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Diverse functions of nuclear non-coding RNAs in eukaryotic gene expression

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1. ABSTRACT

Recent genome-wide analyses revealed that eukaryotic genomes are almost entirely transcribed, generating a large number of short or long non-protein coding RNAs (non-coding RNAs; ncRNAs). Rapidly accumulating experimental evidence suggests that ncRNAs are not just transcriptional noise, but have biological roles in gene expression. In this review, we focus on the functions of nuclear-localized ncRNAs including the spliceosomal small nuclear RNAs. These nuclear ncRNAs play diverse regulatory roles in a wide-range of nuclear reactions, such as transcription, precursor-mRNA (pre-mRNA) splicing, nuclear structure formation, nuclear trafficking, and chromatin remodeling. The regulatory functions of ncRNAs in these reactions are reinforced by target-site recognition through base-pairing or formation of an RNA/DNA triple helix. Recent studies revealed an unexpected linkage between the machineries for RNA interference (RNAi)-mediated gene silencing and pre-mRNA splicing. In addition, the biogenesis of some ncRNAs was found to overlap with the pathway of pre-mRNA splicing. Our understanding of the mechanisms of coordinated gene regulation in the nucleus has increased dramatically through studies on nuclear ncRNAs. A new paradigm of "ncRNA regulation" is now emerging.

2. INTRODUCTION

Recent advances in sequencing and genome-wide microarray technologies have revealed that transcription is widespread throughout the eukaryotic genome (1-5). As protein-coding genes cover only a small fraction of the genome, especially in higher eukaryotes, genomic transcripts contain large numbers of ncRNAs, which are defined as RNAs with no significant protein-coding capacity. NcRNAs originate from intergenic, aberrantly processed or antisense transcripts.

There has been a debate over whether such genomic transcripts are just "transcriptional noise" or RNAs with functional roles (6, 7). It was suggested that, considering the fidelity of the transcriptional initiation by RNA polymerase II, aberrant transcripts are easily generated from eukaryotic genomes as transcriptional noise (8). Also, as shown in the fission yeast *fbp1*$^+$ gene encoding fructose-1, 6-bisphosphatase, the expression of which is robustly induced by glucose starvation, transcription by RNA polymerase II results in chromatin remodeling leading to transcriptional activation (9). Transcription through the promoter region can convert the chromatin to an open configuration, promoting the accessibility of RNA polymerase II and induction of progressive transcription

Table 1. Functions of nuclear ncRNAs described in this review

Name	Category	Function	References
Transcriptional regulation			
Evf2	lncRNA	activation of the transcription factor for the Dlx-5/6 genes	15
$ncRNA_{CCND1}$	lncRNA	repression of transcription of the cyclin D1 gene	16
DHFR ncRNA	lncRNA	regulation of the *DHFR* gene by the promoter interference	17
Chromatin modification			
Kcnq1ot1	lncRNA	paternal silencing of the *Kcnq1* imprinted gene cluster	23
Air	lncRNA	silencing of the *Slc22a* and *Igf2r* genes	29
Xist	lncRNA	X-chromosome inactivation	30
RepA	lncRNA	X-chromosome inactivation	33
Tsix	lncRNA	repression of *Xist*	31, 32
HOTAIR	lncRNA	formation of the heterochromatin at the *HOXD* locus	21
centromeric ncRNA[1]	lncRNA	formation of the heterochromatin at the centromere	56, 57, 73, 74
TER1	lncRNA	telomere RNA involved in the telomere formation	90
Post-transcriptional regulation			
U1, U2, U4, U5, U6	short ncRNA	splicing of the major-type introns	14
U11, U12, $U4_{ATAC}$, $U6_{ATAC}$	short ncRNA	splicing of the minor-type introns	37
Zeb2 antisense transcript	lncRNA	repression of pre-mRNA splicing of *Zeb2*	41
MALAT1	lncRNA	modulation of alternative pre-mRNA splicing	42
NRON	lncRNA	regulation of nuclear transport of NFAT	45
MEN epsilon/beta	lncRNA	formation of the paraspeckles	50
mirtrons	short ncRNA	precursors of miRNAs	94, 95

NcRNAs are grouped by their functions and listed. [1]This lncRNA is processed to siRNAs essential for RNAi-mediated formation of the heterochromatin.

under glucose starvation. In this case, the transcription itself, not the resultant transcripts, has biological meaning (9).

There are also an increasing number of reports that ncRNAs are not just transcriptional noise, but act as functional RNAs to regulate gene expression (for a review, 10). Functional ncRNAs can be categorized into two groups; short ncRNAs, typically ~20-200 nucleotides in length, and long ncRNAs (lncRNAs) ranging from ~200 to 100 kb (10). Their expression is in some cases regulated developmentally and tissue-specifically (11-13).

NcRNAs are localized in the nucleus, cytoplasm or both. Small nuclear RNAs (snRNAs), such as U1 and U2 snRNAs involved in pre-mRNA splicing (14), are well-known functional ncRNAs predominantly located in the nucleus. On the other hand, ribosomal RNAs consisting of RNA components of a ribosome play roles in the cytoplasm. Compared to these "classic" ncRNAs, the functions of nuclear-localized ncRNAs identified recently remain largely to be explored.

In this review, we focus on the functions of long and short ncRNAs localized in the eukaryotic nucleus. Recent studies have revealed several mechanisms by which nuclear ncRNAs regulate gene expression at the transcriptional or chromatin level, as well as post-transcriptional level (Table 1). In addition to providing an overview of these mechanisms, we will discuss current models of the linkage between chromatin regulation and the splicing machinery through ncRNAs.

3. NCRNA-MEDIATED REGULATION OF TRANSCRIPTION

3. 1. Direct modulation of transcription factors

There is emerging evidence that lncRNAs modulate transcription directly, but through different mechanisms. *Evf2* (Embryonic ventral forebrain 2) lncRNA is an alternatively spliced form of *Evf1* lncRNA, which was identified in a screen for genes differentially expressed in embryonic dorsal and ventral telencephalon (15). It is transcribed from the ultra-conserved region between the Dlx-5 and Dlx-6 genes encoding homeodomain proteins. Binding of *Evf2* lncRNA to the transcription factor Dlx-2 induces expression of the Dlx-5/6 genes by increasing the transcriptional activity of the enhancer through promoting efficient Dlx-2 binding (15) (Figure 1A). The *Evf2* lncRNA, thus, functions as a co-activator of transcription factors.

Similarly, recruitment of the TLS (Translocated in liposarcoma) RNA-binding protein to the promoter of the *CCND1* (cyclin D1) gene and its association with CREB-binding protein (CBP) and p300 histone acetyltransferase, which causes repression of *CCND1* transcription, are directed by ncRNAs transcribed from the 5' regulatory regions of the *CCND1* gene (16). TLS is a regulatory sensor for DNA damage and *CCND1* is a cell cycle regulator repressed by DNA damage signals. $NcRNA_{CCND1}$ is expressed in response to DNA damage signals and can associate with the promoter of the *CCND1* gene. In this case, ncRNA serves as an "RNA ligand" to modify allosterically a specific RNA-binding protein to modulate its activity and also serves as a molecular "anchor" to tether TLS to the promoter (16) (Figure 1B).

3.2. Promoter interference

In addition to the "RNA ligand" type, lncRNAs also regulate transcription through "promoter-specific interference" as shown in the repression of the human dihydrofolate reductase (*DHFR*) gene (17) (Figure 1C). LncRNAs transcribed from the upstream minor promoter of the *DHFR* gene are thought to form a stable triplex in the major promoter of the *DHFR* gene, which induces dissociation of the pre-initiation complex from the major promoter and can repress the transcription of the *DHFR* gene (17). As the major *DHFR* promoter region is GC-rich and contains several G-track sequences, a purine-purine-

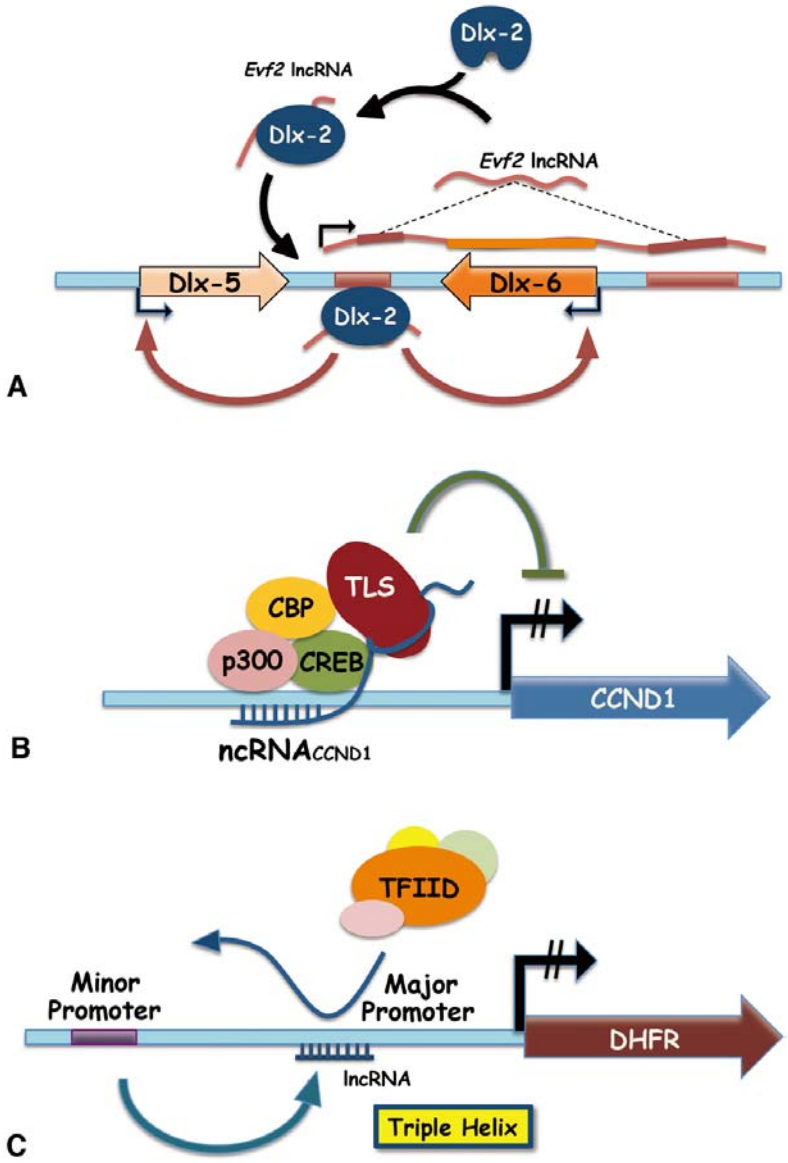

Figure 1. Modulation of transcription factors by lncRNAs. (A) *Evf2* lncRNA physically associates with the homeodomain transcription factor Dlx-2 as a co-activator and activates transcription through efficient binding of Dlx-2 to the enhancer of the Dlx-5 and Dlx-6 genes. (B) LncRNAs transcribed from the upstream region of the cyclin D1 (*CCND1*) gene bind to the RNA-binding protein TLS as a "ligand" and allosterically change its structure. The lncRNAs also bind to the promoter of the *CCND1* gene to tether TLS as an "anchor", which represses the activities of CBP/p300 and transcription. (C) Transcription of the human dihydrofolate reductase (*DHFR*) gene is repressed by a non-coding RNA transcribed from the upstream minor promoter, through formation of the stable RNA/DNA triple-helix that induces dissociation of the transcription complex from the major *DHFR* promoter.

pyrimidine triple-stranded structure (H form) between RNA and DNA (18) is thought to form. The formation of such stable RNA/DNA triple-helix structures seems to be a common mechanism by which regulatory ncRNAs achieve specificity in the recognition of control loci.

4. EPIGENETIC GENE SILENCING DIRECTED BY NCRNAS

In eukaryotic cells, genomic DNA is hierarchically packaged into chromatin with histones and non-histone proteins. Chromatin structures represented by less condensed euchromatin and highly condensed heterochromatin provide for the epigenetic regulation of gene expression. There is growing evidence that a subset of lncRNAs participate in the epigenetic regulation of chromosomal domains (10, 19, 20). *Kcnq1ot1, Air* and *Xist* lncRNAs mediate the epigenetic silencing of multiple genes *in cis* (for a review, 19)(Figure 2A). In contrast, a 2.2-kilo base (kb) *HOTAIR* lncRNA represses transcription *in trans* (21)(Figure 2B). These examples indicate a key role for ncRNAs in the epigenetic regulation of gene expression.

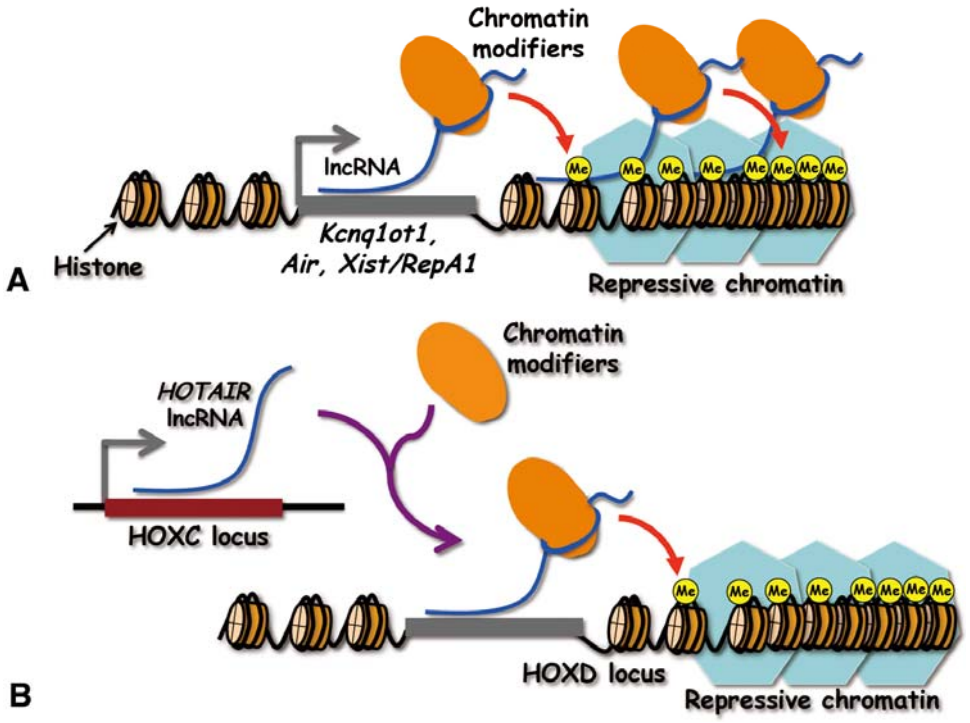

Figure 2. (A) *In cis* recruitment of chromatin modifies. *Kcnq1ot1, Air* and *Xist/Rep1* lncRNAs transcribed from the coding loci are accumulated on a chromatin *in cis* to recruit chromatin modifies, such as histone methyltransferases, Polycomb group proteins and DNA methyltransferases. The chromatin modifiers then facilitate formation of the repressive chromatin by modification of histones and DNA. "Me" indicates methylation of a histone. (B) *In trans* recruitment of chromatin modifiers. *HOTAIR* lncRNA expressed from the *HOXC* locus binds to the *HOXD* locus *in trans* and then recruits the chromatin modifiers to form the repressive chromatin, which inactivates transcription.

4.1. *In cis* recruitment of chromatin modifiers

Several nuclear lncRNAs have been shown to guide the site-specific recruitment of chromatin-modifying enzymes. Although the mechanisms by which these RNAs impart specificity to chromatin-modifying enzymes are still a matter of intense analysis, several models are proposed, which include sequence-specific recruitment through an RNA/DNA triple-helix, allosteric activation of the modifying enzymes, or a scaffold function (22).

Kcnq1ot1 is a nuclear localized lncRNA of 91.5-kb in length (23). It is expressed from the *Kcnq1* gene in an antisense orientation. The promoter for *Kcnq1ot1* is located in the *Kcnq1* imprinting control region. As it is methylated on the maternal chromosome remaining unmethylated on the paternal chromosome, *Kcnq1ot1* lncRNA is expressed from only the paternal chromosome (24). *Kcnq1ot1* lncRNA is involved in the paternal silencing of multiple genes in the mouse *Kcnq1* imprinted gene cluster, which constitutes 8 to 10 genes expressed only from the maternal allele (23, 25-27). *Kcnq1ot1* lncRNA was found to direct the formation of a repressive chromatin domain (or coating) devoid of RNA polymerase II, which consists of a repressive higher order chromatin structure, to inactivate transcription *in cis* (26-28)(Figure 2A). The repressive chromatin domain is formed through the *Kcnq1ot1*-directed recruitment of the histone H3 at lysine 9 (H3K9)- and at lysine 27 (H3K27)-specific histone methyltransferase G9a and Polycomb group proteins (26-28), and through maintaining specific DNA methylation by interacting with the methyltransferase Dnmt1 (25).

The *Air* lncRNA was also shown to play a role in silencing the imprinted *Slc22a* and *Igf2r* genes in placenta (29). It interacts specifically with both the H3K9 histone methyltransferase G9a and the *Slc22a3* promoter. Accumulation of *Air* at the promoter recruits G9a, resulting in allelic silencing. Truncated *Air* did not associate with the *Slc22a3* promoter and caused reduced G9a recruitment and biallelic transcription (29). The *Air* ncRNA can mediate targeted recruitment of repressive histone-modifying activities to silence transcription *in cis* through the *Air*-mediated molecular interaction (Figure 2A).

In female mammals, one of two X chromosomes is inactivated to equalize the gene dosage between the sexes. X-chromosome inactivation is initiated by the expression of a 17-kb lncRNA, *Xist,* which is transcribed from the X-chromosome inactivation (XCI) center and accumulated on the X chromosome *in cis* (10, 30, 31). In addition to *Xist*, *XCI* produces an antisense transcript named *Tsix* (antisense to Xist) (32). *Tsix* is predominantly expressed from the active X chromosome, and functions as a repressor of *Xist* (31, 32). It was recently shown that RepA, a 1.6-kb ncRNA transcribed from the *Xist* locus, recruits the Polycomb Repressive Complex (PRC2) that

catalyzes H3K27 trimethylation to repress one X chromosome from which it is transcribed (33) (Figure 2A). In contrast, the antisense *Tsix* RNA inhibits the interaction of PRC2 with the X chromosome and maintains the chromatin transcriptionally active.

Interestingly, it was shown that *Xist* and *Tsix* form duplexes to be processed to small RNAs (sRNA) on the active X chromosome (34). Processing to sRNAs is dependent on Dicer (ribonuclease III) essential for the RNA interference (RNAi) pathway described later. Depletion of Dicer blocked the accumulation of *Xist* and histone H3 at lysine 27 trimethylation (H3K27me3) on the inactive X chromosome (34). Although there is a contradictory report that Dicer is not essential for the initiation of X-chromosome inactivation in embryonic stem (ES) cells (35), the involvement of the RNAi machinery in X-chromosome inactivation infers the generality of RNAi-mediated chromatin remodeling among species, and during evolution.

4.2. *In trans* recruitment of chromatin modifiers

In addition to dosage compensation of X-chromosomes, lncRNAs also play critical roles in pattern formation and differentiation in mammals (21, 22). Four chromosomal loci termed *HOXA* through *HOXD* are known to be essential for specifying the positional identity of cells in humans (21). Interestingly, *HOTAIR* lncRNA expressed from the *HOXC* locus represses transcription *in trans* across 40 kb of the *HOXD* locus by forming heterochromatin (21) (Figure 2B). *HOTAIR* associates physically with PRC2 and recruits it to the *HOXD* locus for H3K27me3. Depletion of *HOTAIR* using siRNAs caused decreased levels of H3K27me3 and PRC2 binding to the *HOXD* locus, leading to the transcriptional activation of the *HOXD* gene (21). The mechanism by which the *HOTAIR* lncRNA interacts specifically with the *HOXD* locus remains unclear at present. Although *HOTAIR* has little sequence homology with the *HOXD* locus, the formation of an RNA/DNA triple helix for association is an attractive model (22).

5. NCRNAS INVOLVED IN POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

5.1. Fundamental roles of snRNAs in pre-mRNA splicing

In eukaryotic cells, protein-coding genes are interrupted by non-coding sequences known as introns or intervening sequences. Thus, accurate removal of these sequences from pre-mRNAs, that is, pre-mRNA splicing, is essential for eukaryotic gene expression. SnRNAs are a group of metabolically stable ncRNAs predominantly localized in the nucleus (36). They range from 100 to 300 nucleotides in length, account for about 1% of the total mammalian cellular RNA and are characterized by containing modified bases, such as pseudouridine, and a trimethylguanosine cap structure (36). Of those, U1, U2, U4, U5 and U6 snRNAs play essential roles in the splicing of major-type (or U2-type) introns containing GU and AG at the 5' and 3' splice sites, respectively (14). For the removal of minor-type (or U12-type) introns that contain AU and AC at the 5' and 3' splice sites, U11, U12, U5, $U4_{ATAC}$ and $U6_{ATAC}$ snRNAs are required (37).

The splicing reaction is catalyzed in a large complex, the spliceosome, which is composed of four small nuclear ribonucleoprotein particles (snRNPs), U1, U2, U4/U6 and U5 snRNPs consisting of corresponding snRNAs and proteins, and numerous non-snRNP proteins (14). The spliceosome is assembled on a pre-mRNA by the successive binding of these components. Figure 3 shows a splicing pathway of a pre-mRNA with a single major-type intron. At the beginning of the splicing reaction, U1 snRNA and U2 snRNA recognize the 5' splice site and the branch site in a pre-mRNA through base-pairing, respectively, to form complex A. U11 and U12 snRNAs are used for recognition of the splice sites in the minor-type introns. Then, the preformed U4/U6.U5 tri-snRNP binds to complex A to form a pre-catalytic complex B containing all five snRNPs. After that, a conformational reassembly of the spliceosome takes place, which releases U1 and U4 snRNPs from the pre-catalytic complex. Subsequent base-pairing between U6 and U2 snRNAs generates complex B, an active spliceosome, to perform the splicing reaction, which yields mature mRNA and the excised intron with the lariat structure via two successive reactions (Figure 3). The excised intron is then debranched and degraded in the nucleus.

The pre-mRNA splicing reaction is therefore accomplished through dynamic changes in interactions between RNA-RNA and RNA-protein, forming the spliceosome. Differing from the roles of U1 and U2 snRNAs (and U11 and U12 snRNAs) in splice and branch site recognition, several lines of experiments have demonstrated that U6 snRNA (and $U6_{ATAC}$) base-paired with U2 snRNA serves as a catalytic complex in the spliceosome, as a ribozyme (RNA enzyme) (38, 39). Catalysis of the splicing reaction is performed by ncRNAs, not protein factors, in the spliceosome. RNA components in the spliceosome thus play different roles, that is, specific splice site recognition through base-pairing, catalysis of the reaction, and formation of a framework for building each snRNP and eventually the spliceosome.

5.2. Repression of pre-mRNA splicing by antisense ncRNA

In addition to the "classic" spliceosomal ncRNAs (snRNAs) mentioned above, recent analyses uncovered the presence of another class of ncRNAs involved in pre-mRNA splicing and other forms of post-transcriptional gene regulation in eukaryotic cells. A recent genome-wide analysis of transcriptomes revealed that most mammalian genes express antisense transcripts (40), which might play roles in the post-transcriptional regulation of gene expression.

It was shown that an antisense transcript transcribed from the *Zeb2* gene, which encodes a transcriptional repressor of the E-cadherin gene, binds to the 5' splice site of the *Zeb2* intron through base-pairing (41). The association of antisense transcripts with the 5'

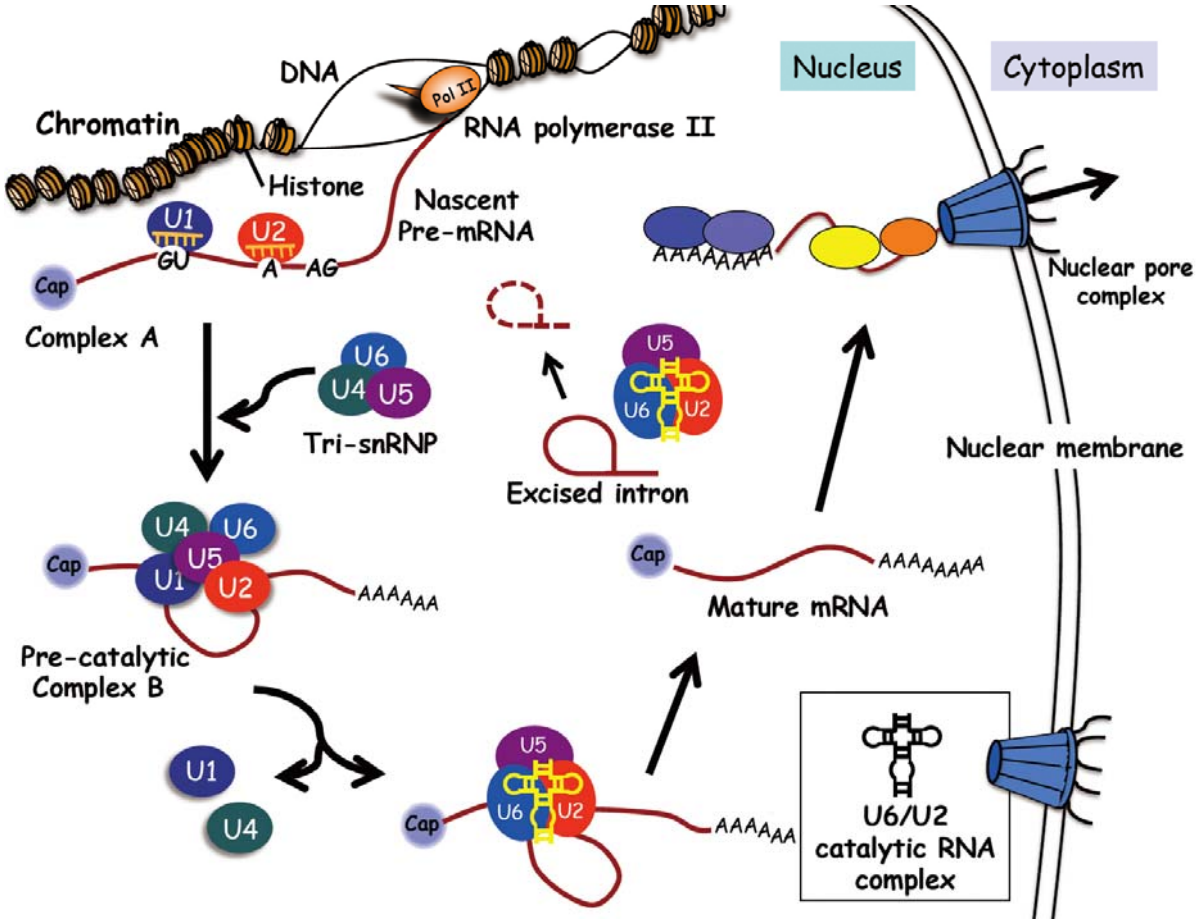

Figure 3. The splicing pathway of a pre-mRNA with a single major-type intron. During the transcription, the 5' splice site and branch site sequences in the nascent transcript are recognized by U1 snRNP and U2 snRNP through base-pairing, respectively, to form complex A. Then, U4/U6.U5 tri-snRNP associates with complex A to form a pre-catalytic complex B. After the release of U1 and U4 snRNPs, conformational change occurs to generate an active spliceosome containing a catalytic U6/U2 base-paired RNA complex. Splicing is performed via a two-step reaction, generating an intron with a lariat structure. After completion of the splicing reaction, the resultant mature mRNA is exported to the cytoplasm through the nuclear pore complex. The lariat intron is debranched and then degraded.

splice site results in repression of *Zeb2* pre-mRNA splicing by preventing recruitment of U1 snRNP to the 5' splice site. As the unspliced intron contains an internal ribosome entry site (IRES) necessary for Zeb2 translation, repression of pre-mRNA splicing by the antisense transcript increases the expression of the Zeb2 protein. The regulation of pre-mRNA splicing, including the modulation of alternative pre-mRNA splicing described below, by masking splice sites or splicing enhancer/ silencer sequences with antisense ncRNAs might be a common mechanism for the post-transcriptional regulation in mammalian cells.

5.3. Modulation of alternative pre-mRNA splicing by ncRNAs

Recently, the nuclear-localized lncRNA MALAT1 (Metastasis-associated lung adenocarcinoma transcript 1) was reported to regulate alternative pre-mRNA splicing by modulating the nuclear distribution and phosphorylation of SR splicing factors (42). Alternative pre-mRNA splicing produces diversity in gene expression by using distinct splice sites or exons, which is regulated in part by SR splicing factors including SF2, in pre-mRNA splicing (for a review, see 43). MALAT1 lncRNA, which was originally identified based on its overexpression in several metastatic cancers, is highly conserved among mammals (42). It is localized in nuclear structures named speckles, and directly associates with SF2, as well as several other splicing factors (42). Although MALAT1 lncRNA is not essential for the integrity of the speckle structure, it is required for the proper enrichment of SF2 and other splicing factors in the nuclear speckles (42). MALAT1 lncRNA was proposed to function as a "molecular sponge" or "molecular anchor" for splicing factors to localize them to the speckles (44). Depletion of MALAT1 lncRNA by antisense oligonucleotides or over-expression of an SR protein affects the alternative splicing patterns (promotion of exon skipping or exon inclusion) of a similar set of pre-mRNAs (42). Depletion of MALAT1 also changed the cellular level and phosphorylation states of SR proteins. Therefore, it is thought that MALAT1

lncRNA regulates alternative splicing by modulating the nuclear distribution of active SR proteins (42, 44).

5.4. NcRNA-mediated regulation of nuclear trafficking

The transport of proteins and RNAs between the nucleus and cytoplasm through the nuclear pores is an important step in the post-transcriptional regulation of gene expression in eukaryotic cells. NRON (Noncoding repressor of NFAT) is a lncRNA involved in the regulation of protein trafficking between the nucleus and the cytoplasm, which was identified in a screening for functional ncRNAs from 512 evolutionarily conserved putative ncRNAs (45). NRON RNA represses nucleocytoplasmic transport of the transcription factor NFAT (Nuclear factor of activated T cells) by interacting with the importin-beta family proteins, which mediate the transport of proteins across the nuclear membrane (45). In this case, ncRNA seems to function as an RNA regulator of a protein complex to modulate NFAT activity by controlling nuclear trafficking.

5.5. NcRNAs as scaffolds for the nuclear structures

The eukaryotic nucleus is divided into dozens of sub-nuclear structures (46). These structures are thought to be involved in the spatial and temporal coordination of gene expression in the nucleus. MENepsilon/beta (Multiple endocrine neoplasia epsilon/beta) is a nuclear-retained lncRNA, like MALAT1. It is located in a specific nuclear structure named the paraspeckle, which is implicated in the nuclear retention of mRNAs that contain inverted repeats such as Alu repetitive sequences (47, 48) or hyper-edited mRNAs (49). p54/nrb and PSF, paraspeckle-specific proteins, selectively bind to MEN epsilon/beta lncRNA. As expected, knockdown of MEN epsilon/beta lncRNA was shown to cause the disassembly of paraspeckles (50), indicating that MEN epsilon/beta lncRNA has a critical structural role in the formation of paraspeckles.

Analysis using a live-cell imaging system revealed that the transcription of MEN epsilon/beta lncRNA is essential for the *de novo* assembly of paraspeckles (51). Also, a recent study by Shevtsov and Dundr (52) showed that the transcription of coding and non-coding RNAs is required for the initial nucleation in the formation of nuclear structures, such as histone locus bodies, paraspeckles and nuclear stress bodies. They demonstrated that transcribed RNAs serve as structural elements in those nuclear structures.

There are several precedents for such "architectural RNAs" for cellular structures. It was previously shown that an RNA/protein complex is essential for mitotic spindle assembly in HeLa cells and Xenopus egg extract (53). In addition, the cytoskeleton and germinal granules in the Xenopus oocyte vegetal cortex were shown to require the non-coding Xlsirts RNA and coding VegT mRNA for proper organization (54, 55). Ablation of these RNAs by the microinjection of antisense RNAs disrupted the cytokeratin cytoskeleton. Therefore, architectural roles of ncRNAs seem to be a general function of lncRNAs in eukaryotic cells.

6. RNAI-MEDIATED FORMATION OF HETEROCHROMATIN

6.1. A self-reinforcing loop of RNAi-mediated formation of centromeric heterochromatin

In addition to transcription, the higher-order structures of chromatin affect other chromosomal processes, such as the segregation of chromosomes. The formation of heterochromatin at the centromere is essential for assembly of the kinetochore and correct segregation of chromosomes.

In *S. pombe*, large stretches of heterochromatin are present at centromeres, telomeres and the silent mating-type locus (for reviews, see 56, 57). The centromere is the chromosomal region for the assembly of the kinetochore, which ensures the equal segregation of chromosomes at cell division through its interaction with the microtubles of the mitotic spindle (58). The centromere in each *S. pombe* chromosome spans 40-100 kb. As shown in Figure 4A, the fission yeast centromere consists of a central core (*cnt*) flanked by innermost repeats (*imr*) and outermost repeats (*otr*) that contain repetitive sequences referred to as *dg* and *dh* repeats, which form the heterochromatin structure (56).

The assembly of centromeric heterochromatin requires the posttranslational methylation of histone H3 at lysine 9 (H3K9me) (59). H3K9me serves as a binding site for the highly conserved HP1 chromodomain proteins (Swi6, Chp1 and Chp2) to form the heterochromatin (59-62). Methylation of H3K9 is catalyzed by the histone methyltransferase Clr4, which forms a multisubunit complex, a Clr4-Rik1-Cul4 complex (CLRC), containing Rik1, Dos1, Dos2 and Cul4 (63-65). CLRC has a Cullin-dependent E3 ubiquitin ligase activity essential for the heterochromatin's formation (63-65). H3K9 methylation is spread *in cis*, depending on Swi6 (66). In addition to H3K9 methylation, the hypoacetylated state is important for the formation of heterochromatin (67, 68).

As histone methyltransferases and deacetylases have no sequence-specific DNA binding ability, a mechanism to recruit these chromatin modifiers specifically to the centromeric region is necessary. There is rapidly emerging evidence that the RNA interference system is involved in the formation of centromeric heterochromatin, especially in the recruitment of the chromatin modifiers to the centromere (69-71). RNAi is required to establish a H3K9me state at the centromere, but not fully required for its subsequent maintenance (72).

RNAi-mediated formation of the heterochromatin in fission yeast is triggered by double stranded ncRNAs (dsRNAs) originating from the centromeric repeat sequences (reviewed in 56, 57, 73, 74) (Figure 4B). The dsRNAs are cleaved by Dicer (Dcr1) to generate small interfering RNAs (siRNAs) of ~22 nucleotides. Resultant siRNAs are loaded onto the RNA-induced transcriptional silencing (RITS) effector complex containing Ago1, Tas3 and Chp1, and used for the specific targeting to homologous nascent transcripts by sequence complementarity. Actually, tethering of the RITS subunit

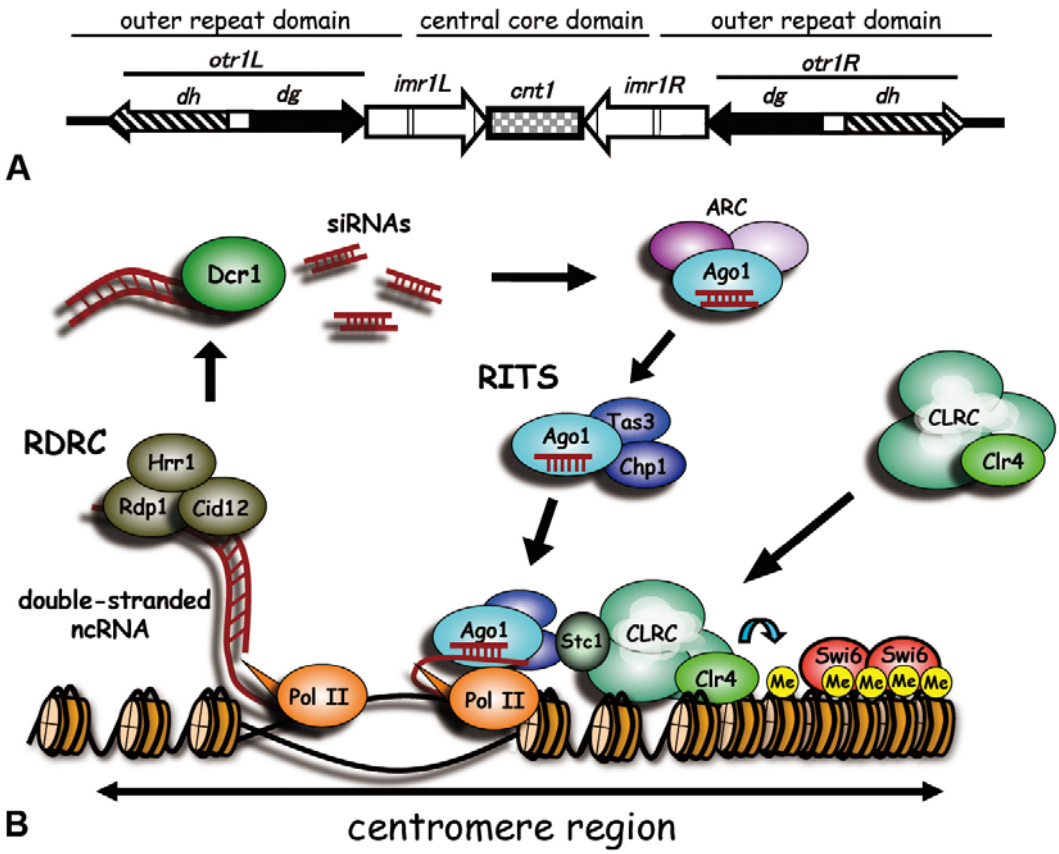

Figure 4. (A) Schematic representation of the structure of the fission yeast centromere1. Vertical lines in the *imr1L* and *imr1R* regions denote clusters of (or single) tRNA genes that function as boundary elements. (B) RNAi-mediated formation of the centromeric heterochromatin in fission yeast. A double-stranded ncRNA originating from the centromeric repeat region is processed into small interfering RNAs (siRNAs) by Dicer (Dcr1). Double stranded siRNAs are firstly integrated into an ARC complex, and then one strand of siRNA is loaded onto an RNA-induced transcriptional silencing (RITS) complex containing Argonaute 1 (Ago1), Targeting complex subunit 3 (Tas3) and Chromodomain protein 1 (Chp1). The siRNA in the RITS complex binds to complementary nascent transcripts from the centromere through base-pairing and tethers the RITS complex to the heterochromatic domain. The RITS complex recruits the CLRC complex containing the histone-methyltransferase Clr4 to methylate the histone H3 at lysine 9 (H3K9). After that, the heterochromatin protein Swi6 binds to H3K9me to form the heterochromatin. The RITS complex also recruits the RNA-directed RNA polymerase complex RDRC for generation of double-stranded ncRNAs, forming a self-reinforcing loop to facilitate heterochromatin formation at the centromere.

Tas3 to the transcripts from the active *ura4*$^+$ reporter gene using a tag system silences *ura4*$^+$ expression (75). The RITS complex stably associates with the heterochromatic domain through the binding of the chromodomain protein Chp1 to H3K9me nucleosomes (76-78), and recruits CLRC containing the histone-modifying enzyme Clr4 through a LIM domain protein Stc1 (79), as well as RNA-directed RNA polymerase complex RDRC for the generation of dsRNAs (70, 80). The dsRNAs synthesized by RDRC are then processed by Dcr1 into siRNAs, forming a self-reinforcing loop to facilitate heterochromatin formation at the centromere (Figure 3B). Through this mechanism, centromeric siRNAs function as specific guides for the recruitment of chromatin modifiers to the centromere.

It appears contradictory that the establishment of heterochromatin requires transcription of the non-coding repeat region in the heterochromatic region. In that situation, transcription factors must access sequences embedded in the repressed heterochromatic region. As mutations in RNA polymerase II subunits inhibit the RNAi-dependent assembly of centromeric heterochromatin, the centromeric repeats seem to be transcribed by RNA polymerase II, like general protein-coding mRNAs (81, 82). Interestingly, centromeric repeats are transcribed during a brief period of the S phase, in which DNA replication occurs, and its transcription is followed by the loading of RNAi and heterochromatin factors to the centromere (83). The RNAi-directed formation of centromeric heterochromatin is thus controlled during the cell cycle.

6.2. Involvement of splicing factors in RNAi-mediated formation of heterochromatin

Recently, several groups reported an unexpected link between the RNAi-mediated formation of

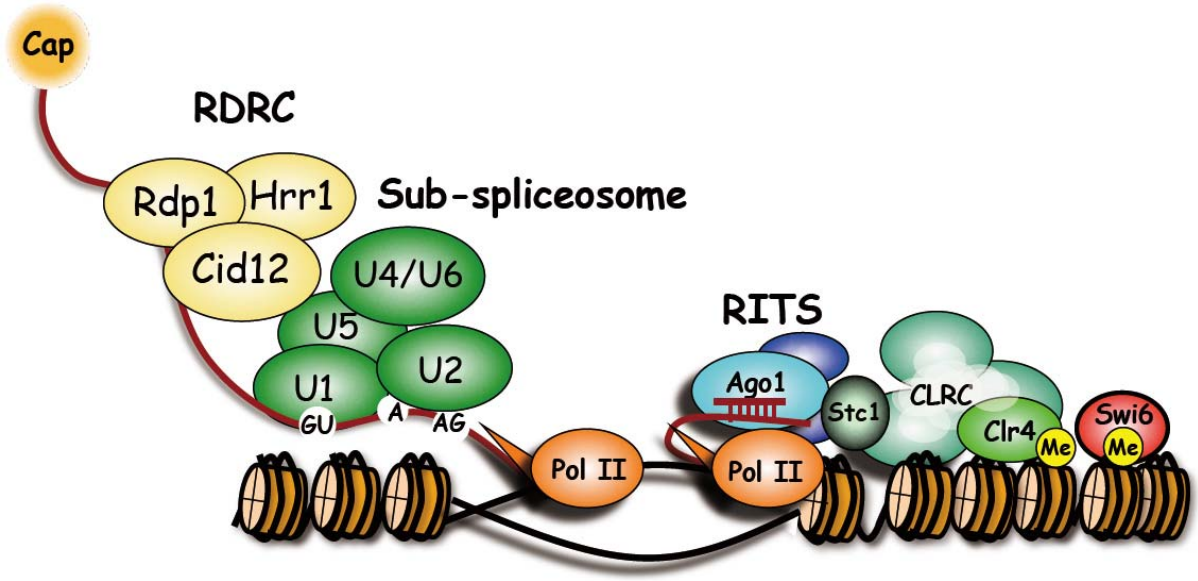

Figure 5. A model for the involvement of the spliceosomal components in the RNAi-mediated formation of centromeric heterochromatin. A subset of spliceosomal factors, including U4 snRNA, recognize the mRNA-type intron in the nascent centromeric ncRNAs and assemble the sub-spliceosome complex as a platform for the recruitment of the RDRC complex. Recruitment of the RDRC complex is directed by interaction of Cid12p with splicing factors, such as Cwf10p. Recruited RDRC then converts the centromeric ncRNAs into double-stranded RNAs, which are used for siRNA generation.

heterochromatin and the splicing machinery (70, 84, 85). The first evidence was obtained from immunoprecipitation experiments performed by Motamedi *et al.* (70). They showed that the subunits of the spliceosome assembled during the splicing reaction are co-precipitated with Cid12p, a component of RDRC (70). The spliceosome subunits were not co-purified with other components of RDRC and RITS, suggesting that Cid12p specifically interacts with the spliceosome. Then, an analysis of *csp* (centromere: suppressor of position effect) mutants defective in centromeric gene silencing (84) revealed that some *csp* mutations are allelic with the splicing mutations *prp* (pre-mRNA processing) (86, 87) and *cwf* (complexed with Cdc5p) (88). The *prp5, prp8, prp10, prp12* and *cwf10* mutants were found to exhibit accumulation of unprocessed ncRNAs from the centromere, decreased generation of siRNAs and defective centromeric heterochromatin (84). In contrast, other splicing mutants, *prp1, prp2, prp3* and *prp4,* showed no defects in the integrity of the centromeric heterochromatin. In addition, an analysis of immunoprecipitates pulled-down with FLAG-tagged Cid12p by liquid chromatography-mass spectrometry (LC-MS)/MS revealed that Cid12p is associated with many splicing factors including Prp5p, Prp10p, Prp12p and Cwf10p. From these results, it was suggested that the spliceosomal complex provides a platform for the recruitment of the RDRC complex, facilitating the formation of centromeric heterochromatin (84). In addition, we analyzed the *prp13* splicing mutant, whose gene encodes U4 snRNA essential for the pre-mRNA splicing reaction, and showed that a mutation in U4 snRNA causes the defective formation of the centromeric heterochromatin, indicating that spliceosomal snRNAs also play essential roles in RNAi-directed gene silencing as well as in the splicing reaction (85).

6.3. A "functional intron" model

The next question to answer is how the spliceosomal complex assembles on the centromeric lncRNAs to recruit the RDRC complex. In an analysis of the cross-talk between heterochromatic gene silencing and the splicing machinery, an intron was found in the centromeric *dg* ncRNA that served for siRNA generation (85). The intron has typical features for introns in protein-coding pre-mRNAs, namely GU and AG at the 5' and 3' splice sites, respectively, and a presumed branch site sequence matching the consensus branch site sequence found in *S. pombe* introns (89). Sequencing of RT-PCR products demonstrated that the *dg* centromeric intron is actually spliced. Based on these results, we proposed a model in which the assembly of the spliceosome or a sub-spliceosome complex on the intron-containing centromeric ncRNAs facilitates the recruitment of the RDRC complex to the ncRNAs, through interaction with Cid12p, to synthesize the dsRNAs for siRNA generation, thereby resulting in the effective formation of heterochromatin (85) (Figure 5).

It is noteworthy that the splicing efficiency of the *dg* centromeric intron is very low (85). In general, introns must be effectively removed from pre-mRNAs to produce functional translatable mature RNAs. However, in the case of centromeric lncRNA, the intron remains in most transcripts and seems to supply an initial binding site for the assembly of a platform to recruit RNAi factors. From this point of view, the intron in the centromeric lncRNA is thought to act as a "functional intron" to facilitate the RNAi-mediated formation of heterochromatin.

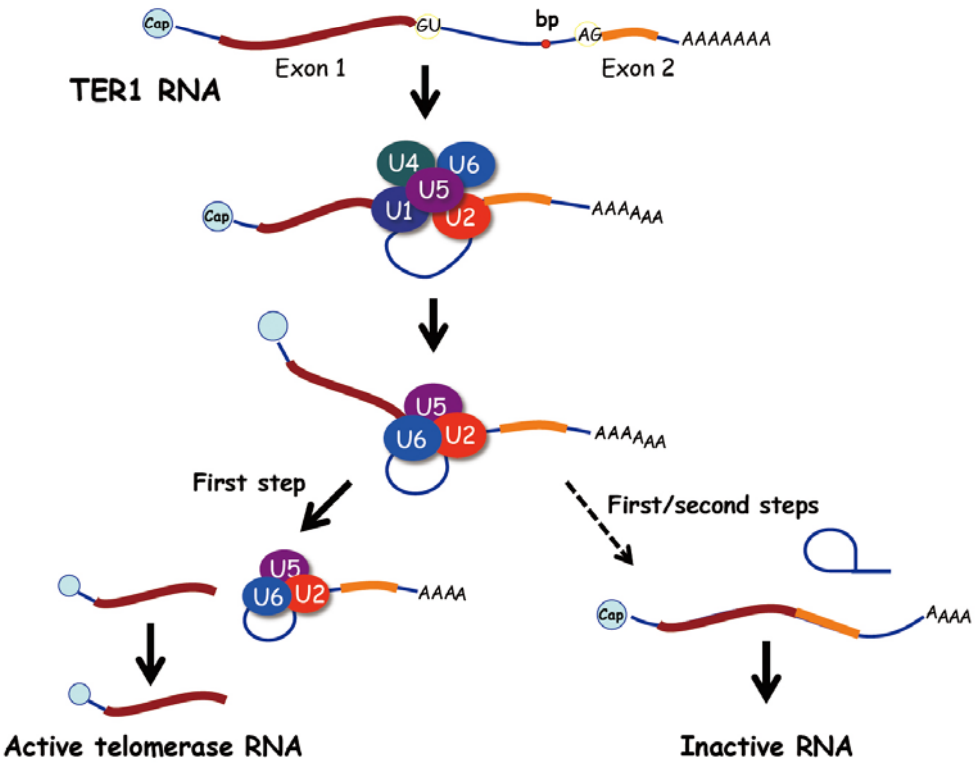

Figure 6. Schematic representation of the 3'-end processing of the telomerase RNA, *TER1*, in *S. pombe.* A transcript from the *ter1*$^{+}$ gene is cleaved by a first step reaction of pre-mRNA splicing to generate a functional telomerase RNA consisting of exon 1. Completion of the splicing reaction through a second step yields inactive forms of *TER1* and causes progressive telomere shortening. Bp indicates a branch point in the intron of the *ter1*$^{+}$ gene.

7. NOVEL ROLES OF THE SPLICEOSOME IN THE BIOGENESIS OF NCRNAS

The relationship between the RNAi-mediated heterochromatic formation and the pre-mRNA splicing machinery suggests that the spliceosome is potentially multifunctional and has evolved to play essential roles not only in pre-mRNA splicing, but also in other biological processes. Actually, the role of the spliceosome (or sub-spliceosome) is not restricted to the generation of centromeric siRNAs. It has been shown that the spliceosome is involved in the 3' end maturation of the telomerase ncRNA essential for maintenance of the chromosome ends (90). The chromosome ends are capped with telomeres, special nucleoprotein structures composed of double-stranded repetitive DNA sequences and a number of telomere-associated proteins, to protect the ends from nucleolytic degradation and DNA repair activities (reviewed in 91). The length of telomeres is maintained by telomerase, a ribonucleoprotein complex with small ncRNA named telomerase RNA and a reverse transcriptase that adds telomeric repeats to the ends of chromosomes. The RNA moiety in the telomerase is used as a template for reverse transcription to extend the 3' single strand of the telomere (92, 93).

Interestingly, in fission yeast, the gene coding for telomerase RNA, *ter1*$^{+}$, contains an mRNA-type intron, and the first step in the splicing reaction was found to generate the mature 3' end of telomerase RNA *TER1* (90) (Figure 6). Inhibition of the first step of the splicing reaction or completion of the splicing reaction resulted in the synthesis of an inactive form of *TER1* RNA, causing progressive telomere shortening. Uncoupling of the first and second steps of the splicing reaction, which does not normally occur in pre-mRNA splicing, is necessary for this mechanism. The splicing machinery seems to regulate the intracellular level of *TER1* RNA.

Another example of a novel role for the spliceosome in the production of functional nuclear ncRNAs was found in microRNA (miRNA) biogenesis (94, 95). The canonical pathway for miRNA biogenesis converts primary hairpin precursors into mature ~22 nucleotide miRNAs through successive cleavage by Drosha and Dicer ribonucleases (96). "Mirtrons" are alternative precursors for miRNA biogenesis bypassing the Drosha cleavage (94, 95). Mirtrons are derived from short hairpin introns present in protein-coding genes. After the formation of the spliceosome, the mirtrons are removed from pre-mRNAs by splicing, and the resultant lariat products are debranched in the nucleus, followed by transportation to the cytoplasm via Exportin-5 and processing by Dicer to yield small regulatory miRNA. The presence of mirtrons in worms, flies and mammals (94) indicates their ancient origin and suggests a relationship between the introns and functional ncRNAs.

8. PERSPECTIVE

Recent advances in analytical technologies in molecular biology have revealed the existence of a large number of long and short, nuclear localized, metabolically stable ncRNAs. In this review, we have highlighted the diverse functions of the nuclear ncRNAs in gene expression. Information on such ncRNA regulation will shed light on the novel regulatory networks in eukaryotic cells, adding another layer of knowledge regarding the control systems for gene expression. In addition, recent studies showed that ncRNAs are relevant to the etiology of human diseases such as thalassemia (97), geographic atrophy (98) and cancer (99, 100). Further research will not only continue to define the molecular mechanisms by which ncRNAs function, their regulatory systems and the interplay among them, but also provide new insights into the pathogenesis of disease. A hidden "ncRNA world" is now being uncovered.

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Abbreviations: ncRNA: non-coding RNA; lncRNA: long non-coding RNA; snRNA: small nuclear RNA; kb: kilobases; *Evf2*: embryonic ventral forebrain 2; TLS: translocated in liposarcoma; CBP: CREB-binding protein; *CCND1*: cyclin D1; *DHFR*: dihydrofolate reductase; H3K9: histone H3 at lysine 9; H3K27: histone H3 at lysine 27; XCI: X-chromosome inactivation; PRC2: polycomb repressive complex; sRNA: small RNA; ES: embryonic stem; RNAi: RNA interference; snRNP: small nuclear ribonucleoprotein particle; MALAT1: metastasis-

associated lung adenocarcinoma transcripts 1; NFAT: nuclear factor of activated T cells; NRON: noncoding repressor of NFAT; MEN:multiple endocrine neoplasia; dsRNA: double-stranded RNA; CLRC: Clr4-Rik1-Cul4 complex; siRNA: small interfering RNA; RITS: RNA-induced transcriptional silencing; Ago1: Argonaute 1; Tas3: targeting complex subunit 3; Chp1: chromodomain protein 1; RDRC: RNA-directed RNA polymerase complex; *prp*: pre-mRNA processing; *cwf*: complexed with Cdc5p; *csp*: centromere: suppressor of position effect; RT-PCR: reverse transcription polymerase chain reaction; miRNA: microRNA

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