The Long Arm of Long Noncoding RNAs: Roles as Sensors Regulating Gene Transcriptional Programs

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SUMMARY

A major surprise arising from genome-wide analyses has been the observation that the majority of the genome is transcribed, generating noncoding RNAs (ncRNAs). It is still an open question whether some or all of these ncRNAs constitute functional networks regulating gene transcriptional programs. However, in light of recent discoveries and given the diversity and flexibility of long ncRNAs and their abilities to nucleate molecular complexes and to form spatially compact arrays of complexes, it becomes likely that many or most ncRNAs act as sensors and integrators of a wide variety of regulated transcriptional responses and probably epigenetic events. Because many RNA-binding proteins, on binding RNAs, show distinct allosteric conformational alterations, we suggest that a ncRNA/RNA-binding protein-based strategy, perhaps in concert with several other mechanistic strategies, serves to integrate transcriptional, as well as RNA processing, regulatory programs.

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1 INTRODUCTION

Although the roles of cognate elements for DNA binding proteins and assorted coactivators and corepressors in organizing and encoding patterns of transcriptional responses are well established (Reviewed in Rosenfeld et al. 2006), the discovery that a huge percentage of the genome is transcribed into noncoding RNAs (ncRNAs), has presented the challenge of determining whether there is a vast functional network of ncRNAs that might regulate gene transcriptional programs (Bertone et al. 2004; Carninci et al. 2005; Cheng et al. 2005; Johnson et al. 2005; Katayama et al. 2005; Carninci et al. 2006; Gustincich et al. 2006; Willingham and Gingeras 2006).

The critical roles of some classes of small ncRNAs (reviewed in Chu and Rana 2007; Kutter and Svoboda 2008; Choudhuri 2009) are beginning to emerge; however, the broad spatial distribution of long ncRNA expression, often at remarkably low levels, has itself raised the challenge to delineate the mechanisms underlying their transcription, regulation, and potential functional roles. The basic questions of “who, when, what, where, and how” are still incompletely defined. Here, we will attempt to organize some of the rapidly expanding information regarding the long ncRNAs, focusing on their roles in transcriptional regulation of nonimprinted genes.

Based on a overview of the cumulative data in the literature regarding ncRNAs, we propose that ncRNAs use several mechanisms to exert important regulatory functions in the control of transcriptional programs (Fig. 1). This large number of ncRNAs have the inherent flexibility to function in “nucleating” molecules for combinatorial complex assembly, ideal for functioning as “sensors” and integrators for a variety of developmental and physiological signals. The alternative recruitment of “corepressors,” “coactivators,” and DNA-modifying complexes in various combinations might additionally be dictated by alterations in strandedness, transcriptional length, or splicing of ncRNAs, functionally modulating their biological actions. We suggest that most of the functions of long ncRNAs relate to their interactions with RNA-binding proteins, most of which contain multiple RNA-binding domains that may recognize distinct “targets.” This implies that many RNA-binding proteins might, as a consequence, show distinct “allosterically dictated” conformations based on the specific RNA sequences they bind—in a sense RNAs would be analogous to the effect of distant “ligands” on nuclear receptors (Glass and Rosenfeld 2000; McKenna and O’Malley 2002). Given that RNA-binding proteins constitute the largest “transcriptional” regulatory family in the genome (Burd and Dreyfuss 1994; Lunde et al. 2007), such an ncRNA/RNA-binding protein-based strategy to

integrate transcriptional programs is very likely a general rule in the transcriptional as well as RNA processing regulation. Taken together, the ncRNA sensor code appears to be a robust and critical strategy underlying a wide variety of gene regulatory programs.

2 CHARACTERIZATION OF LONG NCRNAS

Classification

Compared with small ncRNAs (Montgomery and Fire 1998; Grishok and Mello 2002; Ambros 2001; Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Vagin et al. 2006; Watanabe et al. 2006; Amaral and Mattick 2008; Lee et al. 2009), long ncRNAs are often arbitrarily considered as >200 nt and include long ncRNAs implicated in dosage compensation and imprinting. The most recently identified long ncRNAs in transcriptional regulation of nonimprinted genes are usually shorter than 10 kb. These long ncRNAs can be grouped into a remarkably diverse set of transcripts. One classification has been based on abundance, i.e., the “high abundance” (such as *NEAT1* and *NEAT2*; Hutchinson et al. 2007) and “low abundance” long ncRNAs (such as *CCND1*ncRNA; Wang et al. 2008a). Another classification is by function, i.e., “cis-acting” and “trans-acting” long ncRNAs. The conservative estimated number of long ncRNAs is ~17,000 in the human and ~10,000 in the mouse genome (www.InvivoGene.com). The number is likely to be greatly underestimated, especially, because many primary transcripts are often processed into smaller ncRNAs.

Strandedness

Long ncRNAs can be expressed from either or both DNA strands. Sense–antisense pairing between ncRNAs from two DNA strands has been observed in some cases. For example, massive sense–antisense pairing transcripts are found in *Alu* repeats (Wang et al. 2008b) and UTRs of protein coding genes (Kapranov et al. 2007). The significance of forming a double-stranded (ds) ncRNA is not clear. However, a ds ncRNA will lose the ability to pair with its complementary RNA or DNA sequence. Therefore, we hypothesize that the formation of a ds ncRNA may often act to block/regulate at least some functions of single-stranded (ss) ncRNAs. However, this does not exclude the possibility that ds ncRNAs form to change their affinity for RNA-binding proteins.

Subcellular Localization

In contrast to most mRNAs, which ultimately localize to the cytoplasm after processing, most long ncRNAs are

Figure 1. ncRNA-protein network in gene transcription program regulation. Comparing with the well-established DNA-transcriptional factor network (A), the ncRNA-protein network (B) provides a flexible platform capable of acting as sensors for various signals, integrating multiple regulatory complexes. In *cis* and in *trans* ncRNAs may work synergistically to recruit protein complexes involved in transcription regulation via RNA-binding proteins, as a bridge, to serve as a combinatorial DNA and histone modification code, altering chromosomal architecture.

permanently localized in the nucleus, including polyA-negative long ncRNAs that account for a large portion of the total transcribed sequences (Wu et al. 2008) and long ncRNAs transcribed from intronic regions (Cheng et al. 2005). A subset of long ncRNAs is located in both the nucleus and the cytoplasm (Imamura et al. 2004; Cheng et al. 2005; Kapranov et al. 2007; Wu et al. 2008). There are also some long ncRNAs selectively localized in the cytoplasm (Louroa et al. 2009).

Regulation/Processing

The tissue-/organ-specific expression patterns of many long ncRNAs in development, and the distinct subcellular localization of long ncRNAs, strongly suggest that their expression is under precise control (Amaral and Mattick 2008; Dinger et al. 2008; Mercer et al. 2008). Although some long ncRNAs have been reported to be transcribed by RNA polymerase III (Pol III) (Liu et al. 1995; Nguyen et al. 2001; Yang et al. 2001; Dieci et al. 2007), the majority are transcribed by RNA polymerase II (Pol II). However, it remains largely unknown how long ncRNAs are regulated at the level of their transcription and/or processing. For the ~1600 more abundant (conserved mammalian) long ncRNAs (Guttman et al. 2009; Khalil et al. 2009), it is

reported that the basic “rules” are analogous to those of conventional Pol II transcription units: having histone H3K4 trimethylation mark at their promoters and H3K36 trimethylation marks within transcription body, and forming the so-called chromatin signature of the K4-K36 domain (Fig. 2A) and having a 5' CAP site (Fig. 2B). Moreover, several well-known transcription factors for coding mRNA genes are also found to be present on a large number of ncRNA transcription units (Martone et al. 2003; Cawley et al. 2004; Kim et al. 2005). Other reported factors include Cha4, SAGA and Swi/Snf in *Saccharomyces cerevisiae* and REST in mammalian cells (Reviewed in Neurosci Letter 2009. Aug 11, Epub ahead of print). A central question for those ncRNAs that do not show the conventional H3 trimethylation of K4 and K36 marks and the Cap site (Fig. 2C) is whether this reflects processing from very long ncRNA transcripts that do harbor such sites at their transcription origins, or represents an alternative transcriptional regulatory strategy, such as polymerase entry at nicked sites.

Long ncRNAs are often spliced (Fig. 2D) and appear to use mechanisms similar to those used for miRNA processing. For example, we found that knock-down of the miRNA regulator DROSHA, but not DICER, increased the expression level of *CCND1*ncRNA about four-fold (TW, XS and

Figure 2. Characterization of long ncRNAs. (A) Long ncRNAs can be generated from intergenic K4–K36 domains as exemplified by the *Srpk2* intergenic long ncRNAs, which were identified by hybridizing RNA to DNA tiling arrays (Adopted from Guttman et al. 2009). (B) Expression of promoter-associated long ncRNAs can be identified by their correlation to H3K4 me3 and CAGE-tag (Adopted from Guttman et al. 2009). (C) Some long ncRNAs have no apparent Cap site been identified (such as the *CCND1*ncRNAs). (D) Some long ncRNAs are processed by splicing such as the disease loci in *CDKN2BAS* (*ANRIL*; Pasmant et al. 2007).

MGR, unpublished data), suggesting that *CCND1*ncRNA may be processed in a DROSHA-dependent manner.

3 LONG NCRNA-DEPENDENT RECRUITMENT OF PROTEIN COMPLEXES

One critical function of many, if not all, ncRNAs would appear to reside in their ability to interact with specific regulatory proteins/protein complexes, probably usually involving members of the large families of RNA-binding proteins. Because of RNA sequence and structural flexibility, ncRNAs are well-suited to accommodate binding of multiple complexes. Indeed, many ncRNAs associate with proteins and form complexes that function as a unit, as exemplified by the RNA Pol III-transcribed ribosome, RNA-induced silencing complex (RISC), and signal recognition particle (the conserved SRP, protein-RNA complex;

Batey et al. 2000; Storz 2002). As more and more ncRNAs have been discovered, some generalized rules regarding potentially associated protein complexes are beginning to be discerned.

Studies from several groups suggested a critical role of long ncRNAs in epigenetic regulation by orienting chromatin-modifying factors/complexes to specific locations in the genome and in the nucleus (Sanchez-Elsner et al. 2006; Rinn et al. 2007; Chen et al. 2008; Zhao et al. 2008; Khalil et al. 2009). Initially described factors/complexes that are recruited by long ncRNAs include Ash1 by *TRE1-3* (Sanchez-Elsner et al. 2006) and MSL/MSL2 by *roX* RNA (Li et al. 2008; and reviewed in Scott and Li 2008) in *Drosophila*; the SRC1 complex by *SRA* (Lanz et al. 1999), PRC1 and PRC2 complexes by *Xist* (Plath et al. 2002; de Napoles et al. 2004; Schoeftner et al. 2006), PRC2 complex by *HOTAIR* and *RepA* (Rinn et al. 2007; Zhao

et al. 2008), G9a by *Air* (Nagano et al. 2008), and TLS/CBP/p300 complex by *CCND1*ncRNA (Wang et al. 2008) in mammalian cells. Recently, a genome-wide ChIP-RNA sequencing analysis found that up to 38% of the ~3300 conserved large intergenic ncRNAs are associated with one of the following four chromatin-modifying factors—EZH2, SUZ12, CoREST, and JARID1C/SMCX (Khalil et al. 2009). Besides, the homeodomain protein Dlx-2 has been shown to be recruited to the intergenic enhancer region of *Dlx-5* and *Dlx-6* via a brain specific ncRNA, *Evf2* (Feng et al. 2006).

It is very interesting that most of the reported long ncRNAs-associated proteins are chromatin-modifying factors. Other chromatin/histone-modifying factors, such as LSD1, a component of the CoREST complex (Shi et al. 2005), are likely to combinatorially impose strong effects as well. We propose that the complexity and diversity of ncRNAs promote the formation of chromatin-modifying complexes to establish the “epigenetic” memory. Despite the fact that most of the reported ncRNA-associated chromatin-modifying complexes are involved in gene repression, we suggest that an equally large numbers of long ncRNAs can recruit coactivator complexes, including components of the trithorax/COMPASS/MLL complex (Beisel et al. 2007; Schuettengruber et al. 2007; Shilatifard 2008). An immediate question is how regulatory ncRNAs recruit protein complexes. One possibility is that ncRNAs form RNA:RNA or RNA:DNA structures, which provide sequence specificity and serve as platforms to bind proteins that are not strictly sequence-specific, and thus directing these proteins to target sites (Mattick and Gagen 2001; Mattick 2007). It is also possible that ncRNAs can alter their structure on “ligand” binding and function as “riboswitches” (Wickiser et al. 2005; St Laurent and Wahlestedt 2007). It will be of interest to explore more open questions as: How many distinct complexes are recruited to various ncRNAs? What are the combinatorial “programs” of multiple coregulator complexes that might be required for maintaining epigenetic memory?

4 MECHANISMS MEDIATING TRANSCRIPTIONAL REGULATION BY LONG NCRNAS

Given their widespread distribution, long ncRNAs are likely to play roles in gene repression and/or activation, acting as sensors of various regulatory signals. The central strategy is the use of epigenetic regulation, including histone and DNA methylation, and many other post-translational modifications and remodeling complexes. In addition, long ncRNAs can affect the loading of general transcription factors as well as polymerase, or modulate the activities of specific transcription factors.

Serving as Ligands or Cofactors to Mediate Histone Modification

SRA

Nuclear receptors (NRs) comprise a super family of ligand-dependent transcription factors that regulate metabolism, development and reproduction (reviewed in Glass and Rosenfeld 2000; McKenna and O'Malley 2002). It has been well-established that the activities of NRs are mediated by the ligand-dependent exchange of coactivators and corepressors, which were initially thought to all be proteins. The finding that an ncRNA, *SRA*, functions as a coactivator (Lanz et al. 1999) significantly expanded concepts of mechanisms enabling transcriptional coactivation. *SRA* coactivates a range of NRs, including ER, AR, GR, PR, RAR α , PPAR δ and γ , TR, and VDR (Lanz et al. 1999; Deblois and Giguere 2003; Kawashima et al. 2003; Zhao et al. 2004; Hatchell et al. 2006), and some other classes of transcription factors, such as MyoD (Carette et al. 2006). *SRA* has multiple stem-loops and a series of mutational studies showed that discrete stem-loops are required for the coactivator activity of *SRA*. Many *SRA*-associated RNA-binding proteins have been found to either positively or negatively regulate the coactivator activity of *SRA*. For example, the coactivator activity of *SRA* on *Myo D* is augmented in the presence of DEAD box-containing RNA-binding proteins p68 and p72 (Carette et al. 2006). In the absence of ligand, *SRA* is sequestered by the transcriptionally silent TR α 2 to a repressive protein complex containing RNA-binding proteins SHARP and SLIRP (Shi et al. 2001; Hatchell et al. 2006). SHARP and SLIRP repress the activities of a range of NRs through the recruitment of HDAC and NCoR/SMRT (Shi, Downes et al. 2001; Hatchell, Colley et al. 2006). When the ligand is present, *SRA* is released from TR α 2 and switches between binding corepressor complexes and binding coactivator complexes (e.g., SRC1 and SRC2; Xu and Koenig 2004; Xu and Koenig 2005).

Evf2

Another example for a long ncRNA serving as a transcription coactivator/corepressor is *Evf2* in the developing mouse forebrain. *Evf2* is a long, polyadenylated ncRNA transcribed from an ultraconserved intergenic enhancer region associated with the *Dlx-5/6* locus (Feng et al. 2006) (Fig. 3A). The *Dlx* genes are related to the *Drosophila Distalless* gene (*dll*) homeodomain-containing protein family, and play crucial roles in neuronal development. Ei and eii are intergenic enhancers identified from *Dlx-5/6* loci, regulated by homeodomain protein Dlx-2. *Evf2* is transcribed from the Ei region and its expression is highly correlated

Figure 3. ncRNAs act as co-factors or ligands to regulate transcription. (A) A brain-specific ncRNA, *Evf2* is generated from an intergenic enhancer region of *DLX-5/DLX-6* and promotes the enhancer activity via recruitment of homeodomain protein *DLX-2* (Left). Both *DLX-2* and *MeCP2* are dismissed from the intergenic enhancer region when the expression of *Evf2* is disrupted (Right). (B) Low-copy numbered *CCND1* ncRNAs are induced on DNA damage and *in cis* recruit an RNA-binding protein, *TLS* to inhibit the *CCND1* mRNA expression via inhibiting the HAT activities of *CBP/p300*. *PRC2* complex is recruited to the *CCND1* 5' regulatory region via *TLS*. (C) *HOTAIR* generated from *HOXC* on Chr. 12, acts *in trans* on *HOXD* on Chr. 2 to repress the gene expression via recruitment of *PRC2* complex.

with the expression of *Dlx-5* and *-6*. *Evf2* forms a complex with *Dlx-2* and recruits *Dlx-2* to induce the enhancer activities of *ei* and *eii*, resulting in induced expression of both *Dlx-5* and *-6*. These data suggest that the *Evf2* ncRNA functions as a coactivator molecule, analogous to the functions of *SRA*.

Evf2-null mice were generated by inserting transcription stop sites into *Evf* exon 1 (Bond et al. 2009). ChIP analysis on these *Evf2*-null mice revealed that both *Dlx* and *MeCP2*, a previous known repressor of *Dlx-5*, are dismissed from *ei* and *eii*. *MeCP2*-mediated repression is suggested to be through the recruitment of HDACs. The binding of HDACs, however, is not changed in the absence of *MeCP2* in *Evf2*-null mice. Surprisingly, the levels of *Dlx-5* and *-6* transcripts increased in *Evf2*-null mice. It is unclear how *Dlx-5* and *-6* are transcribed in the absence of *Dlx* binding. It is possible that *Dlx* proteins may only be required for initial activation of *Dlx-5/6* in an *Evf2*-independent manner, whereas subsequent regulation of *Dlx-5/6* by *Dlx* and *MeCP2* is *Evf2*-dependent. Alternatively, other *Dlx*-binding sites compensate in the absence of *Dlx-ei/eii* interactions; or the major role of *Dlx* proteins is to prevent repressors such as *MeCP2* from binding *ei/eii*, rather than acting as direct activators.

CCND1 ncRNA

Promoter-associated ncRNAs can function as ligands to mediate histone modifications, exemplified by our study of *CCND1* ncRNA and members of the TET RNA-binding protein family, including *TLS*, *EWS*, and *TAFII68* (Wang et al. 2008) (Fig. 3B). *TLS*, translocated in liposarcoma, is an RNA-binding protein with RNA-binding domains at its C-terminus. These RNA-binding domains are frequently deleted in human tumors in which the amino terminus of *TLS* is fused with other transcriptional factors, suggesting an important role of RNA-binding domains of *TLS* in human disease. The amino-terminal glutamine-rich domain of *TLS*, on the other hand, is responsible for the interaction with two well-known histone acetyltransferases, *CBP* and *p300*. The interaction between *TLS* and *CBP/p300* results in the substrate-specific inhibition of the HAT activities of *CBP/p300* (Wang et al. 2008). We reported that a series of ncRNAs (*CCND1* ncRNAs) are generated from the 5' regulatory regions of *CCND1*. The *CCND1* ncRNAs are upregulated in response to genotoxic stress, when the *CCND1* mRNA is down-regulated. The induction the *CCND1* ncRNAs recruit *TLS* and cause a close-to-open conformation change in *TLS* that licenses

its amino-terminal binding of CBP/p300, resulting in substrate-specific inhibition of their HAT enzymatic activities, and thus establishing the hypo-acetylation status of the chromatin and repressing of the *CCND1* mRNA expression. Surprisingly, these *CCND1*ncRNAs are at very low abundance (two to eight copies/cell) and are associated with chromatin, functioning *in cis* as ss RNAs to recruit and modulate the activity of TLS. Interestingly, these ncRNAs appear to facilitate the recruitment of PRC1 and PRC2 complexes as well (Fig. 1) (TW, XS, BS, and MGR, unpublished data). In combination with our preliminary data that TLS binds to the EZH2 complex (BS, TW, XS, and MGR, unpublished data), it further suggested that TLS might be required for the recruitment of EZH2 to *CCND1* promoter during repression.

HOTAIR

HOX genes are a group of genes that control the anterior–posterior axis and segmentation during development. Humans contain four clusters of *HOX* genes, *HOXA* on chromosome 7, *HOXB* on chromosome 17, *HOXC* on chromosome 12, and *HOXD* on chromosome 7 (Wellik 2009). Tiling arrays covering all four human *HOX* clusters identified 231 novel ncRNAs, which are spatially expressed along developmental axes and show distinct histone methylation patterns (Rinn, Kertesz et al. 2007). Among these, a 2.2 kb long ncRNA, *HOTAIR*, transcribed from the boundary of two diametric chromatin domains in the *HOXC* locus was preferentially expressed in posterior and distal sites. *HOTAIR* recruits PRC2 complex *in trans* across 40 kb of the *HOXD* locus on chromosome 2, promotes the histone H3K27 trimethylation and results in transcriptional repression of *HOXD* locus (Fig. 3C). Therefore, *HOTAIR* represents an ncRNA that exerts transcriptional repression, at least in part, through recruitment of the chromatin modifying enzyme complex PRC2.

DNA Methylation

Many protein-coding genes have antisense partners, including tumor suppressor genes (Yu et al. 2008). Misregulation of the associated antisense ncRNAs may subsequently silence the tumor suppressor gene (Fig. 4A), leading to oncogenesis (Yu et al. 2008).

P15AS

A long (~200 bp) antisense ncRNA, *P15AS*, was identified from *P15* gene. *P15* is frequently hypermethylated, thus silenced, in leukaemia (Lubbert 2003). It is well established that small ncRNAs, such as microRNA and piRNA, can mediate DNA methylation in many species (Bao et al.

2004; Matzke et al. 2004; Ronemus and Martienssen 2005). The opposite expression patterns between *P15AS* and *P15* raise the possibility that *P15AS* may trigger epigenetic silencing of the *p15* genes in cancer cell lines. Indeed, the activity of the *P15* promoter could not be reactivated by removal of *p15AS* (Yu et al. 2008). In the presence of exogenous *p15AS*, the *p15* promoter showed a marked increase in dimethylation of H3K9 and a decrease in dimethylation of H3K4 in human cancer cell lines and mouse embryonic stem cells (Yu et al. 2008). DNA hypermethylation was induced by *p15AS* when embryonic stem cells were differentiated into embryoid bodies (Yu, Gius et al. 2008). Antisense transcript-mediated silencing could be a general mechanism for the silencing of many other tumor suppressor genes in cancer, many of which have long antisense transcripts (Fig. 4A).

Khps1

Khps1 is an antisense, long ncRNAs generated from the regulatory region (T-DMR) of the sense transcript *Sphk1* (Imamura et al. 2004). When overexpressed, *Khps1* leads to CpG demethylation and simultaneous non-CG methylation in the T-DMR (Imamura et al. 2004), the methylation status of which correlates with the regulation of *Sphk1* expression (Imamura et al. 2001). This RNA-induced CG demethylation and non-CG methylation suggests an intriguing and important connection between ncRNAs and epigenetic regulation (Imamura et al. 2004).

General Transcription Factors and RNA pol II Loading

It is known that RNA has the ability to regulate bacterial RNA polymerase activity (Wassarman and Storz 2000). There are now quite a few cases in which long ncRNAs have been reported to promote or repress transcription by binding to mammalian RNA pol II and modulating the latter's loading on the promoters of regulated genes (Fig. 4B).

Alu RNA

Massive ncRNAs have been found to be transcribed from the *SINEs* retrotransposon elements, including *Alu* repeats in human cells and *SINE B1* and *SINE B2* in mouse cells (Maraia et al. 1993; Kramerov and Vassetzky 2005). These ncRNAs have been suggested to be involved in gene regulation through roles in transcription, mRNA editing, or even miRNA regulation (Reviewed in Hasler and Strub 2006; Hasler et al. 2007). *Alu* RNA and *SINE B2* RNA have been shown to repress mRNA transcription by preventing RNA pol II loading during heat shock (Allen et al. 2004; Espinoza et al. 2004; Espinoza et al. 2007; Mariner et al. 2008; Yakovchuk et al. 2009). Both *SINE B2* and *Alu* RNA

Figure 4. ncRNAs act as transcription regulators *via* diverse mechanism. (A) Antisense ncRNA from tumor suppressor gene (TSG) induces DNA and repressive histone methylation on the promoter of TSG. (B) ncRNA generated from an alternative transcription start site (TSS) plays either a repressive or activating role by modulating the recruitment of general transcription factors on the promoter of its adjacent gene. (C) ncRNA *NEAT1* is specifically expressed in paraspeckles and colocalized with paraspeckle markers (PSF, p54/nrb, PSP1, PSP2 and CFI(m)/68). Knockdown of *NEAT1* by specific siRNA disrupts paraspeckles, whereas overexpression of *NEAT1* increases the number of paraspeckles. (D) ncRNA *NRON* prevents nuclear localization of the transcription factor NFAT.

could directly bind to RNA pol II with a high affinity (Allen et al. 2004; Espinoza et al. 2004; Mariner et al. 2008). Surprisingly, *Alu* RNA contains modular domains, a hallmark of protein regulators of RNA pol II transcription (Mariner et al. 2008). The modular domain of *Alu* RNA can be fused to a RNA pol II-binding *SINE* B1 RNA to mediate transcription repression, in a manner similar to trans-acting protein transcription factors (Mariner et al. 2008). Because *SINE* B2 RNA is able to block the association of RNA pol II

as a DNA/RNA hybrid, it was proposed that the modular repression domains in *Alu* RNA target the DNA-binding channel of RNA pol II and thus prevent the polymerase from forming proper contacts with promoter DNA (Mariner et al. 2008). Indeed, this hypothesis was supported by a series of biochemical assays (Yakovchuk et al. 2009). *Alu* RNA and *SINE* B2 RNA apparently prevented the closed complexes between RNA pol II and the promoter DNA, despite the fact that RNA pol II remains associated

with the promoter DNA. This effect is reversible in the presence of RNase I, but only so before the formation of the closed complex, suggesting these two ncRNAs block the re-engagement of RNA pol II on the promoter DNA (Yakovchuk et al. 2009). Together, these data suggest that ncRNAs derived from *SINE* elements function as trans-repressors of gene expression.

Fbp1+ ncRNA

In the fission yeast *S. pombe*, the expression of *fbp1+* is induced by glucose starvation (Hirota et al. 2008). Glucose starvation also induces at least three large (~ several kb) and rare sense-stranded ncRNA transcripts before induction of the *fbp1+* transcript. The transcription start sites (TSSs) for these ncRNAs are located at ~-1.3 kb to -530 bp from the ATG. During the glucose starvation, the RNA pol II binding sites shift from the 5' to 3' region in the *fbp1+* promoter (from ncRNA to *fbp1+* transcript), paralleling a corresponding alteration of chromatin structure determined by MNase digestion. Disruption of the ncRNA transcripts by inserting a terminator between the ncRNA transcripts and the TATA box resulted in short and premature ncRNA products, failed RNA pol II recruitment, and chromatin remodeling in the downstream region of the terminator, and eliminated *fbp1+* induction in the absence of glucose (Hirota et al. 2008). These results suggest that the RNA pol II-mediated ncRNA transcripts across the *fbp1+* promoter increased access to the RNA polymerase and transcriptional activators through progressive opening of chromatin structure (Fig. 4B). Although they affect the activity of general transcription factors and/or RNA pol II, these long ncRNAs regulate gene transcription in a gene-specific manner. It will therefore be of interest to determine whether additional *cis* acting ncNRAs are involved in these functions.

Transcription Interference

Alternative TSSs have been found on >80% of the tested genes by the Encyclopedia of DNA Elements (ENCODE) project (Trinklein et al. 2003; Carninci et al. 2005; Cooper et al. 2006; Birney et al. 2007; Kawaji et al. 2009). These newly identified TSSs are located at 5' distal or internal to the annotated gene boundary. Long ncRNA transcripts generated from the alternative TSS may affect the transcription of the adjacent mRNA genes by interference with the loading of the general transcriptional factor, such as TBP (Fig. 4B). The examples of this mechanism are a long ncRNA generated from an upstream minor promoter that inhibits the *DHFR* gene encoding dihydrofolate in human (Martianov et al. 2007) and a long ncRNA, *SRG1*, transcribed from

the upstream alternative TSS of the *SER3* gene in *S. cerevisiae* (Martens et al. 2004; Martens et al. 2005).

Regulating the Activity of Transcriptional Factors

By screening a short hairpin RNA library against 512 evolutionarily conserved putative ncRNAs, an ncRNA – *NRON* (noncoding repressor of NFAT), was found to repress the nuclear factor of activated T cells (NFAT) family (Willingham et al. 2005). *NRON* has three splicing variants, with size ranging from 0.8kb to 3.7kb. *NRON* showed tissue-specific expression and it was particularly enriched in lymphoid tissues. Eleven proteins were found to bind to *NRON*, including importin- β and factors directly mediating the nuclear-cytoplasmic transport of cargoes such as NFAT, suggesting that *NRON* may act as a modulator of NFAT nuclear trafficking (Fig. 4D). This was supported by the evidence that the level of nuclear NFAT was greatly elevated in the presence of *NRON* shRNA (Willingham et al. 2005). It is unknown if the role of *NRON* is to repress nuclear import or to promote the nuclear export of NFAT.

The mechanisms of long ncRNA regulating transcription are diverse, many of which are not included in above discussion. For example, a newly identified ncRNA, *HSR1*, has been shown to be involved in regulating the activity of the heat shock transcriptional factor 1 (*HSF1*) (Shamovsky et al. 2006). Because *HSR1* was coimmunoprecipitated from *HSF1*-eEF1A complex, the interaction of *HSR1* and eEF1A was suggested to be required for the activation of *HSF1* during heat shock.

5 BIOLOGICAL ROLES OF LONG NCRNAS

Although still unresolved, the idea that many or even a majority of the ncRNA transcripts are functional has been suggested by the fact that the percentage of the ncRNAs transcribed in the genome is proportional to the complexity of the organism (Taft et al. 2007), and it has received initial experimental support.

Roles of Promoter-Associated ncRNAs

Transcriptome maps in the entire nonrepetitive portion of the human genome revealed many long ncRNAs (and short ncRNAs) around promoter regions (Kapranov et al. 2007). The number of identified promoter-associated ncRNAs continues to grow in human (Calin et al. 2007; Guenther et al. 2007; Kapranov et al. 2007) as well as other species (Davis and Ares 2006; Guenther et al. 2007). Many of these transcripts are under precise control of diverse signals. Another class of less stable ncRNAs, promoter upstream transcripts, was revealed after siRNA deletion

of hRrp40, a core component of the human 3' to 5' exoribonucleolytic exosome, which is one of the major RNA degradation complexes (Preker et al. 2008). These transcripts are reminiscent of the cryptic unstable transcripts in *S. cerevisiae* (Wyers et al. 2005; Neil et al. 2009; Xu et al. 2009), which are located 0.5–2.5 kb upstream of active TSSs and require the downstream promoters. Promoter upstream transcripts partially overlap with promoter-associated ncRNAs, but lack their own known promoters. Both promoter-associated and upstream ncRNAs can be bidirectionally transcribed. These ncRNAs are postulated to have regulatory functions, as suggested by a few ncRNAs that are experimentally supported to regulate transcription of downstream genes, e.g., *CCND1*ncRNA (Wang et al. 2008), *Khps1a* (Imamura et al. 2004), and *DHFR* ncRNA (Martianov et al. 2007), although some events have been suggested to reflect “occlusion” rather than via recruiting RNA-binding proteins or controlling CpG demethylation (Imamura et al. 2004); however, those events are likely to be mechanistically linked. An alternative, nonexclusive function of these long ncRNAs around or upstream of active promoters is to alter chromatin structure through their own transcription.

Roles of ncRNAs as “Transcriptional” Boundary Markers

Almost half of the human protein-coding genes have been suggested to be marked by both promoter- and 3'-associated short ncRNAs (Kapranov et al. 2007; Borel et al. 2008). That 40% of the small ncRNAs could be processed from long ncRNAs as indicated by the genome-wide, high resolution tiling arrays (Kapranov et al. 2007) raises the possibility that at least a portion of the short ncRNAs represent processed products of the long ncRNAs, thus serving as markers of boundaries of mRNA transcription units. In addition, they may function in stabilizing “transcriptional” boundaries (Kapranov et al. 2007), conceptually analogous to the proposed network for boundary elements, such as CTCF (Phillips and Corces 2009).

Roles of ncRNAs as Sensors of Signals

In concert with their ability to recruit specific complexes, long ncRNAs appear to function as sensors of developmental signals and other signaling pathways, such as genotoxic stress. As an example, we have reported that *CCND1* ncRNAs, generated in the regulatory region of the *CCND1*, sense the genotoxic stress signal and up-regulate, followed by *in cis* recruiting TLS, which leads to its changing conformation to be able to bind and inhibit CBP/p300 and results in *CCND1* mRNA repression (Wang et al. 2008).

Although we will not discuss in detail the function of the long ncRNAs in dosage compensation and imprinting in this review, we would like to point out a 1.6-kb long ncRNA (*RepA*) within another long ncRNA, *Xist*, functions as a developmental signal sensor, at onset of X-inactivation when the antisense ncRNA *Tsix* is down-regulated, and recruits the PRC2 complex to *Xist*, which leads to the initiation and spreading of X-inactivation (Zhao et al. 2008). Likewise, preferentially expressed in posterior and distal sites, *HOTAIR* in the *HOXC* cluster targets PRC2 to distant loci in *HOXD* and represses its expression *in trans*, representing an example of long ncRNAs sensing developmental signals that control anterior–posterior axis (Rinn et al. 2007).

Potential Roles in Structural Integrity of Cellular Organelles and Architecture

Analogous to the role of rRNAs in ribosome assembly, long ncRNAs can exert functional roles in specific nuclear organelle assembly, exemplified by the actions of the long ncRNA *NEAT1* (*MEN ϵ*), which is functionally essential for structural integrity of nuclear paraspeckles (Fox et al. 2002; Clemson et al. 2009; Sasaki et al. 2009; Sunwoo et al. 2009). *NEAT1* (*MEN ϵ*) and *NEAT2* are the two (out of three) large, polyadenylated, nuclear enriched ncRNAs identified in two human female cell lines using Affymetrix U133A and U133B expression arrays (Hutchinson et al. 2007). Both genes are located on human chromosome 11, and have no significant homology to each other. However, they both are highly conserved within the mammalian lineage, suggesting a role specific for mammals during evolution. *NEAT2* is colocalized with SC-35+ nuclear speckles/inter-chromatin granules, whereas *NEAT1* (*MEN ϵ*) is localized predominantly to domains adjacent to nuclear speckles—paraspeckles (Fox et al. 2002; Clemson et al. 2009; Sasaki et al. 2009; Sunwoo et al. 2009). Paraspeckles are marked by paraspeckle-associated proteins—PSP1, PSP2, PSF/PSFQ, and p54/nrb, and possibly CFI(m)/68, whose functions have been suggested in transcription, pre-mRNA splicing and nuclear retention of RNA (Fox et al. 2002; Dettwiler et al. 2004; Fox et al. 2005). Knock-down of *NEAT1* (*MEN ϵ*), but not *NEAT2*, specifically eradicated paraspeckles in many cell lines including 293, HeLa, HT-1080, and Tig1, suggesting that *NEAT1* (*MEN ϵ*) RNA is required for paraspeckle formation (Fig. 4C). On the other hand, overexpression of *NEAT1* (*MEN ϵ*) induced more paraspeckle staining (Fig. 4C). In addition, the flexibility of RNA to accommodate a series of complexes and to extend across large physical distances, suggests the potential to play key roles in regulation of nuclear architecture.

6 CONCLUSIONS AND PERSPECTIVES

Together, the cumulative data suggest that ncRNAs exert important regulatory functions in the control of transcriptional programs (Fig. 1). Because of the large number of long ncRNAs, and their functions in “nucleating” molecules for combinatorial complex assembly, the resultant ncRNA-protein interactions are likely to serve as “sensors” for a variety of developmental and signaling pathways, nuclear receptor ligands, and genotoxic-stress. The presence of ss ncRNA versus regions of ds ncRNAs is likely to be dynamically regulated in the environment of the cell. This relationship may dictate the alternative recruitment of “corepressors,” “coactivators,” and DNA-modifying complexes. Thus it is tempting to postulate that the ncRNAs might themselves serve as the critical arbiters of local DNA methylation patterns and even as key arbiters in establishing regions of “epigenetic” memory. Conversely, it is likely that alterations in strandedness/transcriptional length/splicing of ncRNAs functionally modulate their actions. An additional consideration in the actions of long ncRNAs relates to the architecture of most RNA-binding proteins, which contain multiple RNA-binding domains that may recognize distinct “targets.” This implies that many RNA-binding proteins might, as a consequence, show distinct “allosterically-dictated” conformations based on the specific RNA sequences they bind that might regulate interactions with other proteins required for distinct functions—for example at promoters versus during transcriptional elongation/splicing. Given that RNA-binding proteins constitute the largest “transcriptional” regulatory family in the genome (Burd and Dreyfuss 1994; Lunde et al. 2007), ncRNA/RNA-binding protein-based strategy to integrate transcriptional programs is very likely a general rule in the transcriptional as well as RNA processing regulation. Considering the broad expression of ncRNAs, and the ambiguity of any sequence specificity of the reported ncRNAs acting *in trans*, it is even possible that they are recruited by *cis*-acting ncRNAs. Together, the ncRNA sensor code appears to be a robust and critical strategy underlying a wide variety of gene regulatory programs and we are certain to see an explosive increment of knowledge and insights into this area in the coming months.

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