are being fueled by increasingly precise single-cell and spatial multiomics, high-resolution microscopy, single-molecule analysis methods, and advanced computing. Further progress towards elucidating molecular details and functions of lncRNAs will be accelerated as suitable animal models become routinely available, lncRNA nomenclatures are harmonized, and cell-specific and extracellular lncRNAs are comprehensively catalogued.

In sum, we have presented an integrated overview of lncRNA functions that have emerged over the past quarter century. We propose that lncRNAs associate with diverse molecules to build upon genomic processes (e.g., transcription, mRNA turnover, translation) and epigenomic processes (e.g., modifications of DNA, RNA, and histones) a supragenomic level of gene regulation. Beyond nucleic acids and proteins, lncRNAs might associate with molecules like lipids or carbohydrates to further expand their supragenomic influence on cell biology.

We anticipate that future lncRNA research will continue to uncover stunning and unanticipated biology illuminating the broad influence of lncRNAs on protein programs and cell functions. With the landscape of lncRNA biology increasingly coming into view, the diagnostic, prognostic, and therapeutic value of lncRNAs will also become more clearly understood. Judging from the escalation in interest over the past quarter century, the next 25 years of lncRNA research promise to be filled with progress, excitement, and opportunity.

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Box 1: The many monikers of lncRNAs

Throughout the years, lncRNAs have been named depending on features such as the genomic regions from which they originate or their structure, conservation, or function. Below are listed some of the most common types of lncRNAs.

lincRNAs: long intergenic noncoding RNAs

elncRNAs: enhancer-associated lncRNAs

plncRNAs/PROMPTS: promoter-associated lncRNAs

NATs: natural antisense transcripts, antisense lncRNAs

mtlncRNAs: mitochondrial DNA-encoded lncRNAs

snolncRNAs: intron-derived long noncoding RNAs with snoRNA ends

circRNAs: circular lncRNAs

EcircRNAs: circRNAs consisting of exons

IcircRNAs: circRNAs consisting of introns

EIcircRNAs: circRNAs consisting of exons and introns

ceRNAs: competing endogenous lncRNAs

mtcircRNAs: mitochondrial DNA-encoded circRNAs

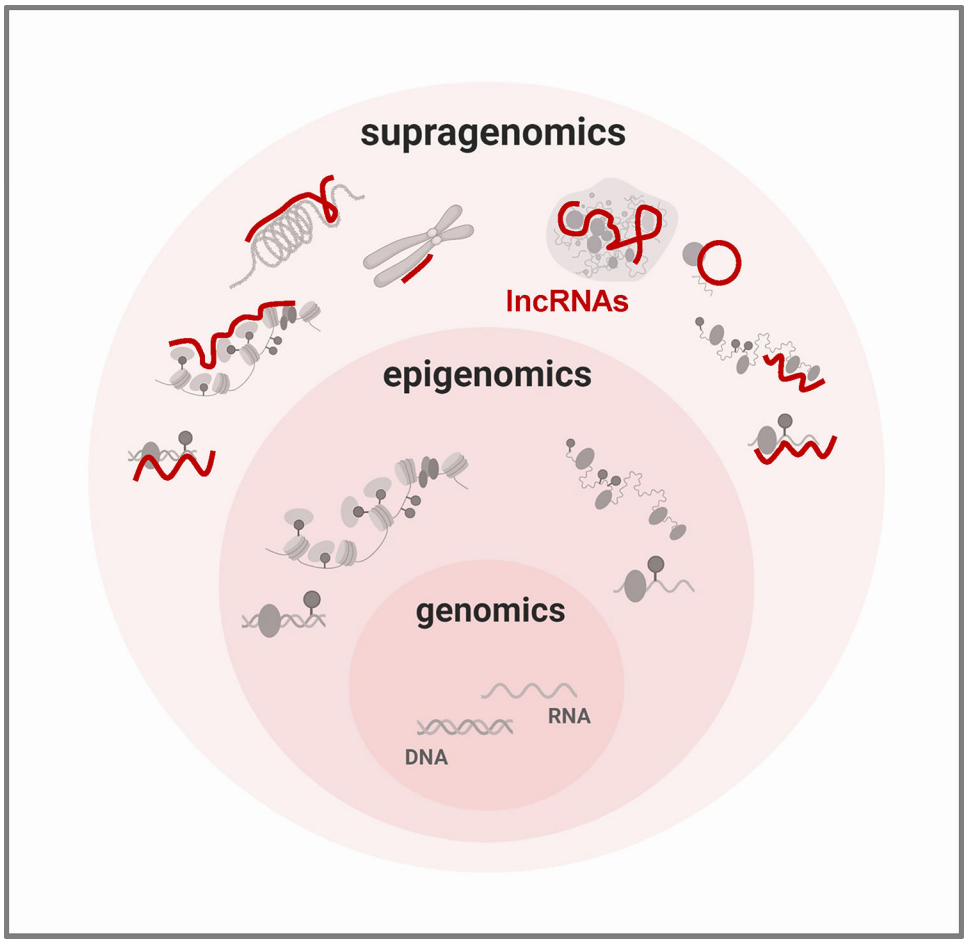

Figure 1. Conceptual summary of supragenomic regulation by lncRNAs.

Vast and heterogeneous, lncRNAs interact broadly with gene regulatory machineries. By providing a supragenomic layer of control built upon genomic and epigenomic processes, lncRNAs modulate many levels of gene regulation, from transcription to protein modification. Figure was prepared using BioRender.

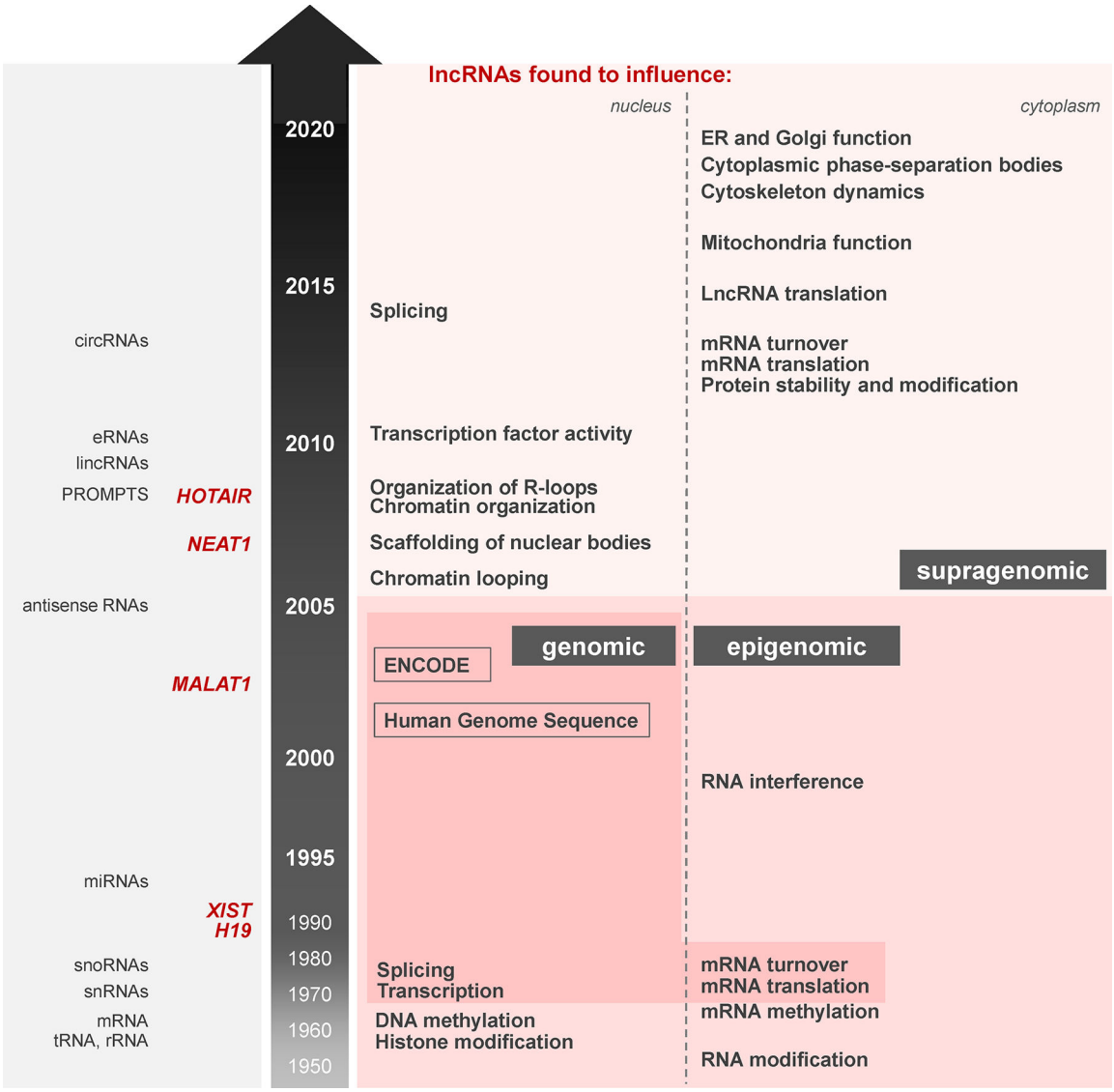

Figure 2. Timeline of lncRNA discoveries over the past 25 years.

Brief timeline of gene expression discoveries with a focus on the most recent 25 years. *Right*. Breakthroughs in genomic regulation [transcription, mRNA turnover, translation (darker shade)] and epigenomic changes [modifications of DNA, RNA, and histones (medium shade)] are highlighted at the bottom of the schematic. Superimposed upon genomic and epigenomic processes, a supragenomic array of regulatory schemes mediated by lncRNAs (light shade) in the nucleus and cytoplasm has emerged over the past 25 years, as discussed in this article. *Left*, key lncRNAs (red font) and RNA families (black font) are listed.

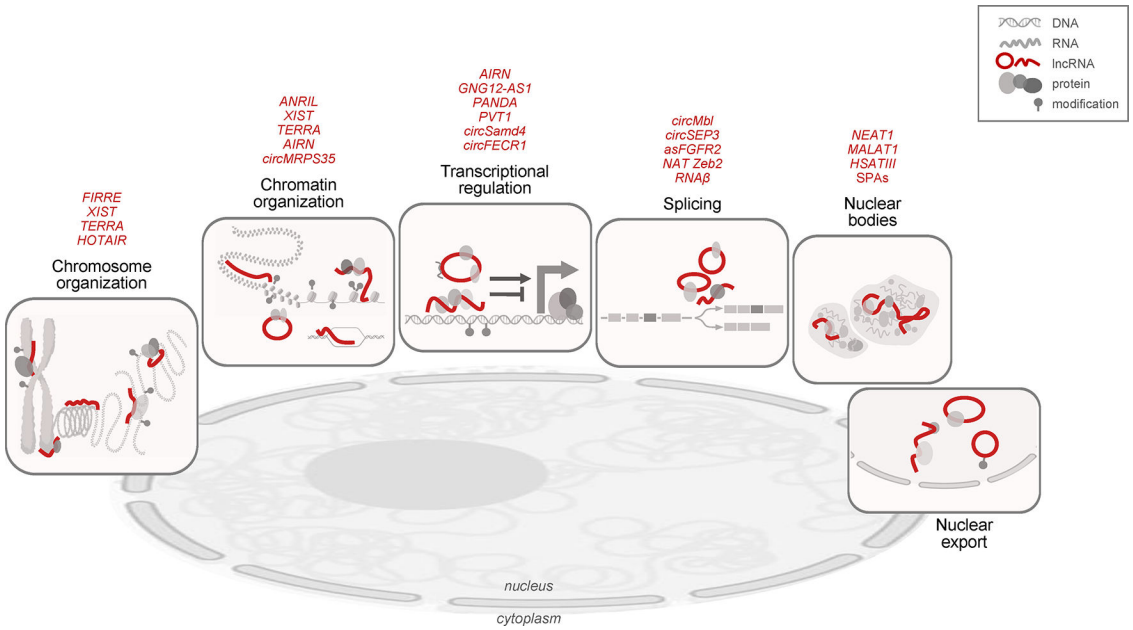

Figure 3. Emerging supragenomic functions of nuclear lncRNAs.

Representative functions of linear and circular lncRNAs in the nucleus (red lines), typically carried out by interacting with proteins and DNA, and modulating their activity. From left to right, lncRNAs can control chromosome organization at a full scale (e.g., X-chromosome inactivation) and at telomeres, in condensed and open chromatin, in chromatin loops, and in contacts across chromosomes. LncRNAs can also influence chromatin organization at a smaller scale by altering local DNA methylation and histone acetylation and/or methylation at nucleosomes, as well as by forming R-loops. LncRNAs directly influence transcription through proteins that bind enhancers or promoters and either promote or suppress the transcription machinery. LncRNAs can influence pre-mRNA splicing by affecting the presence of splicing factors to modulate intron inclusion or exclusion, and can assemble proteins and RNAs in nuclear bodies such as paraspeckles. At the far right, canonical and specialized proteins contribute to exporting linear and circular lncRNAs through nucleopores. Some lncRNAs representing these functions are named above the vignettes; molecules are not drawn at scale. Figure was prepared using BioRender.

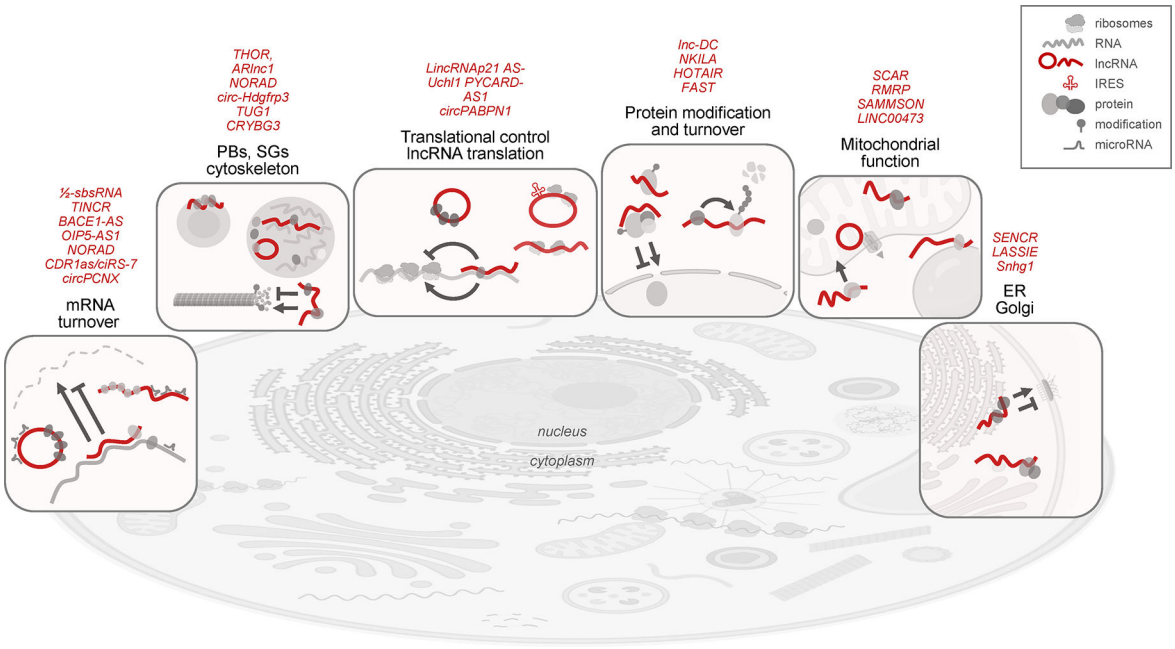

Figure 4. Emerging supragenomic functions of cytoplasmic lncRNAs.

Representative functions of linear and circular lncRNAs in the cytoplasm (red lines), typically elicited by interacting with proteins and altering the stability and localization of mRNAs, as well as the translation and post-translational modification of proteins. From left to right, lncRNAs can promote or repress mRNA decay, either by forming stretches of complementarity with mRNAs or by binding RBPs or microRNAs. LncRNAs associate with RBPs and mRNAs in cytosolic granules (PBs, SGs) to influence mRNA stability and translation, and promote or suppress microtubule polymerization through modification of actin monomers. Some lncRNAs can directly promote or suppress translation of mRNAs through regions of partial complementarity or by sequestering translation regulatory RBPs; moreove, some lncRNAs (linear and circular) may themselves engage with ribosomes and become translated. LncRNAs can affect protein function post-translationally by affecting protein modifications that alter their subcellular distribution or by scaffolding ubiquitin ligases that facilitate protein degradation. In mitochondria, lncRNAs can modulate mitochondrial gene transcription, respiration, and generation of reactive oxygen species; by mediating the interaction with lipid droplets, some lncRNAs can influence mitochondrial fusion and fission. Some lncRNAs associated with ER and Golgi apparatus can help mobilize proteins to the plasma membrane and some ER-associated lncRNAs can promote or reduce adherens junction function. LncRNAs representing these functions are listed above the vignettes; molecules are not drawn at scale. Figure was prepared using BioRender.