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Functional Insights into the Role of Nuclear-Retained Long Noncoding RNAs in Gene Expression Control in Mammalian Cells

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Abstract

The mammalian genome harbors thousands of long noncoding RNA (lncRNA) genes. Recent studies have indicated the involvement of several of these lncRNAs in the regulation of gene expression. LncRNAs play crucial roles in various biological processes ranging from epigenetic gene regulation, transcriptional control to post-transcriptional regulation. LncRNAs are localized in various subcellular compartments and major proportion of these are retained in the cell nucleus; and could be broadly classified as nuclear-retained lncRNAs (nrRNAs). Based on the identified functions, members of the nrRNAs execute diverse roles, including providing architectural support to the hierarchical subnuclear organization and influencing the recruitment of chromatin modifier factors to specific chromatin sites. In this review we will summarize the recently described roles of mammalian nrRNAs in controlling gene expression by influencing chromatin organization, transcription, pre-mRNA processing, nuclear organization and their involvement in disease.

Keywords

long noncoding RNA; nuclear-retained ncRNA; pre-mRNA splicing; MALAT1; nuclear domains; cell cycle; chromatin remodelling

Introduction

The human genome contains less than 2% protein coding sequences, which translate into the requisite number of proteins involved in the myriad of biological functions. The rest of the genomic sequences, till recently were considered as noncoding or “junk”. However, the development of high-throughput sequencing technology has led to the understanding that the majority of the human genome is transcribed to produce non protein coding RNAs (ncRNAs) and constitute the diverse and complex cellular transcriptome (Clark et al., 2011; Kapranov et al., 2007; Wilusz et al., 2009). Interestingly, the complexity of the nuclear transcriptome is fivefold higher compared to the cytosolic transcriptome in terms of transcribed base pairs (Cheng et al., 2005). The ncRNA is classified into broader classes depending upon the length of the RNA sequences. NcRNAs with length > 200 nucleotides are considered as long noncoding RNAs (lncRNAs) whereas the rest of the smaller transcripts are classified as small ncRNAs (Mercer and Mattick, 2013; Wilusz et al., 2009). According to the current lncRNA catalogs, the human genome encodes 10,000–15,000 lncRNAs but they generally display cell type specific expression with only a particular number of them expressed in a specific cell type or specific cell cycle stage. In general,

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lncRNAs are usually expressed at lower levels, compared to protein coding transcripts (Derrien et al., 2012; Sone et al., 2007; Tripathi et al., 2010).

Due to the complex and diverse nature of the transcripts, lncRNAs are classified into different groups. Depending upon the origin of transcripts with respect to the protein coding genes that are adjacent/overlapping with these transcripts, lncRNAs may be placed in one or more groups, i.e.,*sense* (noncoding transcript on the same strand) , *antisense* (noncoding transcript on the opposite strand), *bidirectional* (transcription of lncRNA gene, and a adjacent protein coding gene on the opposite strand in close genomic proximity), *intronic* (noncoding transcript generated from the intronic sequences of another transcript) and *intergenic* (noncoding transcript generated from the genomic region between two protein coding genes) (Carninci et al., 2005; Denoeud et al., 2007; Horiuchi and Aigaki, 2006; Mercer et al., 2009). Retrotransposons and pseudogenes are thought to be involved in the generation and diversification of lncRNAs (Kapusta et al., 2013; Singh and Rath, 2012). LncRNAs are also classified on the basis of their functions, i.e., *cis*-acting (Xist, Tsix, RepA, Kcnq1ot1, Hottip, ANRIL, Evf2, oct4-ps5) and *trans*-acting (pRNA, asOct4-ps5), structural scaffold (NEAT1,TUG1), enhancer (eRNAs, Xite, ncRNA-a), co-activator or co-repressor (SINE B2, SRA, Jpx, pRNA) and decoy (Tsix, PTEN-ps) (Khalil et al., 2009; Kim et al., 2010; Koziol and Rinn, 2010; Mohammad et al., 2009; Orom et al., 2010; Spitale et al., 2011; Wang et al., 2011a) lncRNAs.

The majority of the lncRNAs are transcribed by RNA polymerase II (Guttman et al., 2009; White, 2011). Chromatin of a transcriptionally active lncRNA locus displays marks of histone H3 tri-methylated at lysines 4 (H3K4me3) and 36 (H3K36me3) in their regulatory elements and transcription bodies, respectively, imparting the K4-K36 domain-specific chromatin signature. Several of the transcription factors, those that are involved in the transcription of protein-coding genes, are also shown to be responsible for the transcription of lncRNAs (Kim et al., 2005). The cellular expression of lncRNAs is very much cell type- and developmental stage-specific. Several recent studies indicate that the majority of lncRNAs remain associated with distinct subnuclear structures, including specific chromatin regions. Both the cell type specificity and the distinct localization pattern of lncRNAs suggest that their expression is highly regulated, and they control very specific cellular function(s).

The nucleus, one of the important organelles in a eukaryotic cell, harbours the majority of the hereditable genome (Tripathi and Prasanth, 2011). The nucleus is a very complex, dynamic, but highly organized organelle. It is sub-divided into a number of different nuclear zones/subnuclear organelles, characterized by the absence of a membrane boundary around them and also by the presence of a unique set of proteins and/or RNAs within them. Some of the better defined subnuclear domains include nucleoli, perinuclear compartment (PNC), PML (promyelocytic leukemia) bodies, nuclear speckles, paraspeckles and Cajal bodies (Mao et al., 2011). These subnuclear structures are formed and maintained as stochastic self-organization domains, and are actively involved in distinct cellular function(s) (Dundr and Misteli, 2010), including transcription, DNA replication, RNA metabolism and RNA export (Zhao et al., 2009). Apart from proteins, various lncRNAs localized in the subnuclear domains offer another layer of regulatory mechanism in the nucleus (Mattick et al., 2009; Mercer et al., 2009). These so called nuclear-retained regulatory RNAs (nrRNAs) are shown to be involved in key cellular processes associated with gene expression, including but not limited to epigenetic regulation, chromosomal interaction, transcriptional regulation, RNA processing and nuclear domain structure maintenance. In this review, we discuss the recent findings relating to nrRNAs, their involvement in various cellular processes during development and also their association with diseases.

Chromatin remodelling and epigenetic regulation

It has been previously reported that RNAs are involved in the maintenance of higher order chromatin structure, and most of these RNAs are noncoding, derived predominantly from intronic and intergenic regions of the genome (Bernstein and Allis, 2005). This notion is further supported by the recent data from various research laboratories, demonstrating the ability of nrRNAs to specifically interact with proteins that are involved in chromatin maintenance, modification and gene expression regulation (Batista and Chang, 2013; Chu et al., 2011; Guttman and Rinn, 2012; Mondal et al., 2010). Moreover, by associating with specific chromatin modifying complexes, nrRNAs govern the localization of these modifiers to specific chromatin sites, thereby regulating chromatin modifications and gene expression in an allele- or cell type-specific manner (See Figure 1)(Lai et al., 2013).

X-chromosome inactivation (XCI) in mammals is an example that signifies the distinct role of lncRNAs/nrRNAs in chromatin remodelling, epigenetic modifications and gene repression (Lee, 2011). Among several lncRNAs present in the X-inactivation center (Xic), Xist (X-inactive-specific transcript) was the first-identified and is one of the best-studied nuclear-restricted lncRNAs in mammals [Please see (Augui et al., 2011; Lee and Bartolomei, 2013; Lessing et al., 2013) for an extensive review on X-chromosome inactivation]. Xist is a 17–20 kb long nrRNA expressed from the future inactive X-chromosome (Xi) that spreads all over the surface of Xi *in cis* (Figure 2A) (Lessing et al., 2013). Repeat A (RepA), another lncRNA, is a conserved direct sequence repeat present at the 5’ end of *Xist* gene that interacts with the PRC2 (Polycomb repressive complex 2) (Zhao et al., 2008). PRC2 is held responsible for the tri-methylation of histone H3 at Lys27 (H3K27me3), a transcriptionally repressive mark commonly associated with several of the gene loci, the expression of which are regulated in a cell type- or developmental-specific fashion. The initial binding of Xist with PRC2 is mediated by a 1.6 kb long RepA lncRNA showing homology with the 5’ end of the Xist, and further the allele-specific binding of Xist RNA to Xi is facilitated by the YY1 transcription factor (Jeon and Lee, 2011; Zhao et al., 2008). Other lncRNAs from the Xic, including Jpx and Tsix regulate XCI by influencing Xist expression (Lee et al., 1999; Tian et al., 2010). Jpx is transcribed from the Xi chromosome and acts as a positive regulator to Xist RNA. A recent study revealed that Jpx by binding to CTCF, a factor that is known to repress Xist transcription, evicts the binding of CTCF from the promoter of one of the Xist gene alleles, thereby positively influencing *Xist* gene expression (Sun et al., 2013). On the other hand, Tsix is an antisense lncRNA to Xist RNA, transcribed from the future active X-chromosome and it negatively regulates the expression of Xist RNA. Tsix represses Xist transcription on one of the X-alleles, and thus helps in determining the X-chromosome allele that needs to be activated (Xa). Essentially, Tsix regulates *Xist* transcription at multiple levels; it controls X-chromosome pairing to provide asymmetry in the epigenetic modifications between the two X-chromosomes. Tsix recruits DNA methyltransferase (Dnmt3a) to inhibit *Xist* transcription. Finally, Tsix directly binds PRC2 and also duplexes with Xist-RepA RNA, and thus blocks the PRC2 recruitment to Xist by RepA (Do et al., 2008; Lee, 2012).

Other examples of nrRNAs involved in the allele specific epigenetic regulation of gene expression in *cis* include *Air* and *Kcnq1ot1* (Pandey et al., 2008; Sleutels et al., 2002). Air lncRNA is imprinted; expressed from the paternal allele, and is transcribed from the second intron of mouse insulin-like growth factor 2 receptor (*Igf2r*) gene. Air transcripts interact with the histone methyltransferases (HMT) G9a and recruit them to the promoters of specific genes (*Slc22a3, Slc22a2 and Igf2r*) that need to be silenced (Nagano et al., 2008). Kcnq1ot1 is a 90 kb long lncRNA, transcribed from intron 10 of the imprinted gene *Kcnq1* from paternal allele (Kanduri, 2011). Kcnq1ot1 recruits PRC2 and HMT G9a to the gene-cluster present in the Kcnq1 domain in an allele-specific manner and creates repressive

histone marks i.e., histone H3 tri-methylated at lysine 9 (H3K9me3) and H3K27me3, leading to the silencing of the genes in *cis* (Terranova et al., 2008).

Very recently, another intronic *cis*-acting lncRNA *ANRASSF1* has been characterized, which is transcribed from the opposite strand of the *RASSF1* tumor suppressor gene locus (Beckedorff et al., 2013). *ANRASSF1* specifically localizes within the nucleus and has a shorter life-span (half-life of ~50 min) compared to other well characterized *cis*-acting stable lncRNAs, including *Kcnq1ot1* and *Air* lncRNA. It has been observed that the *ANRASSF1* specifically silences the expression of *RASSF1A* by recruiting PRC2 and repressive histone modifying complexes, but has no effect on the *RASSF1C* and other neighbouring genes.These studies indicate that several of the lncRNAs have the potential to influence the epigenetic environment of genes in a location-specific manner (Beckedorff et al., 2013).

Besides acting in *cis* manner, many nrRNAs also dictate epigenetic modifications and control gene expression in *trans*. For instance, HOTAIR (HOX transcript antisense RNA) antisense lncRNA is transcribed from the HOX-C cluster on chromosome 12. Following transcription, HOTAIR interacts with the PRC2 and LSD1 chromatin modifier complexes utilizing its 5’ and 3’ end, respectively, and recruits them to the Hox-D locus located on chromosome 2 (Figure 1A). This causes H3K27me3 and demethylation of H3K4me2/3 at the Hox-D locus, leading to their silencing (Rinn et al., 2007; Tsai et al., 2010). Besides the Hox-D locus, HOTAIR also targets PRC2 to many other genomic loci for gene silencing by creating repressive histone marks (Chu et al., 2011). Targeted deletion of *Hotair* in mouse leads to derepression of a large number of genes, including genes from the Hox-D cluster and imprinted clusters, and homeotic transformation of spine and malformation of metacarpal-carpal bones (Li et al., 2013a). In addition, aberrant expression of HOTAIR is implicated in cancer progression and metastasis as elevated expression of HOTAIR is reported in primary and metastatic breast cancer, and its depletion leads to decrease in invasiveness (Gupta et al., 2010).

NrRNAs are also known to promote gene activation by recruiting chromatin modifiers to specific chromatin sites. HOXA transcript at the distal tip (HOTTIP) lncRNA is transcribed from the 5’end of the HOXA locus (Wang et al., 2011b) and coordinates the transcription of several of the 5’ HOXA genes *in cis*. HOTTIP recruits WDR5/MLL complex to its target genes at the HOX cluster, facilitating H3K4me3 and transcription. HOTTIP is brought to the proximity of its target genes by chromosome looping (Figure 1A). Similar to HOTTIP, Mistral (Mira) lncRNA is also known to positively influence transcription of genes in the HOX clusters in mouse embryonic stem cells (mES) (Bertani et al., 2011). *Mistral* gene is located in the spacer region separating *Hoxa6* and *Hoxa7* and in mES cells it is specifically activated upon retinoic acid treatment. Mistral recruits MLL1 to chromatin, induces changes in chromatin structure that results in the activation of *Hoxa6* and *Hoxa7* genes (Figure 1A) (Bertani et al., 2011).

In addition to establishing histone tail modifications, lncRNAs also influence other epigenetic modifications, including DNA methylation. For example, an lncRNA transcribed from the CEBPA gene locus regulates local DNA methylation (Di Ruscio et al., 2013). The so-called ecCEBPA (extracoding CEBPA) lncRNA is nuclear enriched and non-polyadenylated. It is transcribed from the upstream region of the *CEBPA* gene and overlaps the entire CEBPA mRNA sequence in the same sense-orientation. Interestingly, ecCEBPA binds to the maintenance DNA methyltransferase 1 (DNMT1) and prevents DNMT1-mediated DNA methylation at the *CEBPA* gene locus. Finally, in addition to ecCEBPA lncRNA, the authors discoverd several RNA species associated with DNMT1 and confirmed their involvement in regulating DNA methylation and thereby modulating gene expression (Di Ruscio et al., 2013).

Transcriptional regulators

Until recently, transcription of RNA was considered to be controlled/regulated by the interplay of the upstream/downstream *cis*-acting regulatory elements and the various interconnected regulatory proteins/factors (Michalak, 2006). However, with the emerging role of lncRNAs and their widespread distribution in the genome, it is interpreted that lncRNAs may play equally vital roles in the activation or repression of cellular genes. Nuclear-restricted lncRNAs have been reported to regulate gene transcription either by modulating the activities of transcriptional factors or controlling the binding of these factors to the promoter region (Chen and Carmichael, 2010; Khalil et al., 2009; Ng et al., 2013). An example of where lncRNAs induce gene transcription is provided by Evf2, a 3.8 kb long, polyadenylated, alternatively spliced nrRNA, transcribed from the ultraconserved intergenic enhancer region of *Dlx-5/6* locus (Feng et al., 2006). *Dlx* genes are related to the *Drosophila* Distalless (DII) genes, and are involved in neuronal development (Panganiban and Rubenstein, 2002). Evf-2 was reported to increase the activity of specific enhancer regions (ei and eii) by interacting and recruiting the homeodomain protein Dlx-2 to ei and eii regions, resulting in the induction of *Dlx-5/6* gene expression (Figure 1B) (Feng et al., 2006). Thus, Evf-2 functions like a transcriptional coactivator that acts in *trans* to regulate the expression of homeodomain genes during development. However, in the *Evf-2* null mice, the expression of *Dlx-5/6* was found to increase despite the absence of Dlx-2 on the ei and eii regions (Bond et al., 2009). In this case, it is not apparent whether the binding of Dlx proteins to some other enhancers compensates for the absence of Dlx-2 on ei, eiiregions or Dlx-2 acts indirectly by preventing the binding of MeCP2, a known repressor of *Dlx-5/6*, to ei/eii regions.

According to the estimation by the ENCODE project, ~80% of genes in a mammalian genome have an alternative transcriptional start site (Birney et al., 2007; Kawaji et al., 2009). In several instances lncRNAs, transcribed from the upstream region of the protein coding genes have been reported to regulate the transcription of their protein-coding partner by preventing the formation of transcriptional complex at the promoter region (Figure 1C) (Batista and Chang, 2013). For example, the *dihydrofolate reductase (DHFR)* gene contains both the minor and major promoter regions. In quiescent mammalian cells, an lncRNA transcribed from the upstream region of minor promoter of *DHFR* gene interacts with the major promoter and general transcriptional factor TFIIB (Martianov et al., 2007). These lncRNAs inhibit transcription of DHFR gene by preventing the association of TFIIB-containing transcriptional complex to the promoter region by forming a stable purine-purine-pyrimidine triple structure with the double stranded DHFR promoter (Martianov et al., 2007).

Linc-HOXA1, located~50 kb away from the Hoxa gene cluster, negatively regulates *Hoxa1* expression in mES (Maamar et al., 2013). Linc-HOXA1 represses *Hoxa1* in *cis* by recruiting the transcriptional cofactor PURB at the *Hoxa1*chromatin site (Figure 1C). Exposure of cells to retinoic acid leads to derepression of the *Hoxa1* transcription, possibly due to reduced interaction of linc-HOXA1 with the *Hoxa1* transcription site (Maamar et al., 2013). NrRNAs are also shown to activate transcription, by recruiting transcription activators or their co-factors to specific gene promoter regions. In this context, a very recent study revealed the role of nrRNA (lncRNA-JADE) in the activation of a gene (*Jade*), an integral component of the histone acetyltransferase (HAT) complex (Wan et al., 2013). In human cells, lncRNA-JADE is induced upon double strand DNA break in an Ataxia-telangiectasia mutated (ATM)-dependent manner. LncRNA-JADE transcriptionally activated *Jade1* by recruiting Brca1 to the Jade promoter and facilitating the interaction between Brca1 and P300/CBP transcriptional co-factor complex. LncRNA-JADE-mediated transcriptional activation of *Jade* facilitated the DNA damage-induced hyperacetylation of histone 4. Interestingly,

LncRNA-JADE is overexpressed in breast cancer samples and its downregulation inhibits tumor growth *in vivo* (Wan et al., 2013).

NrRNAs are also implicated in the transcriptional regulation of cell cycle-regulated genes, especially the ones that are activated upon cellular stress. For example, DNA damage induces several lncRNAs from regions located upstream of the *CCND1* promoter (Wang et al., 2008). These lncRNAs interact with translocated in liposarcoma (TLS) protein, an RNA binding protein and a known inhibitor of CBP/P300 HAT, and facilitate the association of TLS with CBP/P300. TLS in turn represses the CBP/P300 activity on the *CCND1* promoter resulting in the transcriptional inactivation of *CCND1*. LncRNAs transcribed from regions upstream of *CDKN1A* gene promoter are reported to regulate gene expression by recruiting RNA binding proteins and transcription factors to specific chromatin sites (Huarte et al., 2010; Hung et al., 2011). LincRNA-p21, located upstream of the *CDKN1A (p21)* gene, is a p53-responsive gene, and acts as a repressor in the p53-dependent transcriptional responses (Huarte et al., 2010). The tumor suppressor p53 activates the expression of lincRNA-p21 by directly interacting with lincRNA-p21 promoter. LincRNA-p21 in turn inhibits the expression of several of the anti-apoptotic genes that are normally repressed by p53. It has been observed that the interaction between lincRNA-p21 and the heterogeneous nuclear ribonucleoprotein K (hnRNP-K) is required for the genomic association of hnRNP-K with the repressed genes and also for their transcriptional repression (Huarte et al., 2010). PANDA is another p53-induced lncRNA that is also transcribed from the upstream region of *CDKN1A* gene. PANDA is upregulated upon DNA damage, interacts with NF-YA transcription factor and controls the expression of pro-apoptotic genes. PANDA-depleted fibroblasts show increased sensitivity to DNA damage-induced apoptosis (Hung et al., 2011). A very recent study from Huarte's laboratory has reported the involvement of another p53-induced lncRNA in gene regulation (Marin-Bejar et al., 2013). Pint (p53-induced noncoding transcript) is an nrRNA in mouse cells that promotes cell proliferation and post DNA-damage cell survival by regulating the expression of genes involved in TGF-beta, MAP kinase and P53 signalling pathways. Similar to several other known lncRNAs, Pint interacts with PRC2 complex and recruits PRC2 to specific chromatin sites for successful H3K27-trimethylation and transcription repression (Marin-Bejar et al., 2013). These studies have highlighted the crucial roles played by lncRNAs in the expression of cell cycle-regulated genes and other genes that are part of the p53-mediated signaling network.

Recent studies have also documented the involvement of lncRNAs in regulating enhancer functions (Shiekhattar, 2013) (Also please see Darrow and Chadwick in this Special issue). Enhancer elements are distal regulatory sequences, which generally promote the transcription of protein coding genes in an orientation independent manner (Bulger and Groudine, 2011). Enhancers are characterized by higher levels of H3K4me1 and H3K27Ac on their chromatin. They also bind to the p300/CBP transcriptional coactivators in a stimulus dependent manner (Bulger and Groudine, 2011; Creyghton et al., 2010; Heintzman et al., 2007). It has been shown that the enhancers are occupied by RNA pol II that leads to the generation of 0.5 to 5.0 kb long lncRNAs referred as enhancer RNAs (eRNAs) and ncRNA-activating (ncRNA-a; ncRNA-a chromatin retains H3K4me3 modifications and contains weak H3K4me1 marks), some of which are polyadenylated (De Santa et al., 2010; Kim et al., 2010; Orom et al., 2010; Wang et al., 2011a). The transcription of eRNAs is regulated in a stage or tissue-specific manner and their activation is generally correlated with the expression of protein-coding genes located in the nearby regions (Figure 1A) (De Santa et al., 2010; Hah et al., 2013; Kim et al., 2010; Li et al., 2013b). The levels of several eRNAs are found to be increased in cells treated with different stimuli, like endotoxins in human macrophage cells and estradiol in MCF-7 cells (Li et al., 2013b). Li and colleagues (2013) reported that 17β-estradiol (E2)-bound estrogen receptor α (ERα)induces a global upregulation of eRNA transcription on enhancers present near the E2-upregulated coding

genes in MCF-7 cells (Li et al., 2013b). These eRNAs positively influence ligand-dependent ERα-induced gene activation, in part by facilitating the enhancer-promoter chromatin looping. Similarly, induction of Androgen receptor (AR) signalling in prostate cancer cells also activated specific set of eRNAs located at the AR-regulated gene enhancers (Wang et al., 2011a). These eRNAs promoted enhancer-promoter chromatin and function in combination with lineage specific transcription factors like FOXA1 to create a higher order enhancer chromatin network and regulate transcription in a cell type-specific manner (Wang et al., 2011a). Recently, Lai and colleagues (2013) have shown that activator ncRNA-a interact specifically with Mediator co-activator complex during transcription to regulate chromatin localization as well as the kinase activity (towards H3 serine-10) of the Mediator. Further, by using a heterologous reporter system in mammalian cells, the authors revealed the involvement of Mediator components in the ncRNA-a-mediated activation of genes (Lai et al., 2013). Based on genomic analyses, human cells produce several hundreds of enhancer-associated eRNAs or ncRNA-a and recent studies have started to unravel the roles of these transcripts in diverse cellular activities, including p53-mediated gene activation and muscle differentiation (Lam et al., 2013; Melo et al., 2013; Mousavi et al., 2013; Orom et al., 2010).

Post-transcriptional regulators

Post transcriptional gene regulation works at different levels, including pre-mRNA splicing, RNA editing, transport, stability and degradation of mRNA. All of these processes involve complex networks of various regulatory protein complexes/factors that in turn determine the type and amount of a particular protein to be synthesized at specific time/space to govern a given biological process. Recently, few studies have attempted to understand the involvement of nrRNAs in posttranscriptional gene regulation (Yoon et al., 2012a). Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1; also referred to as NEAT 2) is one such example of a lncRNA that is involved in the post-transcriptional gene regulation via controlling alternate splicing of pre-mRNAs (Zong et al., 2011). MALAT1 is a >7 kb long, abundantly expressed nuclear-restricted lncRNA, originally identified as a lncRNA that is overexpressed in early-stage non-small lung cancers (Hutchinson et al., 2007; Ji et al., 2003; Lin et al., 2007). Recent studies show the involvement of MALAT1 in several important cellular processes, including cell cycle progression, serum stimulation-induced gene activation, neuronal synapse formation and cancer cell migration (Bernard et al., 2010; Tano et al., 2010; Tripathi et al., 2010; Tseng et al., 2009; Yang et al., 2011a). MALAT1 RNA is highly conserved among mammals, and predominantly localizes in the nuclear speckles or SC35 domains, a nuclear structure that contains proteins and RNAs involved in pre-mRNA processing (Figure 2B & 2E-G) (Bernard et al., 2010; Hutchinson et al., 2007; Tripathi et al., 2010). MALAT1, though not shown to be involved in the formation and/or maintenance of nuclear speckles, is found to interact with various pre-mRNA splicing factors, including the serine/arginine family of splicing factors (SR proteins). MALAT1-depleted human cells show alterations in the distribution and activity of SR splicing factors, including aberrant changes in alternative splicing of pre-mRNAs (Figure 1D) (Lin et al., 2011; Tripathi et al., 2010). Recently, we have also demonstrated that MALAT1 modulates the expression of genes involved in the cell cycle progression and is required for G1/S and mitotic progression. MALAT1-depleted human diploid fibroblasts activated p53 and its target genes and showed a robust cell cycle arrest. These cell cycle defects were sensitive to the p53 levels, indicating that p53 is a major downstream mediator of MALAT1 activity (Tripathi et al., 2013). Furthermore, MALAT1-depleted cells display reduced expression of B-MYB (Mybl2), an oncogenic transcription factor involved in G2/M progression. This is due to changes in the association of SR splicing factors on B-MYB pre-mRNA resulting in aberrant alternative splicing. Our studies in human cells indicate that MALAT1 promotes cellular proliferation by modulating the expression and/or pre-mRNA processing of cell

cycle-regulated transcription factors (Tripathi et al., 2013). A recent study from Rosenfeld laboratory suggested a role for MALAT1 in cell cycle progression through its involvement in regulating E2F1 transcription factor activity (Yang et al., 2011a). MALAT1-depleted cells showed proliferation defects and an inability to activate E2F target genes upon the addition of serum. The authors described that MALAT1 influenced the interaction of unmethylated polycomb protein, Pc2with E2F1 in serum-activated cells, and such an association facilitates E2F1 SUMOylation, leading to the activation of serum-induced genes (Yang et al., 2011a). Based on all these results it is evident that MALAT1 regulates cell proliferation by interacting with several proteins, including chromatin modifiers and pre-mRNA splicing factors.

Surprisingly, we and others have recently reported that the *in vivo* MALAT1 knockout (KO) mouse is viable and fertile and MEFs from the knock out (KO) mouse did not show any defects in alternative splicing and SR protein activity (Eissmann et al., 2012; Nakagawa et al., 2012; Zhang et al., 2012). These results implicate that MALAT1 is largely dispensable in mice but plays an important role in particular cell types under specific physiological conditions. The cell type- or organism-specific phenotype observed upon depletion of a particular ncRNA is not specific to MALAT1, as earlier studies had reported similar results for other ncRNAs and protein-coding genes (Berthet et al., 2003; Concepcion et al., 2012; Ortega et al., 2003; Schorderet and Duboule, 2011).

An elegant study from Chen and colleagues revealed the role of a novel class of nuclear-retained lncRNAs in splicing regulation (Yin et al., 2012).The so called ‘sno-lincRNAs’ are transcribed from intronic sequences from several genomic sections, including the region in chromosome 15 that has been implicated in Prader-Willi Syndrome (PWS). Sno-lincRNAs are devoid of 5’caps and poly (A) tails and are processed by the snoRNA machinery. The sno-lincRNAs from PWS region accumulate near the sites of their transcription and establish a domain, where the splicing factor Fox2 is accumulated. Functional studies indicate these sno-lincRNAs interact with Fox2, appear to function as a Fox2 ‘sink’, and thereby influence Fox2-mediated pre-mRNA splicing (Figure 1D) (Yin et al., 2012).

Gomafu/RNCR2/MIAT (myocardial infarction associated transcript), a ploydenylated nrRNA with neuron-restricted expression, is another example of an lncRNA that is implicated in posttranscriptional gene regulation (Ishii et al., 2006). Gomafu/MIAT localizes to a novel nuclear compartment, which does not coincide with any of the known nuclear domains (Sone et al., 2007). Gomafu is expressed in differentiating neurons and oligodendrocytes while in progenitor cells its expression is observed following lineage specification (Mercer et al., 2010). Gomafu RNA binds to the branch point-interacting SF1 splicing factor with high affinity utilizing the tandem repeats within the transcript. Gomafu is speculated to influence pre-mRNA splicing efficiency by sequestering the cellular pool of SF1 within the nucleus (Sone et al., 2007; Tsuiji et al., 2011). A general theme emerging from all these studies is that several of the nrRNAs influence pre-mRNA splicing, primarily by titrating the cellular pool of splicing or other pre-mRNA processing factors.

The antisense lncRNAs are known to influence alternative splicing of their sense RNA partners. This has been observed in case of alpha-Thyroid hormone receptor gene (ErbAα), where its antisense transcript RevErbAα dictates the differential synthesis of the alternatively spliced Tra1 and Tra2 mRNAs (Hastings et al., 2000; Hastings et al., 1997) through yet unknown mechanisms.

LncRNAs in nuclear organization

Recent studies indicate that lncRNAs interact and recruit proteins that are involved in the organization and maintenance of specific subnuclear domains (Dundr and Misteli, 2010; Shevtsov and Dundr, 2011). Many of these proteins include RNA processing factors that are involved in RNP metabolism. Proteins present in these discrete domains exchange between the domains with the help of the nuclear domain-resident lncRNAs (Mao et al., 2011). For example, Shevtsov and Dundr (2011) showed that several types of RNAs, both coding and noncoding, can function as structural elements and facilitate in the nucleation of nuclear bodies. They demonstrated transcription as a driving force in the nuclear body formation and RNA acting as a scaffold in the formation of these membraneless structures (Shevtsov and Dundr, 2011). NEAT1 (nuclear enriched abundant transcript 1) or MENε/β (multiple endocrine neoplasia ε/β) or VINC (virus induced noncoding transcript) is a well characterized lncRNA, which acts as an important structural component in the formation and maintenance of paraspeckle domains (Figure 2C) (Fox and Lamond, 2010). The *NEAT1* gene encodes two major isoforms i.e., NEAT1_1/MENε (3.7 kb) and NEAT1_2/MENβ (23 kb), both differ at their 3' end. The long NEAT12 isoform is essential for the formation of paraspeckle while the short NEAT11 though increases the number of paraspeckles when overexpressed, is not found to be sufficient for the maintenance of paraspeckles (Bond and Fox, 2009; Clemson et al., 2009; Sasaki et al., 2009; Sunwoo et al., 2009). The paraspeckle contains several RNA binding proteins, including DBHS (Drosophila Behaviour Human Splicing) family proteins i.e., PSPC1, PSF (SFPQ) and $p54^{nrb}$ (NONO) (Fox et al., 2005; Passon et al., 2012). Members of this family, especially NONO, are known to interact with Adenosine-to-Inosine (A-to-I) edited RNAs and thought to influence nuclear retention and paraspeckle localization of A to I edited transcripts (Chen and Carmichael, 2008; Prasanth et al., 2005). Several studies have suggested a role for nuclear paraspeckles and its constituents in the nuclear retention of RNAs (Chen and Carmichael, 2008; Chen and Carmichael, 2009; Prasanth et al., 2005). The mouse-specific CTN-RNA (CAT2 transcribed nuclear RNA) is one such nrRNA that is A-to-I edited at its 3'UTR, interacts with NONO and localizes to paraspeckle (Figure 2D). Upon interferon-γ and lipopolysaccharide treatment, CTN-RNA is post transcriptionally processed at its 3'UTR to produce a smaller transcript that is further transported to cytoplasm for translation (Prasanth et al., 2005). A similar mode of gene regulation was also reported for the synthesis of migration stimulating factor (MSF) upon TGFβ1 treatment (Kay et al., 2005). These examples signify a novel mechanism of gene regulation, where an RNA under normal conditions is sequestered in specific sub-nuclear compartments like paraspeckles and is exported to the cytoplasm upon a specific signal as part of the 'quick response' mechanism. Another study from the Carmichael laboratory showed a correlation between the presence of NEAT1, paraspeckle and the nuclear retention of A-to-I edited RNAs in human embryonic stem cells (hES) (Chen and Carmichael, 2009). This study found that hES cells do not express NEAT1 transcripts, therefore, lack paraspeckles and as a result, the hyper A-to-I edited mRNAs transport efficiently to the cytoplasm. However, following differentiation of hES cells to trophoblasts, these cells express NEAT1 and form distinct paraspeckles. Interestingly, differentiated ES cells containing intact paraspeckles showed nuclear retention of A-to-I edited transcripts, further supporting the involvement of NEAT1 and paraspeckle in the nuclear retention of edited RNAs. Future studies will determine the exact role of paraspeckle and A-to-I editing in the nuclear storage and signal-induced cleavage of transcripts.

LncRNAs are also known to sequester proteins in subnuclear domains, as part of the cellular stress response. For example, certain environmental stresses like changes in pH during hypoxia or acidosis result in the nucleolar immobilization of proteins, including VHL (von Hippel-Lindau), a factor that under normal oxygen tension facilitates the degradation of HIFα (hypoxia-inducible factor-α). Similarly, other cellular stresses and oncogenic signals

induce the sequestration of HDM2 (MDM2) into the nucleolus (Audas et al., 2012). However, the underlying mechanism responsible for the stress-induced association of factors to nucleolus was not clearly understood. A recent study revealed the role of several lncRNAs transcribed from the intergenic spacer (IGS) region of the rDNA repeats in the stress-induced nuclear detention of proteins (Audas et al., 2012). Unique IGS RNAs are induced upon specific cellular stress and they interact with proteins containing specific peptide codes referred to as nucleolar detention sequence. Interestingly, altering the levels of one type of IGS RNA changes the nuclear detention activity of other IGS RNAs indicating that different members of IGS RNAs function independently and do not influence the function of one another.

NrRNAs and Disease

Since many of the nrRNAs are involved in the fine tuning of chromatin reprogramming, epigenetic modification, transcriptional and post-transcriptional gene regulation, their aberrant expression in the cell alters gene expression that leads to diseases like cancer. Several studies showed higher expression of some of the lncRNAs in cancerous cells or tissues; and their induced expression in mouse models result in tumor formation and metastasis, while some other lncRNAs act as tumor suppressors (Prensner and Chinnaiyan, 2011). As discussed earlier, HOTAIR interacts with PRC2 and LSD1/CoREST/REST complex leading to epigenetic changes by H3K27 methylation and H3K4 demethylation (Rinn and Chang, 2012). Its higher expression has been reported in several type of cancers like breast cancer, hepatocellular carcinoma, colorectal cancer, pancreatic and gastrointestinal stromal cancer (Gupta et al., 2010; Kim et al., 2013; Kogo et al., 2011; Niinuma et al., 2012; Yang et al., 2011b). It has been observed that the over-expression of HOTAIR in cancer samples leads to poor prognosis while its loss decreases the invasiveness of cancerous tissues. Another lncRNA ANRIL (antisense noncoding RNA in the INK4 locus) transcribed from the p15/CDKN2B-p16/CDKN2A-p14/ARF gene cluster is associated with breast cancer, basal cell carcinoma, gliomas, intracranial aneurysm and coronary artery disease (Burd et al., 2010; Pasmant et al., 2011; Shete et al., 2009; Stacey et al., 2009; Turnbull et al., 2010). ANRIL functions as a repressor of tumor suppressor gene $p15^{INK4B}$ by interacting with SUZ12 and recruit PRC2 to the$p15^{INK4B}$ transcription site (Kotake et al., 2011). Nuclear-localized MALAT1 over expression is observed in lung, liver, breast, pancreas, prostate, colon and cervical cancer samples (Guo et al., 2010; Ji et al., 2003; Lin et al., 2007; Schmidt et al., 2011). A study by Tano and colleagues suggested that MALAT1 regulates the activity of genes (CTHRC1, CCT4, HMMR and ROD1) that are involved in cell motility thereby influencing cancer metastasis (Tano et al., 2010).

Recently, Calin laboratory has described the involvement of a novel nuclear-retained lncRNA (CCAT2) in metastatic progression and chromosomal instability in colon cancer (Ling et al., 2013). CCAT2 transcript maps to the highly conserved 8q24.21 region and is overexpressed in the microsatellite-stable colorectal cancer samples. CCAT2 promotes tumor growth, as subcutaneous transplantation of CCAT2-overexpressing cells resulted in larger tumors in the immuno-compromised mice compared to empty vector-transduced cells. Furthermore, CCAT2 lncRNA induces the expression of MYC by regulating the activity of TCF7L2 transcription factor, an essential protein that is required for the transcriptional activation of genes involved in WNT signaling pathway. These data support a novel role for CCAT2 lncRNA in regulating MYC and WNT signaling pathways (Ling et al., 2013).

PCAT-1 is one among several lncRNAs that show elevated expression in prostate cancer tissue samples (Prensner et al., 2011). *In vitro* and *in vivo* studies indicate that PCAT-1 facilitates cancer cell proliferation. Functional studies suggest that PCAT-1 acts as a transcriptional repressor of genes involved in mitosis and cell cycle progression. The same

investigators have recently identified another prostate cancer-associated lncRNA; Schlap1 (second chromosome locus associated with prostate-1), that is specifically overexpressed in a subset of prostate cancers (Prensner et al., 2013). Overexpression of *Schlap1* in cancer samples predicts poor outcomes, including metastasis and prostate cancer-specific mortality. Functional studies indicate that Schlap1 negatively regulates the genome-wide association and function of SWI/SNF chromatin remodelling complex (Prensner et al., 2013).

Conclusion

Recent studies have highlighted the important roles played by lncRNAs in key biological/cellular processes. Majority of the lncRNAs remain in the nucleus, and this provides a better opportunity for nuclear-retained lncRNA or nrRNAs to regulate several of the nuclear functions in a sequence-specific manner, and also to function as structural elements to maintain proper nuclear territories/domains. In addition, regulatory RNAs are retained within the nucleus and are exported out to the cytoplasm upon specific signals/stimuli (Carrieri et al., 2012; Kay et al., 2005; Prasanth et al., 2005). Antisense ubiquitin carboxy terminal hydrolase L1 (AS Uchl1) is one such nuclear-retained lncRNA that is complementary to the mRNA, which encodes Uchl1. AS Uchl1 interacts with Uchl1 mRNA, enhances translation of Uchl1 mRNA by facilitating the formation of active polysomes on Uchl1 mRNA. Interestingly, when cells are inhibited of mTORC1 activity, AS Uchl1 lncRNA is exported out of the nucleus to the cytoplasm. This in turn increases Uchl1 mRNA translation (Carrieri et al., 2012).

Similar to what is known for proteins, lncRNAs are also shown to execute more than one mutually exclusive function. For example, human translational regulatory lncRNA (treRNA) earlier referred as ncRNA-a7, is known to function as an enhancer-like RNA to regulate *Snail* transcription in the nucleus (Orom et al., 2010). It is also involved in the translational repression of specific cellular mRNAs in the cytoplasm (Gumireddy et al., 2013; Orom et al., 2010). TreRNA specifically downregulates the expression of epithelial marker E-cadherin by altering the polysomal distribution on E-cadherin mRNA. TreRNA mediates its function through its association with an RNP complex consisting of several RNA-binding proteins (hnRNP K, FXR1 and FXR2), PUF60 and SF3B3 (Gumireddy et al., 2013). Similarly, LincRNA-p21, in addition to regulating the transcription of genes in the p53 signaling pathway, was also recently shown to repress translation of specific mRNAs (Yoon et al., 2012b). The human genome contains several thousands of lncRNAs. Given the complexity provided by several of the characterized lncRNAs, it is highly likely that many nuclear-retained lncRNAs with additional functions in gene regulation will be identified in the near future. Future studies will also determine how these lncRNAs are being regulated and also their involvement in several human diseases.

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Abbreviations

ANRIL	Antisense nocoding RNA in the INK4 locus
AR	Androgen receptor
A-to-I	Adenine to Inosine

CCAT2	Colon cancer associated transcript 2
CEBPA	CCAAT/Enhancer Binding Protein (C/EBP), Alpha
DBHS	Drosophila Behaviour Human Splicing
DHFR	Dihydrofolatereductase
ecCEBPA	extracoding CEBPA
eRNA	enhancer RNA
ER α	Estrogen receptor α
H3K4me3	Histone 3 Lysine 4 rimethylation
H3K27me3	Histone 3 Lysine 27 trimethylation
HAT	Histone acetyl transferase
hES	Human embryonic stem cells
HMT	Histone methyltransferase
hnRNP-K	heterogeneous nuclear ribonucleoprotein K
HOTAIR	HOX transcript antisense RNA
HOTTIP	HOXA transcript at the distal tip
Igf2r	Insulin-like growth factor 2 receptor
IGS	Intergenic spacer
lncRNA	Long noncoding RNA
MALAT1	Metastasis-associated lung adenocarcinoma transcript 1
mES	Mouse embryonic stem cells
MIAT	Myocardial infarction associated transcript
ncRNAa	Noncoding RNA activator
nrRNA	Nuclear-retained RNA
PRC2	Polycomb repressive complex 2
PWS	Prader-Willi Syndrome
RepA	Repeat A
Schlap1	Second chromosome locus associated with prostate-1
TCF7L2	transcription factor 7-like 2
treRNA	Translational regulatory lncRNA
Uchl1	Ubiquitin carboxyterminal hydrolase L1
VHL	von Hippel-Lindau
XCI	X-chromosome inactivation
Xi	Inactive X-chromosome
Xic	X-chromosome inactivation center
Xist	X-inactive-specific transcript

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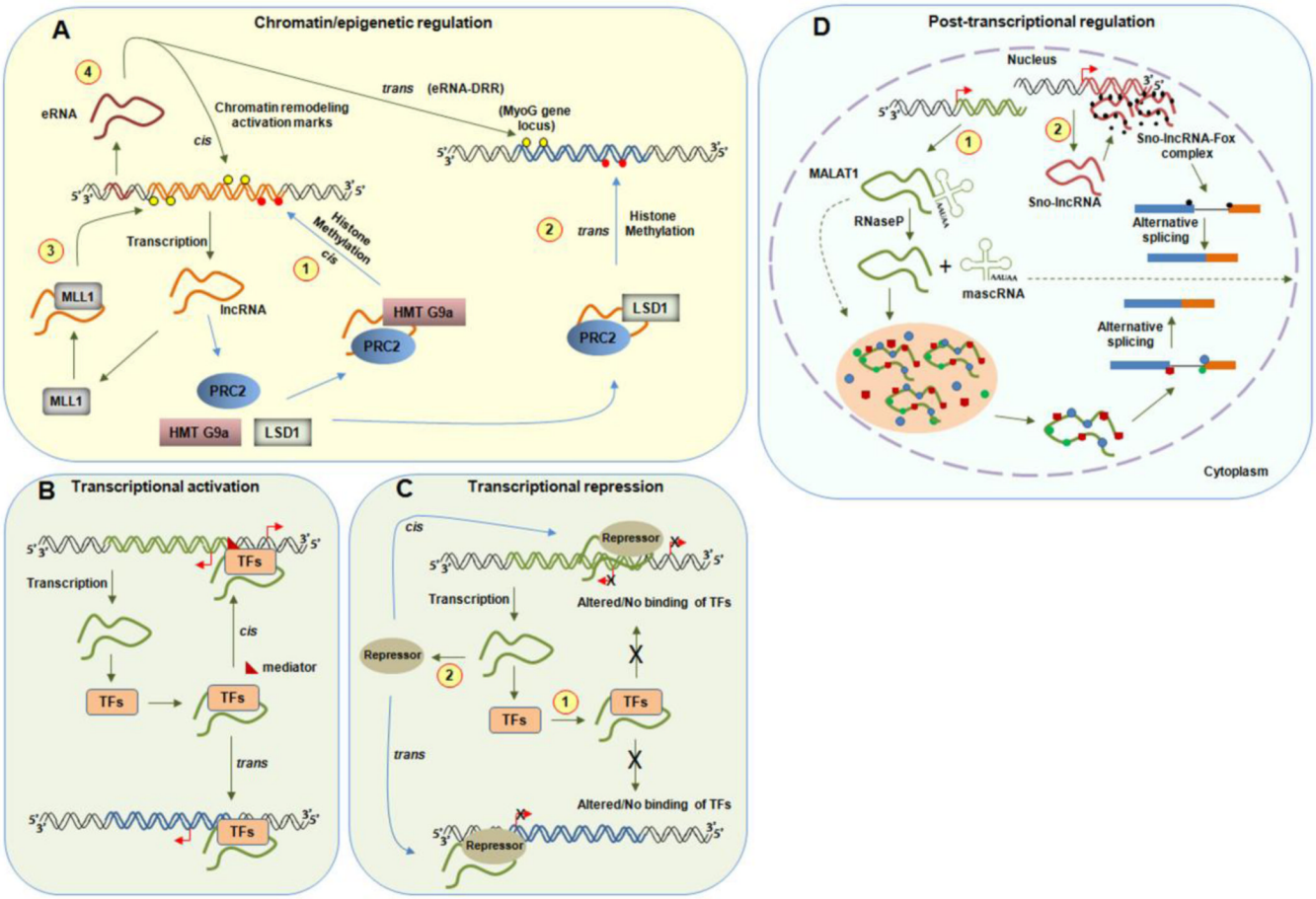

Figure 1. Functions of nuclear retained lncRNAs

(A) Model showing chromatin/epigenetic regulation by lncRNA. LncRNA interacts with different chromatin modifying complexes and recruits them to specific chromatin target site. (1) shows the deposition of repressive transcription marks (red circles) by histone methylation in *cis* (*Air, Kcnq1ot1, ANRASSF1)* and (2) in *trans* (*HOTAIR*) leading to transcriptional inactivation. (3) represents the activation of transcription (yellow circle) by deposition of active chromatin marks through the recruitment of specific histone modifiers (Mistral and HOTTIP). (4) eRNAs also enhances the transcription both in *cis* and *trans* by chromatin modifications.**(B)** LncRNAs activate transcription both in *cis* and *trans* by recruiting transcription factors (TFs) and mediators. **(C)** LncRNA represses transcription (shown by cross or 'x' at the transcription start site) by (1) inhibiting/altering the binding of transcription factors at the initiation complex and also by (2) recruiting repressors at the site of transcription. **(D)** Post-transcriptional regulation by lncRNAs. (1) MALAT1 is cleaved by RNaseP to generate a longer MALAT1 lncRNA and a 61 nucleotide mascRNA, which is transported to the cytoplasm; dotted arrow). MALAT1 binds to pre mRNA splicing factors (shown in red, blue and green) in the nuclear speckles and regulates their distribution and splicing activity. (2) Sno-lncRNA interacts with FOX proteins (black circles) and could participate in the FOX-mediated alternative splicing.

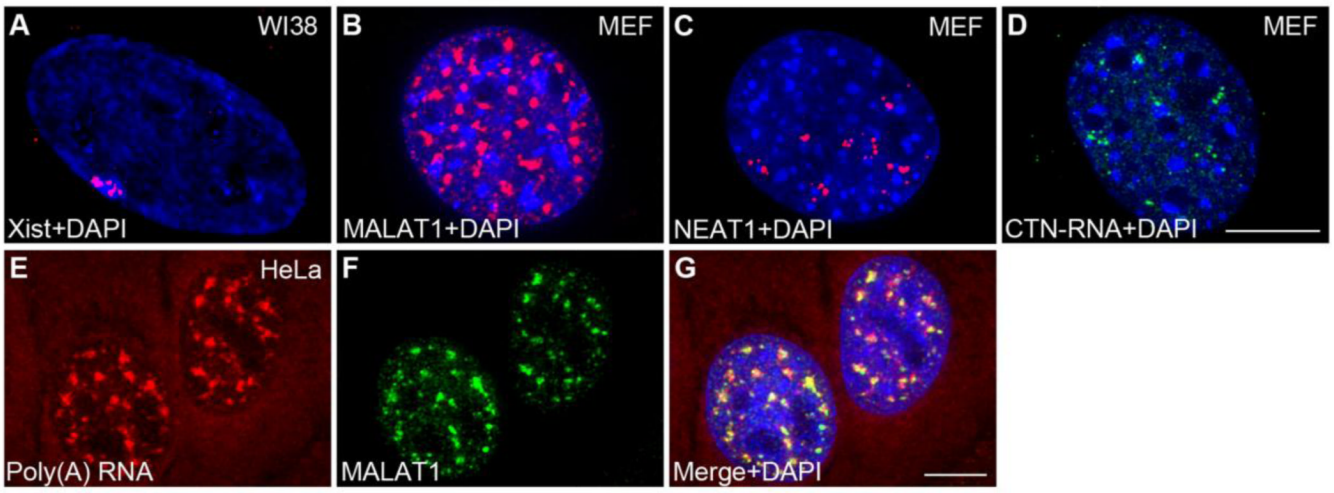

Figure 2. Nuclear-retained regulatory RNAs

(A) RNA-FISH using probe against the XIST RNA (red) reveal the localization of XIST RNA in the inactive X-chromosome. **(B)** RNA-FISH analysis reveal nuclear speckle localization of MALAT1 RNA (red) and **(C)** Paraspeckle localization of NEAT1 RNA (red) and **(D)** CTN-RNA (green). **(E–G)** Dual RNA-FISH analyses demonstrate the enrichment of poly adenylated RNA (**E**, red) and MALAT1 RNA (**F**, green) in the nuclear speckles. Total Poly (A) RNA is detected using fluorescently-labeled oligo (dT) probes. DNA is counterstained with DAPI (Blue). Bar 5μm.