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Long non-coding RNA-mediated mechanisms independent of the RNAi pathway in animals and plants

Phil Chi Khang Au,* Qian-Hao Zhu, Elizabeth S. Dennis and Ming-Bo Wang

Commonwealth Scientific and Industrial Research Organisation Plant Industry; Canberra, ACT Australia

Key words: long non-coding RNA, chromatin modifications, transcriptional gene regulation, translational gene regulation, transcription factor regulation

Abbreviations: *CCND1*, *cyclin D1*; CGR, CR20-GUT15-related; *COLDAIR*, *cold assisted intronic non-coding RNA*; *COOLAIR*, *cold induced long antisense intragenic RNA*; *DHFR*, *dihydrofolate reductas*; DNMT3b, DNA methyltransferase 3b; *FLC, flowering locus C*; *Gas5*, *growth arrest-specific 5*; GR, glucocorticoid receptor; GREs, glucocorticoid response elements; H3K9, histone 3 lysine 9; H3K27, histone 3 lysine 27; H3K27me3, histone 3 lysine 27 trimethylation; hnRNP-K, heterogeneous nuclear ribonucleoprotein K; *HOTAIR*, *HOX* antisense intergenic RNA; HSF, heat shock transcription factor; *HSR1*, *heat shock RNA 1*; lncRNA, long non-coding RNA; LSD1, lysine-specific demethylase 1; MEN, multiple endocrine neoplasia; miRNA, micro RNA; MtRBP1, *Medicago truncatula* RNA binding protein 1; NAT, natural antisense transcripts; NFAT, nuclear factor of activated T cells; ncRNA, non-coding RNA; *NRON*, *non-coding Repressor Of NFAT*; PRC2, polycomb repressive complex 2; SINE, short interspersed elements; siRNA, small interfering RNA; *SRA*, *steroid receptor RNA activator;* SRAP, steroid receptor RNA activator protein; SRC-1, steroid receptor coactivator 1; *SRG1*, *SER3 regulatory gene 1*; TF, transcription factor; TLS, translocated in liposarcoma

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Recent advances in the field of RNA research have provided compelling evidence implicating long non-coding RNA molecules in many diverse and substantial biological processes that include transcriptional and post-transcriptional regulation of gene expression, genomic imprinting, modulation of protein activity and subcellular localization and cellular structural maintenance. While long non-coding RNAs have been most extensively studied in animal species, studies of long non-coding RNA in plants begin to emerge showing some conservation of mechanisms. This review aims to provide an overview of significant and recently identified long non-coding RNA-mediated mechanisms in both animal and plant species.

Introduction

The mechanisms mediated by non-coding (nc) RNA enhance the complexity of eukaryotic organisms. This is a plausible explanation to the surprising finding that different orders of eukaryotes have approximately the same number of protein coding genes but vastly different phenotypic complexity.[1,2] In contrast, the number of non-coding genes increases proportionally with increasing developmental complexity with 98% of the human transcriptome represented as ncRNAs.[2,3] ncRNAs are not translated into proteins and were believed to represent transcriptional noise with the exception of those that play specific roles in transport, splicing and synthesis of proteins including ribosomal RNA and transfer RNA. Over the last decade, studies of ncRNA have shed light on a series of novel mechanisms reshaping our understanding of the RNA world and provided compelling evidence of cellular function. The nature, structure and function of ncRNAs can vary and are diverse. Expression can be tissue- and cell-specific, exhibiting subcellular localization and is regulated in a spatial and temporal manner. Most ncRNAs studied in eukaryotic species are associated with transcriptional and post-transcriptional regulation including imprinting, X-chromosome inactivation and targeted mRNA degradation. ncRNAs also function as molecular cargos to target protein subcellular localization, induce changes in protein conformation and are implicated in cellular structural maintenance. Mechanisms that induce gene silencing through the RNA interference pathways (small interfering RNA and micro-RNA directed silencing) have been extensively studied in both animals and plants and are well reviewed in many publications.[4,5] In contrast, the study of long ncRNAs (lncRNAs) is still at its infancy and many lncRNAs remain uncharacterized. lncRNAs are generally considered as transcripts longer than 200 nucleotides (nt) that can bear many signatures of mRNA including 5'capping, splicing and polyadenylation but have few or no ORFs.[6,7] Here, we review the mechanisms predominantly mediated by lncRNAs (**Table 1**) that are unrelated to the RNA interference pathway in animals and plants; therefore ncRNAs involved in the siRNA and miRNA pathways including primary transcripts of miRNAs will not be discussed. A new class

*Correspondence to: Phil Chi Khang Au; Email: Phil.Au@csiro.au
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Table 1. Summary of ncRNAs and methodologies employed to deduce functions in animals and plants

	Species	ncRNA	Function	Methodology	Reference
Animals	Human	ncRNA from upstream *DHFR*	Forms triplex with the major promoter of *DHFR* and associate with TFIIB to prevent *DHFR* expression	siRNA knockdown; ChIP; RNA-IP	40
	Human	*licnRNA-p21*	Mediate global gene repression via association with nuclear ribonucleoprotein K	ChIP; RNA-IP; siRNA knockdown; overexpression	43
	Human	*Gas5*	Inhibit expression of glucocorticoid-responsive genes by binding to the DNA-binding domain of GR transcription factor	siRNA knock-down; overexpression; RNA-IP; FISH; ChIP	48
	Human	NATs	Regulation of gene expression by association with sense RNA to regulate translation	Overexpression	51
	Human	ncRNAs from 5' of *CCND1*	Allosterically alter the structure of TLS leading to repression of *CCND1*	siRNA knockdown; ChIP; RNA-IP	66
	Human	*SRA*	Required to confer functional specificity of nuclear receptor coactivator SRC-1	Mutagenesis; overexpression; RNA-IP	67
	Human	*Alu*	Conformational inactivation of RNA Pol II	ChIP; mutagenesis	77
	Human	*HSR1*	Activate the DNA-binding activity of HSF	siRNA and antisense knockdown	85
	Human and mouse	*HOTAIR*	In trans epigenetic regulation of the HOXD gene clusters	siRNA knockdown	12, 13
	Human and mouse	*NRON*	Repress NFAT activity by preventing nuclear accumulation	siRNA knockdown	65
	Mouse	*Air*	Regulate genomic imprinting *Igf2r*, *Slc22a2* and *Slc22a3* genes	Mutagenesis; FISH; ChIP; RNA-IP	10, 19
	Mouse	*Kcnqlot1*	Regulate genomic imprinting of the Kcq1 gene cluster	ChIP; mutagenesis; RNA and DNA FISH	11, 18, 143
	Mouse	*Xist*	Control of epigenetic dosage compensation by silencing one of the two X chromosomes	Mutagenesis; subcellular fractionation	23, 24
	Mouse	*RepA*	Control of *Xist* expression by PRC2-mediated H3K27me3 of *Xist* promoter	shRNA knockdown; overexpression; FISH; ChIP	15, 26
	Mouse	*Evf-2*	Modulate transcription factor Dlx-2 activity to induce adjacent gene expression	siRNA knockdown; overexpression; ChIP; mutagenesis	46
	Mouse	*B2*	Conformational inactivation of RNA Pol II	Overexpression; RNA-IP; mutagenesis	75, 76
	Mouse	*MEN ε* and *MEN β*	Required for formation and maintains structural integrity of nuclear paraspeckels	siRNA knockdown; overexpression; FISH	90–92
	Drosophila	3' UTR of *oskar* mRNA	Assemble cytoplasmic complexes	Mutagenesis	89
	Xenopus	*5S* ribosomal RNA	Masks the nuclear localization signal on TFIIA to prevent nuclear localization	Mutagenesis; microinjection	63
	Xenopus	*Xlsirts*	Required for organization and integrity of oocyte cytokeratin cytoskeleton	Antisense knockdown, FISH	88
	Saccharomyces cerevisiae	ncRNA from 3' end of *PHO5*	Antisense intergenic transcription of ncRNA in PHO5 leads to activation of the coding gene via chromatin changes involving nucleosome eviction	Mutagenesis; ChIP	57
	Saccharomyces cerevisiae	*SRG1*	Transcriptional interference: transcription of *SRG1* affects association of transcription initiation complex to transcribe adjacent *SER3* gene	ChIP; mutagenesis	38
	Fission yeast	*ade6-M26* locus ncRNA	Transcription of this ncRNA induces chromatin changes to enable meiotic recombination	Mutagenesis; ChIP	144
	Fission yeast	ncRNAs from upstream *fbp1*	The act of transcription of ncRNAs initiate the opening of the chromatin to allow transcription of downstream *fbp1*	ChIP; mutagenesis	55
	Fission yeast	*MeiRNA*	Associate with its protein form for subcellular nuclear localization	Mutagenesis; FISH	58
	C. elegans	*Rncs-1*	Associates with and inhibit RDE-4/Dicer complex	Overexpression; mutagenesis	86

Abbreviations used: ChIP, chromatin immunoprecipitation; RNA-IP, RNA-immunoprecipitation; FISH, fluorescence in situ hybridization; siRNA, small interference RNA; shRNA, small hairpin RNA, RdDM, RNA-directed DNA methylation.

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Table 1. Summary of ncRNAs and methodologies employed to deduce functions in animals and plants[15]

	Species	ncRNA	Function	Methodology	Reference
Plants	Arabidopsis	*COOLAIR*	Associated with silencing of *FLC*	Mutagenesis; ChIP	104
	Arabidopsis	*COLDAIR*	Recruits PCR2 to silence *FLC*	ChIP; RNA-IP; siRNA knockdown	105
	Arabidopsis	*IPS1/At4*	Target mimicry: sequesters *miR399* to prevent complementary *PHO2* mRNA degradation	Overexpression; mutagenesis	109
	Arabidopsis	Pol V transcribed ncRNA	Allows the association of AGO4 to ncRNA at RdDM target gene locus	Mutagenesis; RNA-IP; ChIP	145, 146
	Maize	*Zm401*	Regulate expression of genes essential for pollen development	Overexpression; siRNA knockdown	112, 113
	Medicago truncatula	*Enod40*	Directs subcellular localization of MtRBP1 during specific stages of root nodule organogenesis	Immunofluorescence	114

Abbreviations used: ChIP, chromatin immunoprecipitation; RNA-IP, RNA-immunoprecipitation; FISH, fluorescence in situ hybridization; siRNA, small interference RNA; shRNA, small hairpin RNA, RdDM, RNA-directed DNA methylation.

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of lncRNA-mediated mechanism, namely "Long non-coding RNA-mediated modulation of proteins involved in transcription" is discussed.

lncRNA Mechanisms in Animals

lncRNA-mediated chromatin modifications. In animals and plants, chromatin modifications such as methylation, acetylation and phosphorylation of histones, are crucial for tissue-specific gene expression and for genome reprogramming during development.[8,9] A combination of these histone modifications determines the accessibility of the DNA to binding factors; thus the active or inactive status of the embedded genes. These histone modification patterns are believed to be established by ubiquitously expressed chromatin modifying complexes; how these enzymatic complexes are recruited to distinct and specific sites under different cellular contexts has not been fully decoded. Recent investigations on X chromosome dosage compensation, imprinting and homeotic gene expression predominantly in mammalian species revealed the association of lncRNAs with these complexes and showed that lncRNAs may play a crucial role in organizing chromatin structure, and in establishing and maintaining the epigenetic landscape during these biological processes.[10-15] Disruption to these chromatin modifications could lead to dysregulation of developmental processes and to various diseases.[16,17]

Several lncRNAs have been identified in imprinted gene clusters in mammals, and at least two of them, *Air* and *Kcnq1ot1*, have been shown to play a critical role in establishing monoallelic expression patterns of imprinted genes.[18,19] It seems that they achieve their role by binding to chromatin modifying complexes and guiding them to specific genomic locations for proper histone modifications required for silencing of maternal or paternal alleles.[10,11] *Air* is transcribed by the promoter located within intron 2 of the *Igf2r* gene and is predominantly expressed from the paternal allele, resulting in silencing of paternal *Igf2r* and two other cis-linked genes, *Slc22a2* and *Slc22a3*, in mouse placenta.[19] *Air* was found to interact with the *Slc22a3* promoter chromatin and the H3K9 histone methyltransferase G9a in placenta. *Air* accumulates at the *Slc22a3* promoter correlating with localized H3K9 methylation.[10] Truncation of *Air* results in the loss of G9a accumulation at the *Slc22a3* promoter and biallelic transcription of *Slc22a3*, suggesting that *Air* specifically recruits G9a to the promoter region of *Slc22a3* and leads to targeted H3K9 methylation and allelic silencing of paternal *Slc22a3*.[10] Similarly, *Kcnq1ot1* is expressed from the paternal allele and is responsible for silencing of *Kcnq1*, which is antisense to *Kcnq1ot1*, and a cluster of genes flanking the *Kcnq1* locus in placenta.[11,18] *Kcnq1ot1* interacts with G9a and components (EZH2 and SUZ12) of the Polycomb Repressive Complex 2 (PRC2) responsible for H3K27 trimethylation (H3K27me3) in a lineage-specific manner. *Kcnqlot1* recruits these chromatin modifying complexes to the *Kcnq1* domain to establish repressive chromatin marks.[11,20] *Air* co-immunoprecipitates with G9a while *Kcnq1ot1* can be pulled-down by antibodies against G9a, EZH2 or SUZ12 protein, suggesting that these lncRNAs associate with these chromatin modifers.[10,11] However, it is unclear whether the association is direct or recruited through association of lncRNAs with other unidentified protein factors.

Interaction between lncRNAs and chromatin modifying complexes is also essential for X-chromosome inactivation, a mechanism by which one of the two X chromosomes is epigenetically silenced in female mammalian embryos to ensure that females have the same dosage of X-linked genes as the males.[21] At least seven lncRNAs, including *Xist* and *RepA*, participate in this process.[22] *Xist* is transcribed only from the future inactive X chromosome (Xi) and is responsible for binding PRC2, and for spreading PRC2 and its H3K27 trimethylase activity throughout the Xi to finally transcriptionally silence the entire Xi.[22-25] Paradoxically, transcriptional induction of *Xist* is due to *RepA*-mediated recruitment of PRC2 and H3K27me3 of the *Xist* promoter.[15,26] *RepA* is transcribed from the conserved 5' end of *Xist* and is in the same orientation as *Xist*. It consists of 7.5 tandem repeats of a 28-nucleotide (nt) sequence that folds into two conserved stem-loop structures.[27] RNA gel shift analysis showed that *RepA* RNA directly interacts with EZH2 of PRC2 through its 28-nt stem-loop structures.[15] A more recent investigation confirmed the

interaction between *RepA* and PRC2 and found that binding also occurs with the SUZ12 component of PRC2; this association is more efficient in the presence of the full-length *RepA* that can form the two long stem-loop structures.[28] Although more studies are required to determine the exact secondary structure of *RepA* that interacts with PRC2, these studies together with the result of another recent study by Kanhere et al. clearly show that secondary structures of ncRNAs might play an important role in binding and/or recruitment of chromatin modifying complexes, such as PRC2, to specific genomic locations for deposition of H3K27me3 and transcriptional gene silencing.[29]

In addition to in cis action, lncRNA-mediated chromatin modifications can also occur in trans. In mammals, *HOX* transcription factors are clustered on four chromosomal loci, *HOXA–HOXD*, and are essential for specifying the positional identities of cells.[30] Maintenance of *HOX* expression patterns is under complex epigenetic regulation, including lncRNA-mediated chromatin remodelling. Hundreds of lncRNAs have been identified in the human *HOX* loci including *HOTAIR* (*HOX* Antisense Intergenic RNA), a 2.2-kb lncRNA generated from the *HOXC* locus that functions to repress in trans transcription across 40-kb of the *HOXD* locus, located at a different chromosome. *HOTAIR* achieves its role by recruiting and guiding PRC2, through interacting with SUZ12 and EZH2, to the *HOXD* locus to establish the H3K27me3 silencing marks.[12] Using a series of *HOTAIR* deletion mutants, a 5' domain of ~300 bp of the *HOTAIR* transcript was found to be able to retain the PRC2 binding activity of the full-length *HOTAIR*.[13] This study also showed that a 3' domain of *HOTAIR* interacts directly with Lysine-Specific Demethylase 1 (LSD1)-containing CoREST repressor complexes, which are found at the genomic regions flanking *HOXD*.[31] LSD1 mediates enzymatic demethylation of H3K4me2 and is required for proper repression of *HOX* genes in Drosophila.[32,33] These results indicate that *HOTAIR* serves as a scaffold for these two distinct histone modification complexes, which enable *HOTAIR*-mediated assembly of PRC2 and LSD1, and coordinates targeting of PRC2 and LSD1 to chromatin for coupled H3K27 trimethylation and H3K4me2 demethylation.[13] In addition, computational analysis and RNA footprinting showed that the PRC2 and LSD1 binding domains of *HOTAIR* possess distinct secondary structures,[13] supporting the notion that secondary structure of lncRNAs might be an important determinant in recruitment of chromatin modifying complexes. Importantly, results of this recent study not only provided evidence for direct interaction between lncRNA and enzymatic complexes but suggest that lncRNAs may serve as modular scaffolds to assemble chromatin modifying complexes and to specify complex patterns of chromatin states at specific genes.

A growing body of evidence supports the notion that lncRNAs are key components in the regulation of chromatin states and epigenetic inheritance. First, lncRNAs are pervasively transcribed in the genome as shown by whole genome tiling array and RNA-seq studies.[34,35] Second, lncRNAs have been found to directly or indirectly interact with a growing number of enzymatic complexes involved in chromatin modifications, such as PRC1, PRC2, CoREST, SMCX and G9a.[10-12,14,15,36] Third, ~20% of approximately 3300 lncRNAs identified from various human cell types and ~13% of lncRNAs expressed in HeLa cells were found to be associated with PRC2 and CoREST, respectively.[36] The finding that approximately 40% of the lncRNAs associated with CoREST are also associated with PRC2 suggests that a number of lncRNAs can serve as scaffold to assemble two or more chromatin modifying complexes to regulate expression of the same target genes. It is clear that lncRNAs play an essential role in mediating epigenetic changes by recruiting chromatin remodelling complexes to specific genomic loci and several possible mechanisms by which lncRNAs tether or guide chromatin modifying complexes to their specific destinations have been proposed.[12,37] More studies are required to uncover the exact mechanism controlling the interaction between lncRNAs and chromatin modifying complexes.

lncRNA-mediated transcriptional and translational regulation. While lncRNA-mediated chromatin modifications are potent mechanisms in the regulation of gene transcription, other mechanisms of gene regulation mediated by lncRNA have also been described. In *Saccharomyces cerevisiae*, the transcription of lncRNA *SRG1* (*SER3 regulatory gene 1*) in the regulatory region of the *SER3* gene represses *SER3* transcription during growth in rich medium.[38] This mechanism of gene silencing is termed "transcriptional interference" which may occur due to interference between transcriptional machineries processing along adjacent sequences;[39] thus the active transcription of *SRG1* prevents the association of transcriptional initiation elements to adjacent sequences of close proximity.

A similar mechanism of gene regulation has been described by Martianov et al. In serum-starved U2OS cells, a lncRNA transcribed from the minor (upstream) promoter of the *DHFR* (*dihydrofolate reductase*) gene binds to TFIIB (Transcription Factor IIB) to prevent its association with the major (downstream) promoter of the *DHFR* gene resulting in gene repression.[40] Moreover, the lncRNA can also form a triplex with the major promoter of the *DHFR* gene, further preventing association of TFIIB to this promoter region. More recently, Schmitz et al. showed that the formation of a DNA:RNA triplex in the presence of a lncRNA that is complementary to the rDNA (ribosomal DNA) promoter facilitates the recruitment of DNMT3b (DNA methyltransferase 3b) to catalyse DNA methylation at that particular locus in an unknown mechanism in mice.[41] Such a mechanism for controlling promoter usage could be widespread as many triplex structures exist in eukaryotic chromosomes.[42] However, it remains to be determined whether lncRNA-mediated formation of triplex structures in promoters is a common mechanism and whether this mechanism is conserved in plants. Notably, it is also unknown whether sequestration of TFIIB affects the expression of other unrelated genes that require TFIIB for transcription.

A long intergenic ncRNA named *licnRNA-p21* is induced by p53 upon DNA damage in human cells to mediate global gene repression via association with heterogeneous nuclear ribonucleoprotein K (hnRNP-K), also a component of the p53 pathway.[43] p53 is a tumor suppressor known to be involved in the maintenance of genomic integrity and functions to trigger a transcriptional response leading to cell arrest and/or apoptosis.[44,45] Association of *licnRNA-p21* with hnRNP-K modulates

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hnRNP-K localization to the promoter region of target genes to confer transcriptional repression.[43]

While the mechanisms of intergenic lncRNA transcription discussed so far appear to negatively regulate gene expression, there is also evidence that lncRNAs function can confer gene activation consequences. In mice, the lncRNA *Evf-2* functions as a cofactor to modulate transcription factor activity. This lncRNA is transcribed from an ultra-conserved enhancer and recruits transcription factor Dlx-2 to the same enhancer to induce expression of adjacent protein-coding genes.[46] Consistent with this, a recent study which used GENCODE annotation of the human genome to identify novel lncRNAs suggests that lncRNAs can function as enhancers to increase transcription of neighboring genes.[47] The study however excluded the analysis of lncRNAs derived from intergenic or overlapping protein-coding gene regions indicating that this mechanism of gene enhancement could be more common. Although the exact mechanism remains to be elucidated, involvement of transcription factor enhancement by lncRNA similar to the role of lncRNA *Evf-2* is a possibility.

The *Gas5* (*Growth arrest-specific 5*) gene encodes a lncRNA that suppresses the expression of a subset of genes during nutrient starvation in HeLa cells. *Gas5* RNA binds to the DNA-binding domain of GR (Glucocorticoid Receptor) transcription factor, preventing its association with the GREs (Glucocorticoid Response Elements) located in the regulatory regions of glucocortocoid-responsive genes.[48] This modulation of GR transcriptional activity appears to influence cell survival and metabolic activities during starvation.

Natural antisense transcripts (NATs) have been demonstrated in numerous studies to possess the ability to hybridize to overlapping genes and generate endo-siRNA in Drosophila and mice.[49,50] In addition, some NATs regulate the translation of target mRNAs in rats and humans.[51-54] The most recent demonstration of this mechanism by Beltran et al. showed that the binding of a NAT complementary to the 5' splice site of *Zeb2*/*Sip1* mRNAs, prevents spliceosomal removal of the internal ribosome entry site sequence situated within an intron at the 5' untranslated region; thus allowing translation of this mRNA necessary for epithelial-mesenchymal transition in humans.

In some cases, the act of lncRNA transcription itself is sufficient to cause gene regulation/chromatin modification rendering the lncRNA as mere non-functional by-products of transcription. Examples of this phenomenon have been demonstrated in yeast and human leading to silencing or activation of neighboring genes via chromatin remodelling. The step-wise transcription of several lncRNAs from the upstream promoter region of the *fbp1* locus in the fission yeast (*Schizosaccharomyces pombe*) is required to initiate the opening of the chromatin in a progressive manner towards the mRNA transcription start site;[55] thus allowing transcription of the downstream gene. Similarly, the transcription of lncRNAs at the *ade6-M26* locus induces changes in the chromatin during meiosis enabling meiotic recombination in human.[56] In response to phosphate starvation, antisense transcription of a lncRNA from the 3' end of the *PHO5* gene in yeast induces the expression of the protein coding gene, *PHO5*.[57] The authors concluded that because *PHO5* RNA is highly unstable and is rapidly degraded by exosomes, the non-coding transcript is unlikely to possess a functional role but instead proposed the idea that the kinetics of *PHO5* activation is affected by intergenic transcription of the ncRNA which in turn leads to nucleosome eviction allowing a 600 bp region to become fully accessible for RNA Pol II transcription.[57]

lncRNAs function as molecular cargos to target protein subcellular localization. The activity of many proteins required for cell cycle progression and gene transcription can be modulated by limiting their subcellular localization. This mechanism of control has been demonstrated and can be regulated by lncRNAs. In the fission yeast, *Mei2p*, an RNA-binding protein required for pre-meiotic DNA synthesis and meiosis I is transported from the cytoplasm to the nucleus via association with its RNA intermediate, the *MeiRNA*.[58] Mutated Mei2p with lower ability to bind to *MeiRNA* remained cytoplasmic. In the absence of *MeiRNA*, Mei2p transgene product containing an added nuclear localization signal is able to translocate to the nucleus and promote meiosis I, suggesting that the role of *MeiRNA* is to act a chaperon to guide Mei2p nuclear import.[59] The authors suggest that this mechanism of subcellular localization provides an explanation for the puzzling finding that ongoing transcription is required for accumulation of certain proteins in the nucleus.[59] Indeed, such an explanation is logical but may not be applicable to germ cells that display little or no transcriptional activity such as those in mammalian species.[60-62]

Conversely, cytoplasmic localization of proteins regulated by lncRNA has also been observed. Transcription of *5S ribosomal* RNA relies on TFIIA (Transcription Factor A). Following transcription, the *5S ribosomal RNA* binds to TFIIA and is escorted to the cytoplasm. The binding of *5S ribosomal* RNA masks the nuclear localization signal on the TFIIA protein, resulting in cytoplasmic retention during oocyte development in the Xenopus.[63]

The subcellular localization of a transcription factor, NFAT (nuclear factor of activated T cells), important for T cell-mediated immune response,[64] is regulated by the lncRNA *NRON* (*Non-coding Repressor Of NFAT*). *NRON*, expressed in a number of mouse and human tissues, binds to members of the nucleocytoplasmic trafficking machinery by inhibiting NFAT nuclear accumulation. This specifically represses NFAT activity and prevents the expression of genes mediated by NFAT.[65]

lncRNAs as molecular chaperons to confer protein conformational activity. The folding structure of proteins deduced by post-translational modifications including phosphorylation can affect their active or inactive states. The discovery that lncRNAs are also capable of changing the conformational activity of many critical protein factors has added another level of complexity to our understanding of protein regulation. Wang et al.[66] showed that under DNA damage signalling conditions, ncRNAs transcribed from the 5' regulatory regions of the *CCND1* (*Cyclin D1*) gene in human cell lines function to allosterically modify the structure of an RNA-binding protein named TLS (Translocated in LipoSarcoma) by releasing it from its inactive conformation. TLS is not only modified by the ncRNA but is also guided to the promoter region of the *CCND1* gene to inhibit the histone acetyltransferase activities of CREB-binding protein and p300, resulting in repression of *CCND1* expression.[66] This is consistent

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with the role of CDND1 as a cell cycle regulator known to be repressed by DNA damage signals.

Similarly in human cells, Lanz et al. showed that a lncRNA known as *steroid receptor RNA activator* (*SRA*) is required to confer functional specificity of a ribonucleoprotein complex known as SRC-1 (Steroid Receptor Coactivator 1),[67] a nuclear receptor coactivator.[68] However, unlike the previous study which resulted in gene repression, *SRA* association with the SRC-1 results in the activation of hormone related nuclear receptors which then functions to direct the assembly and stabilization of a preinitiation complex for transcriptional activation at the promoter of targeted genes associated with hormonal changes.[69] This mechanism appears to be tissue-specific as *SRA* is only expressed in several tissues, particularly in the brain.[67] Expression analysis in muscle cells and RNA interference showed that *SRA* is a coactivator of MyoD transcription factor during skeletal muscle differentiation.[70] A coding *SRA* has also been described; this RNA differs from the non-coding *SRA* by an extended exon-1 containing methionine codons necessary for translation.[71] The SRA protein (SRAP) also functions as a coactivator of hormone related nuclear receptors and many other transcription factors including transcription factor IIB.[72-74] The role of SRA and SRAP in the activation of nuclear receptors has been implicated in prostate cancer.[73,74] It is unknown whether *SRA* can associate and regulate its protein form.

lncRNAs also have the ability to affect the transcriptional machinery as a whole by binding to RNA polymerase II (RNA Pol II), causing global gene repression. The *Alu* RNA and *B2* RNA are transcribed from short interspersed elements (SINE) during heat shock in human and mouse cells, respectively.[75-77] These RNA molecules, although not evolutionarily related and share no sequence homology, are both able to bind to RNA Pol II causing general repression of transcriptional activity, suggesting that ncRNAs with diverse sequences can possess conserved functions. Therefore, information from the primary sequence of these ncRNAs is insufficient to allow prediction of function. Interestingly, *scAlu* and *B1* RNA, which are the short form of *Alu* and a homologue of *B2*, respectively, are able to bind to RNA Pol II but cannot induce transcriptional repression.[77] This suggests that neither sequence specificity nor the binding to the RNA Pol II itself is sufficient to inhibit gene transcription. The repressive component (the regulatory domain) lies in two separate regions in the *Alu* RNA which are different to the regions required for binding (the binding domain) to RNA Pol II.[77] The authors suggest that it is the structural conformation of those two regulatory domains and not the sequence that confers transcriptional repression. SINE transcripts also increase during other cellular stresses and during viral infection,[78,79] suggesting that they may also modulate transcription in a variety of other biological responses. Heat shock induced transcriptional repression appears to also exist in many other eukaryotic species including Drosophila[80,81] and plants.[82-84] Paradoxically, some genes including those that encode heat shock proteins are transcriptionally activated during heat shock, suggesting that an underlying mechanism must exist to overcome SINE RNA mediated gene repression at those specific gene loci. Shamovsky et al. demonstrated that the activation of heat shock proteins in mammalian cells rely on the trimerization of a heat shock transcription factor (HSF) with a ncRNA named *HSR1* (*Heat Shock RNA 1*) and translation elongation factor eEF1A in a ribonucleoprotein complex.[85] This association renders the transcription factor active in DNA-binding activity.

In *C. elegans*, a starvation induced lncRNA, *Rncs-1* (*RNA non-coding, starvation upregulated*), affects the processing of siRNAs by inhibiting the activity of the RNase III catalytic enzyme, Dicer.[86] This impaired activity is due to the branched structures present in its 300 nt double-stranded RNA structure that presumably allows its association with the RDE-4/Dicer complex but prevents Dicer cleavage; thus inhibiting Dicer processing of other double-stranded RNA to siRNAs necessary for target messenger RNA downregulation.

Structural lncRNAs. Our discussion on ncRNA-mediated mechanisms so far implicated their function as riboregulators affecting the activity and localization of proteins as well as their direct or indirect role in gene regulation. We now discuss evidence in which lncRNAs function as structural RNAs. Many mRNAs, in addition to encoding proteins, are known to be involved in the cytoplasmic localization of RNA structures in Xenopus oocytes, important for the determination of cell fate during cleavage development.[87] However, many more examples in which RNAs function in establishing structural cellular integrity begin to emerge, particularly in those involving lncRNAs.

Studies of cytoplasmic RNA function in oocytes showed that the lncRNA *Xlsirt*s along with *VegT* mRNA are responsible for the organization of the vegetal cortex of Xenopus oocytes. Deletion of either transcript results in disruption of the cytokeratin cytoskeleton, essential for the proper formation of germinal granules and the subsequent development of the germline.[88]

In Drosophila oocytes, the 3' untranslated region of the *oskar* mRNA possesses a function independent of its protein, osk, by binding to and assembling cytoplasmic complexes essential for oocyte development.[89] *Oskar* mutant oocytes arrest early during oogenesis; this arrest can be rescued by the expression of the 3' untranslated region of *oskar* RNA alone.[89]

In mice, three independent studies have found that two lncRNAs, *MEN epsilon* (also known as *NEAT 1*) and *MEN beta*, transcribed from the MEN (Multiple Endocrine Neoplasia) I locus associate with protein components of nuclear body paraspeckles and are involved in conferring structural integrity of these nuclear organelles.[90-92] Knock-down of these RNAs disrupts paraspeckle formation,[91,92] while overexpression of *MEN epsilon* induces an increase in paraspeckle number.[90] Paraspeckles have been suggested to be important for nuclear storage of RNA and previous studies showed that RNase treatment causes their disruption,[93,94] further confirming the involvement of RNA in their formation and maintenance. A recent study showed that *MEN epsilon* interacts with paraspeckle proteins through distinct regions in the 5' and 3' ends of the transcripts.[95]

lncRNA mechanisms in plants

The plant genome is enriched with highly transcribed pseudogenes, ncRNAs and transposable elements including SINE

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elements,[96,97] suggesting that ncRNA mechanisms are conserved in plants. However, research on lncRNAs in plants is still in its infancy. To date, apart from the identification of lncRNAs that represent the primary transcript precursors for miRNAs,[98] few lncRNAs have been isolated in plants and are generally uncharacterized.

The mechanism of chromatin modification induced by lncRNAs-mediated recruitment of the PRC2 complex has recently been explored in plants. In Arabidopsis, there is more than one PRC2 complex formed by combinations of different EZH2 and SUZ12 homologues with other subunits of PRC2.[99] During prolonged cold periods in Arabidopsis, the *FLC* (*Flowering Locus C*) locus controlling flowering time undergoes epigenetic silencing, resulting in repression of *FLC* expression and early flowering.[100-103] Swiezewski et al. demonstrated the expression of antisense transcripts named *COOLAIR* (*Cold induced Long Antisense Intragenic RNA*) derived from the *FLC* locus upon exposure to cold.[104] More recently, a sense non-coding transcript named COLDAIR (Cold Assisted Intronic non-coding RNA) derived from a cryptic promoter within intron 1 of the *FLC* locus is also induced during prolonged exposure to cold.[105] The authors of both independent studies suggest that *COOLAIR* and *COLDAIR* function to recruit PRC2 to induce histone modifications at the *FLC* locus, reducing the level of *FLC* expression. The latter study confirmed physical association of the lncRNA, COLDAIR with PRC2 using RNA immunoprecipitation.[105]

Genome-wide analysis of the Arabidopsis genome has identified the expression of overlapping NATs corresponding to a significant proportion of Arabidopsis transcriptome.[106,107] Although the role of NATs in translational induction by association with the sense mRNA as demonstrated in animals has yet to be described in plants. Studies of NATs in Arabidopsis have demonstrated other means of gene regulation in which the formation of double stranded RNA with the complementary sense RNA recruits them into the siRNA pathway.[108]

Also in Arabidopsis, the induced expression of ncRNAs of the *IPS1/At4* family during phosphate starvation responses results in the accumulation of the *PHO2* mRNA, a target of miR399 (micro-RNA 399). Franco-Zorrilla et al. showed that a conserved motif of 23 nt in this ncRNA family is complementary to miR399 but has critical mismatches at positions 10–11, required for miRNA guided cleavage.[109] Therefore, *IPS1/At4* RNAs are not cleaved by miR399 but instead sequester miR399 to inhibit its effect on *PHO2* mRNA, in a mechanism known as target mimicry. *PHO2* RNA encodes an E2 ubiquitin conjugase-related protein that negatively affects shoot phosphate content and remobilization in an unknown mechanism.[110]

In *Cucumis sativus*, a lncRNA named *CsM10* was isolated using differential display reverse transcription PCR, which showed differential expression patterns in different tissues, seedling developmental stages and photoperiods.[111] *CsM10* harbors a 179 bp sequence with high sequence homology to a family of abiotic stress-associated ncRNAs known as the CR20-GUT15-Related (CGR) family, suggesting a role in the regulation of gene expression. More studies are required to elucidate its exact function.

In maize, a putative lncRNA, *Zm401*, is expressed specifically in pollen. Forward and reverse genetic studies deduced a function for *Zm401* in regulating the expression of critical genes necessary for pollen development including *ZmMADS2*, *MZm3-3* and *ZmC5*.[112,113] *MZm3-3* was upregulated in *Zm401* mutants while *ZmMADS2* and *ZmC5* were both downregulated.[113] Overexpression of *Zm401* severely affects pollen development due to abnormal tassels and degenerate anthers.[112] How this lncRNA can mediate the downregulation of certain genes but the upregulation of others is intriguing and remains to be elucidated. One possible mechanism may involve the association of specific domains of the transcript with different members of transcriptional protein complexes.

lncRNA-mediated subcellular localization of proteins has also been described in plants. The lncRNA *Enod40* directs the re-localization of MtRBP1 (*Medicago truncatula* RNA Binding Protein 1) from the nucleus to cytoplasmic granules during specific stages of legume (*Medicago truncatula*) root nodule organogenesis.[114]

Perspectives

Eukaryotic organisms are equipped with the intrinsic abilities to cope with a large range of environmental changes/stresses by producing molecules to alter cellar activities correspondingly.[115] These molecules include hormones that play diverse physiological roles in the regulation of basal- and stress-related homeostasis and can alter the expression level of cell survival and apoptosis-associated genes in response to external and internal cues.[116-118] In animals, much evidence provided here shows the induction of lncRNA during stress conditions, suggesting that lncRNA expression is generally related to the organisms' response to environmental changes (**Table 2**). Similarly, the production of non-coding siRNAs derived from NAT expression in plants is often induced by abiotic (salt) and biotic (bacterial) stress.[108,119] Plants have also evolved to utilize their RNAi machinery as a defence mechanism against viral infections.[120,121] While mammalian organisms have exceeded this complexity and evolved an immune system to target foreign pathogens, it appears that they still retain the mechanism that utilizes ncRNA in response to stress conditions.

A large proportion of functions mediated by these lncRNAs appear to involve the regulation of proteins involved in transcription, particularly transcription factors, whether it is the mRNA form (e.g., *Zeb2* mRNA and NAT association) or the protein form, including those that play critical roles in the maintenance of hormonal balance to induce cellular survival such as nuclear receptor transcription factors. This gene regulation mechanism has not been classified in the current literature; we therefore attempt to define it as "Long non-coding RNA modulation of proteins involved in transcription" (**Table 2 and Fig. 1**). There is evidence of miRNA-mediated regulation of transcription factors at the post-transcriptional level particularly during cell differentiation in animals and plants.[122-124] However, the regulation of transcription factors at the post-translational level, which is a prevalent lncRNA mechanism in animals, may represent a

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Table 2. Summary of induced lncRNAs and their role in the modulation of proteins involved in transcription

	Species	lncRNA	Mechanism	Stimulus	Reference
Animals	Human	*Gas5*	Inactivation of GR transcription factor	Nutrient starvation	48
	Human	*SRA*	Activation SRC-1 transcription factor	Hormonal changes	67
	Human	*HSR1*	Activate HSF transcription factor	Heat shock	85
	Mouse	*Evf-2*	Activate Dlx-2 transcription factor	Development	46
	Mouse	*B2*	Inactivate RNA Pol II	Heat shock	75, 76
	Human	*Alu*	Inactivation of RNA Pol II	Heat shock	77
	Human	*DHFR* ncRNA	Inactivate transcription factor II B	Serum-starvation	40
	Xenopus	*5S* ribosomal RNA	Inactivate transcription factor IIA	Oocyte development	63
Plants			?		

more direct and efficient mechanism of regulating target gene expression than through the miRNA and siRNA pathways which require multiple processing and association events; this may be particularly true when epigenetic changes need to occur immediately following environmental stress. Therefore, it is surprising that lncRNA-mediated mechanisms described in plants, although at a much lower extent than in animal species, have not yet shown evidence of interplay between lncRNAs and transcription factors to regulate gene expression.

Nevertheless, some mechanisms such as lncRNA-mediated chromatin modification and protein subcellular localization do appear to be conserved in plants, although these plant lncRNAs are not orthologues of those that play similar roles in animals. Therefore, it remains to be determined whether animal and plant lncRNA orthologues play homologous roles in similar mechanisms. Transcriptional interference in which the transcription of lncRNA upstream of the coding region interferes with the transcription of the coding gene could also be a widespread phenomenon. Ponting et al.[39] suggested that because promoter sequences of lncRNAs are more conserved than the sequences of transcripts,[6,125] the act of transcription itself can possess a greater and more widespread biological role than the transcript sequence. We have discussed earlier that the formation of a DNA:RNA triplex in promoters could possibly be a widespread mechanism conserved in mammals but this remains to be examined in plant species.[42]

In addition to the discovery of novel mechanisms for newly identified lncRNAs, recent studies in mammals have identified regulatory mechanisms mediated by known ncRNAs (60–300 nt) that were previously thought to function mainly as housekeeping RNA. For instance, it is well established that small nuclear RNAs (snRNAs) play fundamental roles in regulating transcription by RNA Pol II and in processing of the transcripts.[126] In 2002, Kwek et al. showed that the association of *U1* snRNA with TFIIH (Transcription Factor II H) positively regulates the rate of transcription initiation by RNA Pol II,[127] a new role for *U1* snRNA that is in addition to its known role in RNA processing. On an interesting note, recent studies in animals have demonstrated that some small nucleolar RNAs (snoRNAs) and transfer RNA are potentially precursors of miRNA incorporated into the RNA interference pathway.[128-136] In Arabidopsis, the association of snoRNA with Argonaute 7,[135] a key component of the RNAi machinery, suggests that similar mechanisms may also be conserved in plants. These studies add convincing evidence to support the notion that ncRNAs have muti-functional, structural and regulatory abilities.

It is generally considered that the ultimate goal of ncRNAs is to fine tune the expression of genes. As lncRNAs appear to regulate many transcription factors, we are beginning to see their impact in relation to cancers where a slight shift in the expression level of oncogenic genes can affect cellular proliferation. In fact, miRNAs,[137,138] subunits of PRC, *Gas5* and *CCND1* are some of many factors implicated in tumor progression in mammalian species.[139]

Studies of *rncs*, *Alu*, *B1* and PRC2-associated lncRNAs provide compelling evidence that the function of lncRNA imposed on proteins is not due to sequence-specificity but the structure of the RNA complexes. Further research on RNA structure and function will allow the development of computational programs to predict RNA function based on structures, opening up a new platform of computational biology, similar to that developed for protein function prediction based on amino acid sequence motifs.

As we increase our knowledge of ncRNA-mediated mechanisms that enhance the complexity of eukaryotic organisms, we are one step closer to understanding our unique origin. Implementation of this knowledge will undoubtedly improve treatments for various stress induced conditions and diseases. New strategies in molecular and computational approaches are being developed to tackle the difficulty in studying ncRNAs.[140-142] We believe that a shift in focus, studying lncRNAs in plants can reveal many more surprises as plants can provide a more efficient model for the functional study of ncRNAs. The ease of maintenance and short life cycles combined with the great diversity of environmental conditions under which plants can grow make them ideal models for ncRNA research that will certainly improve our understanding of the RNA world.

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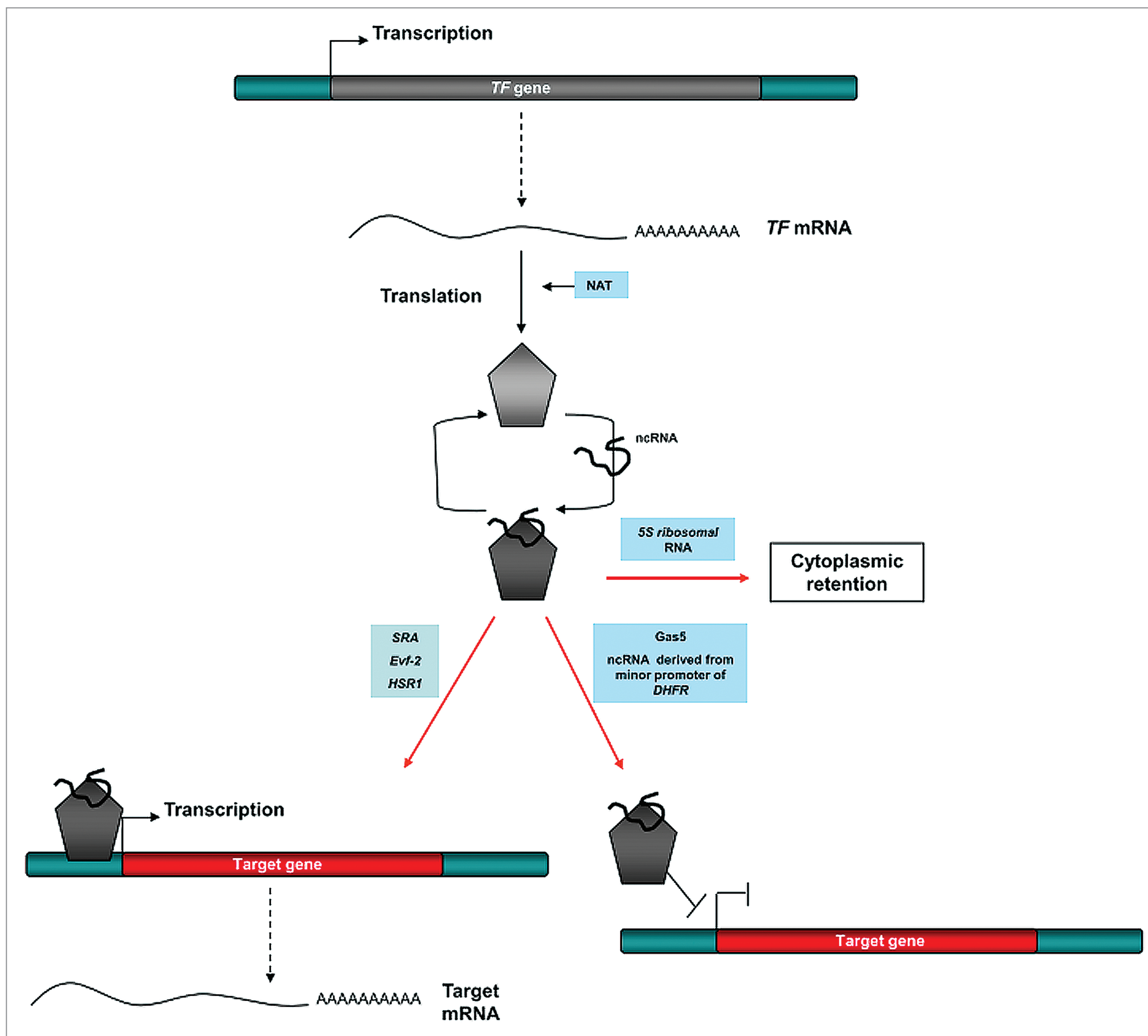

Figure 1. Schematic overview of lncRNAs and their translational and post-translational regulation of transcription factors (TF) from examples derived in eukaryotes. NAT (Natural antisense transcripts) association with a *TF* transcript induces its translation. The cytoplasmic localization of a TF protein is directed by its association with 5S ribosomal RNA. The activation and inhibition of target genes through mechanisms involving association of different lncRNAs with TF is shown.

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