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REVIEW

# Stable intronic sequence RNAs (sisRNAs): a new layer of gene regulation

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**Abstract** Upon splicing, introns are rapidly degraded. Hence, RNAs derived from introns are commonly deemed as junk sequences. However, the discoveries of intronic-derived small nucleolar RNAs (snoRNAs), small Cajal body associated RNAs (scaRNAs) and microRNAs (miRNAs) suggested otherwise. These non-coding RNAs are shown to play various roles in gene regulation. In this review, we highlight another class of intron-derived RNAs known as stable intronic sequence RNAs (sisRNAs). sisRNAs have been observed since the 1980 s; however, we are only beginning to understand their biological significance. Recent studies have shown or suggested that sisRNAs regulate their own host's gene expression, function as molecular sinks or sponges, and regulate protein translation. We propose that sisRNAs function as an additional layer of gene regulation in the cells.

**Keywords** Non-coding RNA · sisRNA · Intron

**Abbreviations**

| | |
|---|---|
| AID | Activation-induced cytidine deaminase |
| ASO | Antisense oligodeoxynucleotides |
| CB | Cajal Body |
| $C_H$ | Heavy chain constant |
| cis-NAT | cis-natural antisense transcript |
| CSR | Class switch recombination |
| EBV | Epstein–Barr virus |
| GV | Germinal vesicle |
| HSUR | *Herpesvirus saimiri* U RNA |
| HSV | Herpes simplex virus |
| Ig | Immunoglobulin |
| IgH | Immunoglobulin heavy chain |
| LAT | Latency-associated transcript |
| miRNA | microRNA |
| ncRNA | Noncoding RNA |
| Pre-mRNA | Precursor messenger RNA |
| Pre-rRNA | Precursor ribosomal RNAs |
| PWS | Prader–Willi syndrome |
| rancRNAs | Ribosome-associated ncRNAs |
| rga | regena |
| RNP | Ribonucleoprotein |
| RNAi | RNA interference |
| S regions | Switch regions |
| scaRNAs | Small Cajal body associated RNA |
| sisRNA | Stable intronic sequence RNA |
| snRNA | Small nuclear RNA |
| snRNP | Small nuclear RNP |
| snoRNA | Small nucleolar RNA |
| sno-lncRNA | snoRNA-related long ncRNA |

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## Introduction

The discoveries of several classes of intron-derived RNAs suggest that introns are not merely junk. Instead, they play an important role in gene regulation. In this review, we will first briefly look at intronic RNA history, as well as the currently known classes of intron-derived RNAs. We will then look at a novel class of intron-derived RNA known as stable intronic sequence RNAs (sisRNAs) and review their known or suggested biological functions in the cell.

Introns were discovered in 1977, independently in the laboratories of Philip Sharp and Richard Roberts [1, 2].

Using adenoviruses, both groups observed that mRNAs transcribed from the adenovirus DNA were not exact copies of one another. Instead, there were several stretches of sequences found in the DNA, which were absent in the resulting mRNA. Soon after, work from several other laboratories suggested that such an arrangement was also present in the eukaryotic genome [3–10]. In 1978, Walter Gilbert famously named these stretches of sequences, which split the gene into pieces, as 'introns' (short for intragenic regions), whereas the regions that are expressed were termed as 'exons' [11]. Most eukaryotic genes are initially expressed as precursor messenger RNAs (pre-mRNAs), containing both introns and exons. The introns are then spliced out, leaving behind the exons that are ligated together to form the mature mRNA.

Introns can be spliced out either through self-splicing or via the spliceosomal machinery. Self-splicing introns are catalytic RNAs or ribozymes, which have the ability to catalyze their own splicing. They are divided into three classes: Group I, II and III introns. The three groups of self-splicing introns differ mainly in the mechanisms utilized to carry out the splicing [12–14]. In this review, we will focus on introns spliced by the spliceosomes, because they account for the splicing of majority of eukaryotic introns [15]. The spliceosome is a multi-subunit ribonucleoprotein (RNP) complex made up of many different proteins and five small nuclear RNAs (snRNAs). The five snRNAs are U1, U2, U4, U5, and U6 [16–19]. The spliceosome is a dynamic complex with its different components changing throughout the whole splicing process. First, the U1 small nuclear RNP (snRNP) recognizes and binds to the 5′ splice site, while the U2 snRNP associates with the branch site. Subsequently, the U4, U5 and U6 snRNPs bind to the intron, after which RNA–RNA and RNA–protein interactions of the spliceosome undergo major structural rearrangements, destabilizing the U1 and U2 snRNPs from the complex, and eventually leading to the activation of the spliceosome. The 2′ hydroxyl of a specific nucleotide found at the branch site (commonly adenosine) becomes nucleophilic and carries out a nucleophilic attack on the phosphodiester bond at the 5′ splice site. As a result, the 5′ end of the intron becomes covalently linked to the branch site nucleotide forming a lariat, whereas the adjacent exon is now 'free' at its 3′ end. The 3′ hydroxyl of that 'free' exon then attacks the phosphodiester bond at the 3′ splice site, releasing the lariat intron from its neighbouring exon. The two adjacent exons are then ligated together (Fig. 1). The spliceosome finally disassembles and its components are ready for the next round of splicing. It has been shown that after splicing, the intronic lariat is debranched and degraded rapidly [20]. Hence, intronic RNAs are commonly deemed as 'junk' and devoid of any functions.

Although introns were discovered almost 40 years ago, there is still a debate over their evolutionary origins. There are two opposing hypotheses: "introns-early" [21, 22] and "introns-late" [23, 24]. Proponents of the introns-early hypothesis, also known as the exon theory of genes, believe that the very first exons were minigenes, coding for very short polypeptide structures. Such minigenes predated cellular life. Over the course of evolution, these minigenes were assembled together via introns, which were random nucleotide sequences introduced at the ends of these minigenes. These intronic sequences allowed for exon shuffling, which eventually resulted in the formation of whole genes comprising of several minigenes or exons. Modern organisms that have genomes containing almost little to no introns, such as prokaryotes, were thought to have lost their introns over evolutionary time. This is a result of the pressure to simplify their genomes and to reduce replication time, therefore allowing for competitive growth advantage. On the other hand, the introns-late supporters believe that introns arose much later, during the evolution of eukaryotes. Prokaryotes have no spliceosomal introns, apart from some that have a few non-spliceosomal (self-splicing) introns. Prokaryotes do not have a nucleus and thus transcription and translation are coupled and occur in the cytoplasm. If prokaryotes were to contain spliceosomal introns, a problem would arise as splicing would not be able to be completed prior to translation. Eukaryotes, on the other hand, have evolved to compartmentalize their genetic material into a nucleus, and hence such a splicing problem would not occur. The evolution of a nucleus allowed for eukaryotes to evolve and possess spliceosomal introns. Currently the consensus on the origin of introns is a mixture of both hypotheses. Spliceosomal introns appeared suddenly at the time eukaryotes first emerged and these introns most likely derived from self-splicing introns, which were present much earlier in precellular life [25].

Regardless of how or when introns were introduced into the genome, an important question is why introns have been so ubiquitous throughout the genomes of higher eukaryotes? In 1994, it was proposed that RNAs derived from introns are not just junk but could have evolved functions in the cell [26]. The discoveries of small nucleolar RNAs (snoRNAs), small Cajal body associated RNAs (scaRNAs) and microRNAs (miRNAs) have offered support to this hypothesis [27]. These classes of intron-derived RNAs have been shown to play various roles in gene regulation.

snoRNAs are an abundant group of non-coding RNAs (ncRNAs) that are important for rRNA processing and maturation [28, 29]. A minority of snoRNAs are involved in the endo- and exo-nucleolytic reactions to remove the spacer regions found in precursor ribosomal RNAs (pre-

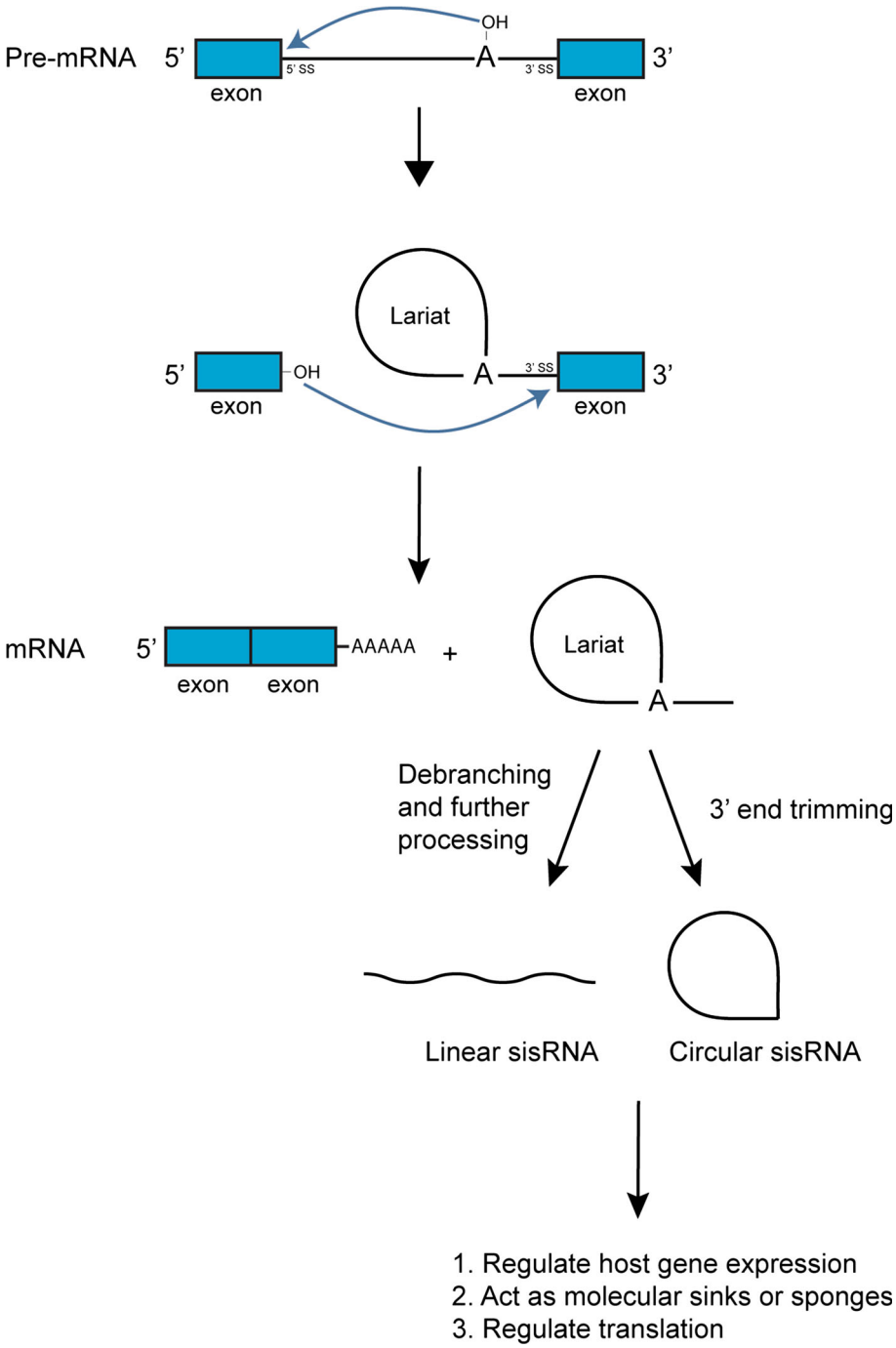



**Fig. 1** Biogenesis of linear and circular sisRNAs. During splicing, branch point nucleotide adenosine forms a 2′–5′ phosphodiester bond with the 5′ end of the intron, generating a lariat intermediate. The free 3′ OH group of the exon then cleaves the 3′ end of the lariat intermediate and joins the two exons together with a lariat as a by-product. The lariat can either be debranched by a Lariat debranching enzyme into a linear sisRNA or be trimmed at the 3′ end by an exonuclease into a circular sisRNA. Exons and introns are in *blue* and *black*, respectively

rRNAs), whereas the majority of snoRNAs act as guides for the covalent modification (pseudouridylation and 2′-*O*-methylation of ribose groups) of several regions of the rRNAs.

scaRNAs are structurally and functionally similar to snoRNAs. However, instead of localizing in the nucleolus, they localize in the Cajal body (CB), a nuclear body involved in the biogenesis of spliceosomal snRNAs. At the Cajal bodies, scaRNAs guide the covalent modifications of the snRNAs [30].

miRNAs are short approximately 22-nucleotide RNAs, which regulate gene expression. They generally function either though RNA silencing or inhibition of protein synthesis [31, 32]. In plants, miRNAs base pair with their targets with perfect or near perfect complementarity to silence their target mRNAs through the RNA interference (RNAi) machinery [33]. On the other hand, in animals, most miRNAs base pair with their target mRNAs with imperfect complementarity and repress the translation of target mRNAs [34].

## Discovery of sisRNAs

As mentioned previously, most introns are rapidly degraded after splicing. Their rapid turnover has a direct implication on both transcription and splicing, thus affecting many other processes in the cell [35]. Firstly, the degradation of introns would free up nucleotides that could be used during subsequent rounds of transcription. Secondly, splicing factors that remain bound to the spliced intronic lariat are released and would then be available for the splicing machinery. It has been reported that the accumulation of intronic lariats is indeed detrimental to a cell [36, 37]. This observation suggests that introns that remain relatively stable upon splicing may not be just artefacts, as this would have been selected against during the course of evolution. The phenomenon of stable intronic RNA is not a recent observation: this was initially observed in viruses in the 1980's, then in *Xenopus*, mammalian cells and *Drosophila* (Table 1). However, these unusually stable intronic RNAs were thought to be the rare exceptions.

In 2012, Joseph Gall's laboratory reported the first genome-wide identification of intronic RNAs in the oocyte germinal vesicle (GV or nucleus) of *Xenopus tropicalis* [38]. These intronic RNAs were only detected after acquiring pure nuclear RNA by dissecting the GVs and manually removing the nuclear envelopes, thus preventing contamination from the highly abundant cytoplasmic mRNAs. These intronic RNAs were termed 'intronic sequences' since they can be shorter than the full-length introns they originated from. These intronic sequences are being produced by more than 90 % of the genes transcribed during oogenesis. They observed that these intronic sequences in the GV were highly stable and could still be detected after 12 h to 2 days upon inhibition of transcription by actinomycin D treatment. For that, they named these stable intronic sequence RNAs (sisRNAs). Two years later, sisRNAs were also found to be present in the cytoplasm of the *X. tropicalis* oocytes [39]. These cytoplasmic sisRNAs were identified by removing the whole GVs and extracting the cytoplasmic RNA. Cytoplasmic sisRNAs are resistant to RNase R treatment, suggesting that they are circular molecules (lariats without tails). Compared to nuclear sisRNAs, cytoplasmic sisRNAs were observed to be mainly derived from shorter introns, and were located in relatively fewer introns. They noted that both nuclear and cytoplasmic sisRNAs could persist in the mature oocytes even from the early stages of embryogenesis until the blastula stages, when zygotic transcription starts, suggesting that this store of sisRNAs may have a role to play throughout the early development of *X. tropicalis*.

We classify sisRNAs as intronic RNAs that are spliced from its primary transcript and remain stable in the cell (Fig. 1). We review the known or suggested biological functions of sisRNAs in the cell: regulating host gene expression, functioning as molecular sinks and, regulating protein translation. We propose that sisRNAs are biologically active ncRNAs that act as an additional layer of gene regulation in the cell.

## sisRNAs regulate host gene expression

Recently, the laboratory of Jun Wei Pek reported a sisRNA, named *sisR-1*, in *Drosophila melanogaster,* which they showed to regulate the expression of its host gene [40]. They initially identified a total of 34 candidate sisRNAs after deep sequencing analysis of rRNA-depleted total RNA extracted from 0 to 2 h embryos. 0–2 h embryos were used because RNAs present in these embryos would have been stable for no less than 10–12 h since zygotic transcription only begins 2 h after egg laying in *Drosophila*. Of these 34 candidate sisRNAs, some are resistant to RNase R treatment, suggesting that both circular and linear sisRNAs are present. Upon further examination of a few of these candidate sisRNAs, they showed that sisRNAs are not just oocyte-specific, but are present throughout development as well as in adult tissues. *sisR-1* is a linear sisRNA derived from the fourth intron of the *regena* (*rga*) gene locus. In addition, a cis-natural antisense transcript (cis-NAT), which they named *ASTR*, is also found in the same locus. Both *ASTR* and *rga* showed analogous expression patterns during embryogenesis. When they knocked down *ASTR* via shRNA treatment, it led to a significant decrease in the expression levels of *rga* pre-mRNA, suggesting that the expression of *rga* is promoted by *ASTR*. On the other hand, shRNA-mediated knockdown of *sisR-1* led to an increase in the expression of both *ASTR* and *rga*. However, when both *sisR-1* and *ASTR* were knocked down simultaneously, there was no longer an increase in expression of *rga*. Thus, they proposed that *sisR-1* regulates the expression of its own host gene by repressing the cis-NAT *ASTR* (Fig. 2a). Hence *sisR-1* is involved in a negative feedback loop, which promotes the robust decrease in expression of its host gene during development.

Another example of a sisRNA that is involved in the regulation of its host gene is the intronic switch RNA. B cells undergo immunoglobulin (Ig) heavy chain (IgH) class switch recombination (CSR) upon stimulation by antigens [41]. CSR is an intrachromosomal deletional recombination within the switch (S) regions of the IgH locus facilitated by the activation-induced cytidine deaminase (AID) [41]. During CSR, the default heavy

**Table 1** List of sisRNAs discovered

| Species | Host gene locus | Conformation | Localization | Tissues | Functions | References |
|---|---|---|---|---|---|---|
| Human | Genome-wide | Circular | Nuclear | Cell lines | ND | [43] |
| | *ANKRD52* locus (*ci-ankrd52*) | Circular | Nuclear | Cell lines | Regulates host gene expression by interacting with RNA Polymerase II | |
| Human | 15q11-q13 region of chromosome 15 (sno-lncRNA) | Linear | Nuclear | Cell lines | Acts as molecular sink to sequester Fox2, altering alternative splicing | [44] |
| Human, mouse | T cell receptor-β locus | Lariat | Nuclear and cytoplasmic | T cells | ND | [76, 77] |
| Human, rat | Pem homeobox locus | Lariat | Nuclear and cytoplasmic | Cell lines | ND | [35] |
| Mouse | κ locus | ND | Nuclear | Cell lines | ND | [78] |
| Mouse | *Igh* locus (intronic switch RNA) | Linear | ND | Cell lines | Regulates host gene expression by acting as guides to target AID to its host gene locus, promoting CSR | [42] |
| *Xenopus laevis* | Simian virus 40 | Lariat | Nuclear | Oocytes | ND | [79] |
| *Xenopus tropicalis* | Genome-wide | Linear and lariat | Nuclear and cytoplasmic | Oocytes, embryos | ND | [38, 39] |
| *Drosophila melanogaster* | *delta* locus | ND | Nuclear | Embryos | ND | [80] |
| *Drosophila melanogaster* | tRNA locus | Circular | ND | Larvae, pupae and adults | ND | [81] |
| *Drosophila melanogaster* | Genome-wide | Linear and circular | ND | Embryos, larvae, pupae and adults | ND | [40] |
| | *regena* locus (*sisR-1*) | Linear | Nuclear and cytoplasmic | Embryos | Regulates host gene expression by repressing the cis-NAT *ASTR* | |
| Human and murine cytomegalovirus | Immediate-early transcript | Lariat | Nuclear | Human and mouse cell lines | Murine sisRNA promotes viral progression from acute to persistent phase of infection | [82, 83] |
| Adenovirus 2 | E2A region | Linear and lariat | Nuclear | Human cell lines | ND | [84] |
| Herpes simplex virus | Latency-associated transcript (2-kb LAT intron) | Lariat | Nuclear | Monkey kidney cells, human cell lines, human and mouse neurons | Regulates translation of Hsp70 by altering the 60S ribosomal subunit, promoting host cell survival upon reactivation | [59–61, 64–66] |
| Epstein–Barr virus | W repeat region (*ebv-sisRNA-1*) | Linear | Nuclear | Human B cells | Acts as molecular sink to sequester miR-142-3p, preventing the repression of the EBV lytic gene product | [45, 48] |
| | W repeat region (*ebv-sisRNA-2*) | Linear | ND | Human B cells | ND | |

*ND* not determined

chain constant ($C_H$) region $C_\mu/C_\delta$ is replaced to one of the other downstream $C_H$ regions ($C_\gamma$, $C_\alpha$ or $C_\varepsilon$), 'switching' the B cell from expressing IgM/IgD to IgG, IgA or IgE respectively. In a recent study by the laboratory of Jayanta Chaudhuri using CH12 B lymphoma cells, a cell line that switches at a high rate, it was found that intronic RNA transcripts coming from the S regions of the IgH locus are involved in CSR [42]. These intronic switch RNAs are spliced from the primary germline transcripts of the IgH locus, and were found to be debranched. Through RNA pull-down assays, they showed that these linear sisRNAs are able to bind to AID. They suggested that these sisRNAs bind to AID by forming G-quadruplex structures. Due to complementary sequences between the intronic switch RNA and the S region, these sisRNAs regulate its own host genes by acting as guides, which

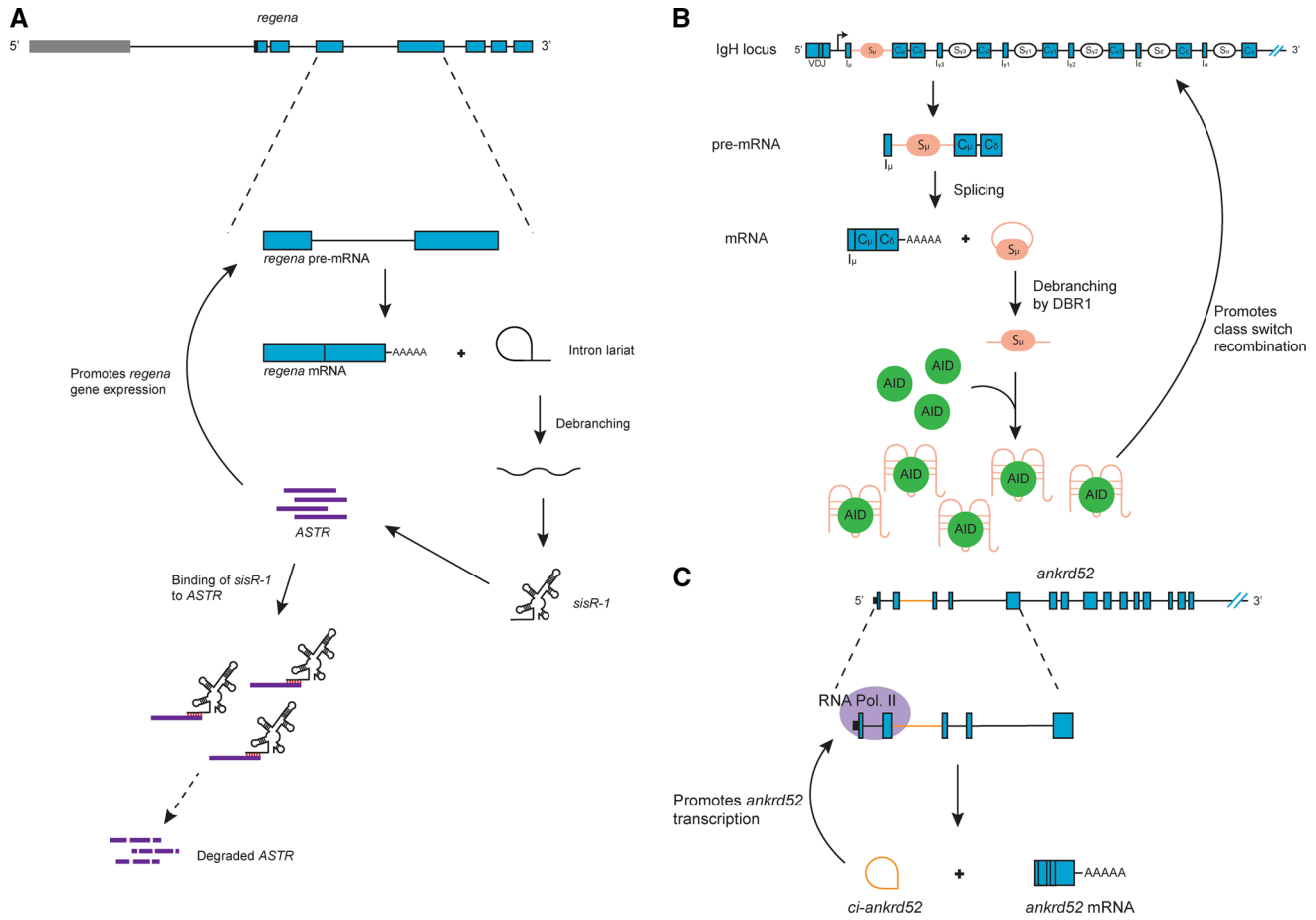



**Fig. 2** sisRNAs regulate host gene expression. **a** Model of a *Drosophila* sisRNA, *sisR-1*, biogenesis and its regulation on its parental gene *rga* through repression of *ASTR*. **b** Model for the regulation of IgH CSR in B cells by the intronic switch RNA, acting as guides to target AID to the IgH locus. **c** Model of a circular intronic RNA, *ci-ankrd52*, and its regulatory role on its parental gene expression via interactions with RNA Pol. II

target the AID to the IgH locus for efficient CSR to take place (Fig. 2b).

*ci-ankrd52*, a circular sisRNA, has also been observed to regulate its host gene expression. Using a custom computational framework, Ling-Ling Chen and Yang Li's laboratories identified several hundred intronic RNAs in the non-polyadenylated RNA fraction extracted from HeLa and H9 cells [43]. RNase R treatment of these identified sisRNAs revealed that both linear and circular forms were present. They suggested that a consensus motif near the 5′ splice site and the branchpoint of the circular sisRNAs might explain why these sisRNAs are not debranched and remain circular. One such circular sisRNA is *ci-ankrd52*, which originates from the second intron of the *ANKRD52* gene and is localized in the nucleus. To study its function, they used antisense oligodeoxynucleotides (ASOs) modified with phosphorothioate to knockdown *ci-ankrd52* expression. They observed that the expression of its host gene *ankrd52* was significantly reduced upon the knockdown of *ci-ankrd52*. When they examined the nuclear localization of *ci-ankrd52* using in situ hybridization, they observed that *ci-ankrd52* specifically colocalized to the transcription sites of its host gene locus. They further showed that *ci-ankrd52* is associated with the elongation RNA Polymerase II complex. Taken together, this study suggests that *ci-ankrd52* regulates the efficient transcription of its host gene through its positive interaction with RNA Polymerase II (Fig. 2c).

The three sisRNAs described above all act in cis to regulate the genes from which they are derived from. *sisR-1* regulates *rga* by repressing its cis-NAT, *ASTR*; the intronic switch RNA regulates its IgH gene locus by targeting AID to the S region of the locus, while *ci-ankrd52* regulates its host gene *ankrd52* by localizing and recruiting RNA Polymerase II to its sites of transcription. An interesting theme emerges whereby introns are utilized to self-regulate their parental gene. Upon transcription and splicing, two species of transcripts (mRNAs and introns) are generated, and in most cases the mRNAs are the major effectors by being translated into proteins. As introns are

being produced at equal molar ratio as the exons in a primary transcript, spliced introns are therefore a direct readout of the transcription rate of a particular gene. Thus, cellular mechanisms that utilize or monitor introns to modulate feedback loops may allow for a more direct and efficient way to regulate the expression of a particular gene. On the other hand, sisRNAs may also act in trans to modulate the expression of other gene loci in the nucleus.

## sisRNAs as molecular sinks or sponges

Deep sequencing analysis of rRNA-depleted non-polyadenylated RNA fraction from HeLa and H9 cells revealed a class of sisRNAs with ends corresponding to regions mapping back to snoRNAs, which Ling-Ling Chen and Gordon Carmichael's laboratories termed snoRNA-related long ncRNA (sno-lncRNA) [44]. In H9 cells, the most abundant sno-lncRNAs expressed was observed to be from the imprinted 15q11–q13 region, a genomic region on chromosome 15 implicated in Prader–Willi syndrome (PWS). They further showed that this group of linear sisRNAs is produced via the same mechanism involving snoRNPs and they suggested that their stability is due to the snoRNP components that remain associated to them. sno-lncRNAs localize to the nucleus, however, they do not accumulate in nuclear bodies associated with snoRNAs (nucleolus and CB), suggesting that these transcripts are functionally different from snoRNAs. They further observed that these sisRNAs associate with an alternative splicing factor Fox2 in the nucleus. Conversely, they showed that Fox2 is also strongly enriched in nuclear regions containing these sisRNAs. When they knocked down these sisRNAs using phosphorothioate ASOs, only a slight change in global gene expression was observed. When they focused on genes that are known to be regulated

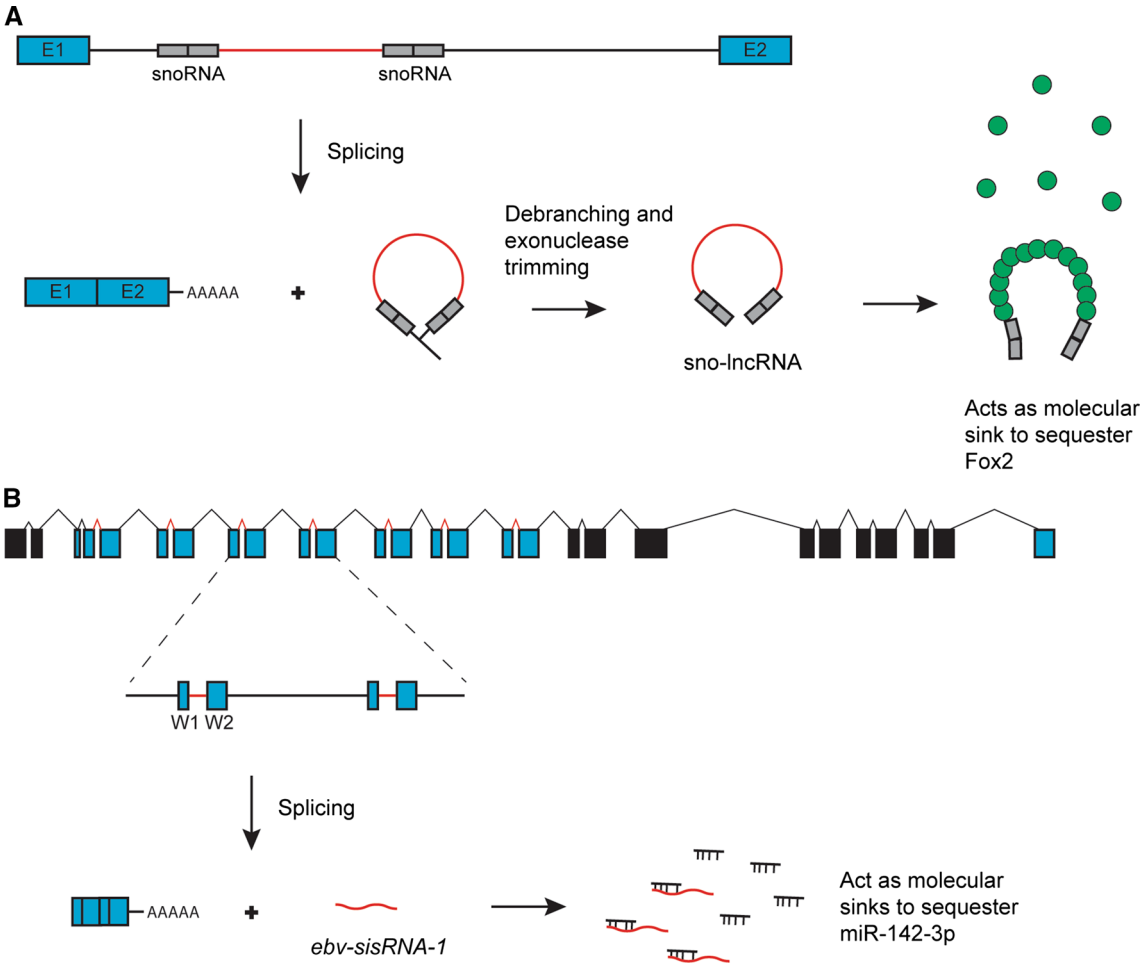
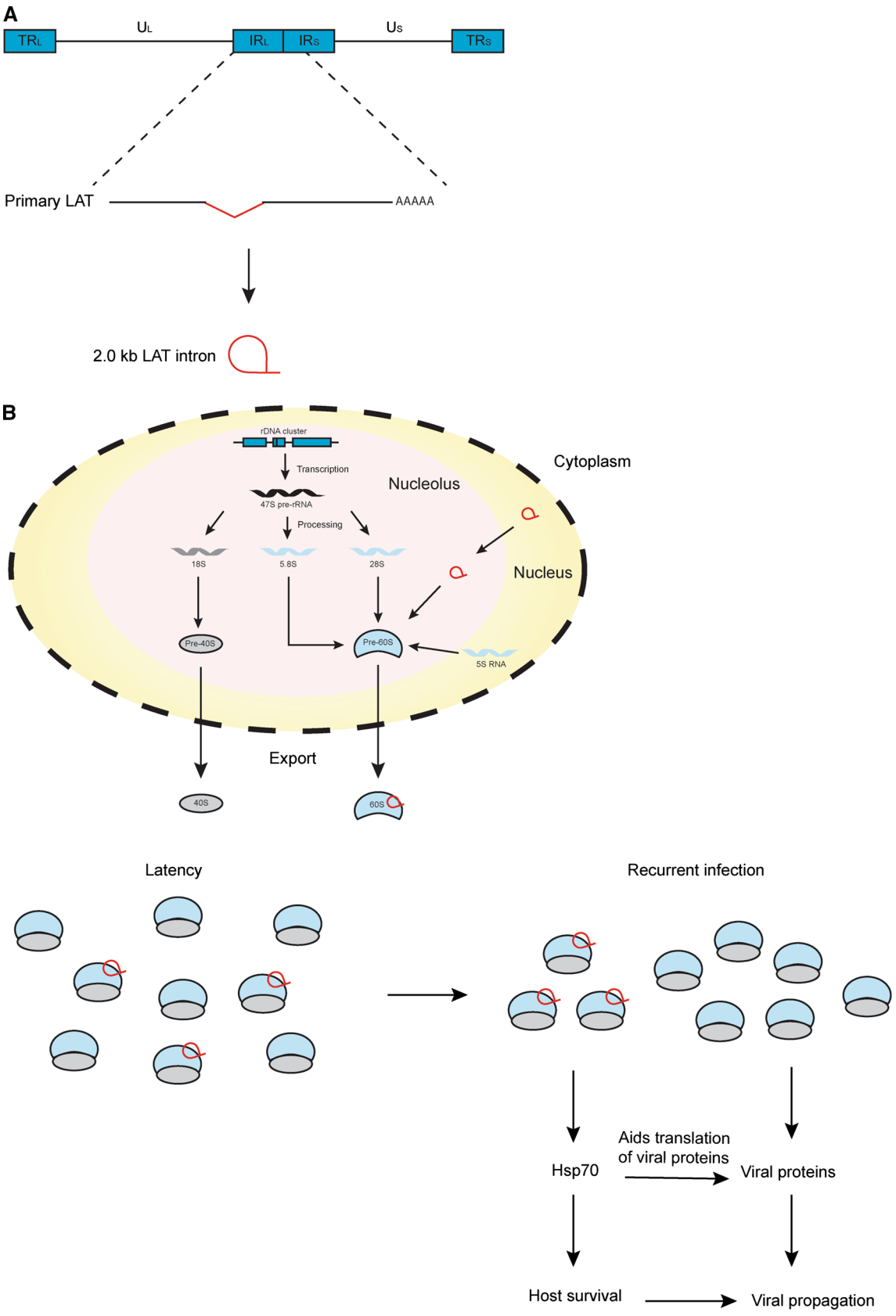



**Fig. 3** sisRNAs act as molecular sinks or sponges. **a** Model of sisRNAs from the PWS region, sno-lncRNAs, sequestering Fox2 proteins and altering Fox2-regulated splicing. **b** Model of a viral sisRNA, *ebv-sisRNA-1*, acting as a molecular sink against miR-142-3p, preventing the repression of the EBV lytic gene product


A
TRL
UL
IRL
IRS
US
TRS
Primary LAT
AAAAA
2.0 kb LAT intron
B
rDNA cluster
Transcription
47S pre-rRNA
Processing
18S
5.8S
28S
Nucleolus
Cytoplasm
Nucleus
Pre-40S
Pre-60S
5S RNA
Export
40S
60S
Latency
Recurrent infection
Aids translation
of viral proteins
Hsp70
Viral proteins
Host survival
Viral propagation

◀**Fig. 4** sisRNAs regulate protein translation. **a** Linear map of the HSV genome. The 2-kb LAT intron is processed from the primary LAT transcript which is transcribed from the internal repeat long ($IR_L$) and short ($IR_S$) regions on the genome. **b** Model for the regulation of translation of Hsp70 by the 2-kb LAT, ensuring the survival of the host cell during reactivation from latency. $U_L$ unique long, $U_S$ unique short, $TR_L$ terminal repeat long, $TR_S$ terminal repeat short

by Fox2, they observed a change in their splicing profile. They summarized and postulated that these sisRNAs, which originate from the PWS region act as molecular sinks to sequester Fox2, and as a result, alter Fox2-regulated splicing (Fig. 3a).

In 2013, the laboratory of Joan Steitz discovered a sisRNA, *ebv-sisRNA-1*, encoded by the Epstein–Barr virus (EBV) [45]. EBV is a human herpes virus, with a linear double stranded DNA genome. EBV has been implicated in several human cancers and autoimmune diseases. Its viral life cycle is divided into a lytic and a latent phase. During initial infection, EBV goes through a lytic replication phase upon entry into host epithelial cells, after which it migrates to the B cells. The virus remains in B cells, cycling through periods of latency and lytic reactivation [46]. During latency, the virus expresses genes that allow infected B cells to escape detection by the host immune system [47]. *ebv-sisRNA-1* was discovered through a small RNA-sequencing analysis of nuclear RNA extracted from human B cells, which have been stably infected with EBV, and are in the latency III program of gene expression. *ebv-sisRNA*-1 is a linear nuclear-enriched sisRNA derived from a small intron in the W repeat region. Using the program RNAduplex, it was predicted that *ebv-sisRNA-1* was able to form stable hybrids with human miRNAs, of which 4 of these miRNAs have been known to be expressed in B cells [48]. Most of the base pairing between the four miRNAs and *ebv-sisRNA-1* are $\sim$100 % conserved. One of this miRNA, miR-142-3p, is known to target and repress an EBV lytic gene product [49]. It is possible that *ebv-sisRNA-1* may affect the levels or activity of miR-142-3p via one of the following mechanisms. Its base pairing with perfect seed complementarity to miR-142-3p can lead to the subsequent degradation of the miRNA. Alternatively, it may act as a molecular sink, sequestering miR-142-3p and preventing it from repressing the EBV lytic gene product (Fig. 3b). It is also possible that the interaction between *ebv-sisRNA*-1 and the other three miRNAs might be achieved through similar mechanisms for the same purposes. Thus, EBV can employ one of these strategies to evade detection by host immune cells.

Here we show two sisRNAs that can act as molecular sinks or sponges to sequester their targets and prevent them from carrying out their biological functions. sno-lncRNAs from the PWS region are sisRNAs that sequester Fox2 proteins and thus alter Fox2 mediated splicing; while the *ebv-sisRNA-1* may sequester an miRNA, miR-142-3p, and prevent it from repressing the EBV lytic gene product. One example of a ncRNA that acts similarly to sno-lncRNAs from the PWS region is *MALAT1*, a lncRNA that has been shown to be implicated in human cancers [50]. *MALAT1* was shown to interact with the splicing factors called SR proteins, and it was suggested to be a protein sponge, regulating the cellular distribution of SR proteins in the nucleus, and as a result modulating alternative splicing of pre-mRNAs [51].

Several ncRNAs have also been proposed to behave as miRNA sponges, similar to *ebv-sisRNA-1* [52]. One such ncRNA is *PTENP1*, a pseudogene of the tumor suppressor PTEN. *PTENP1* was shown to regulate the expression of PTEN by competing for the same set of miRNAs that interact with PTEN. Thus, *PTENP1* was coined as a competing endogenous RNA or ceRNA [53, 54]. Other examples of miRNA sponges are *Herpesvirus saimiri* U RNA (HSUR) 1 and 2, which are ncRNAs encoded by the *Herpesvirus saimiri*, a virus under the same family as EBV [55]. These two ncRNAs were shown to share complementarity and coimmunoprecipitated with three miRNAs expressed in T cells. Interestingly, one of these miRNA is miR-142-3p, the same miRNA that is proposed to be sequestered by the *ebv-sisRNA-1*, suggesting a similar mechanism being deployed by these two viruses to evade detection by the immune system.

However, it is important to take into account the abundance of both the sisRNA/ncRNA and its target miRNA, as well as the tissues and cell types that they are expressed in, when assessing the sponge effect of sisRNAs/ncRNAs on miRNAs in vivo to ensure the biological significance of such an assessment [56, 57].

## sisRNAs regulate protein translation

The herpes simplex virus (HSV) encodes a sisRNA known as the 2-kb latency-associated transcript (LAT) intron. HSV is a herpes virus, and similar to EBV, it also establishes latent infections in its host. During latency, the virus resides in the trigeminal ganglia of the infected host. LATs are the only transcripts produced by the virus during latency, which are transcribed and spliced from a primary 8.3-kb transcript [58] (Fig. 4a). The 2-kb LAT intron is the most abundant LAT species produced during latency and was first discovered by Lawrence Feldman's laboratory in 1991 [59]. The intron was detected through Northern blot analysis of total RNA extracted from HeLa cells and was demonstrated to be a stable intron localizing to the nucleus. Several years later, John Taylor's group showed that this sisRNA exists as a lariat by studying the trigeminal ganglia

of latently infected mice and infected monkey CV-1 cells [60]. A year later, Nigel Fraser's laboratory revealed that the lariat branches at a Guanosine, which was suggested to account for the stability of the lariat by preventing it from being debranched [61].

LAT-null HSV mutants (deletion of the LAT promoter and the 5′ portion of the 2-kb LAT) did not show any other deficiencies apart from being unable to reactivate from latency [62]. It was demonstrated that the LAT can inhibit apoptosis and promote cell survival, possibly explaining the importance of LAT during reactivation [62, 63]. In 2006, Nigel Fraser's group showed that SY5Y human neuroblastoma cells transfected with the 2-kb LAT intron were protected from cold shock and this was due to an accumulation of Hsp70 [64]. The upregulation of Hsp70 during cold shock was not observed at the transcriptional level. Due to the sisRNA's association with ribosomal proteins, the authors postulated that the sisRNA upregulates Hsp70 expression at the translational level [65]. A year later, they showed that this sisRNA associates with the forming 60S ribosomal subunit in the nucleolus [66] They hypothesized that the 2-kb LAT intron possibly interferes with the processing of the 28S rRNA, thus altering the protein compostion of the 60S ribosomal subunit. Taken together, they proposed a model in which upon the processing of the sisRNA from its primary LAT transcript, it gets transported to the nucleolus, possibly in a manner similar to snoRNAs. There it associates with the 60S ribosomal subunits and together, they get exported to the cytoplasm. These altered 60S ribosomal subunits form a modified pool of ribosomes, which would then be utilized by the infected cell to make sure that there is a stable expression of Hsp70 (Fig. 4). This strategy is advantageous for the virus as it ensures the survival of the infected cells during times of stress when it reactivates from latency, as well as in aiding in the translation of viral proteins.

It is interesting to find out how these altered 60S ribosomal subunits and the resulting modified pool of ribosomes are able to specifically affect the translation of Hsp70, and if a similar mechanism exists in other organisms. Recently, it was discovered that there are ncRNAs that are able to bind to ribosomes and affect protein synthesis. In *Saccharomyces cerevisae* undergoing hyperosmotic stress, an 18-mer ncRNA originating from the *TRM10* mRNA was observed to associate with the 60S ribosomal subunit [67]. In the halophilic archaeon *Haloferax volcanii*, a 26-mer ncRNA originating from the 5′ ends of valine tRNA associates with the 30S small ribosomal subunit during environmental stress [68]. However, for both these ncRNAs, the resulting effect of their association with ribosomes is a reduction in the efficiency of global protein translation. These translational regulators were classified as ribosome-associated ncRNAs (rancRNAs) [69]. Although there is an obvious length difference between the 2-kb LAT intron and these two short rancRNAs, all three transcripts work similarly towards protecting the cell from stress and promoting cell survival.

## Future perspectives and challenges

It is still very early for the sisRNA field and we are just beginning to answer many questions regarding sisRNA biogenesis and functions in the cell.

- How are sisRNAs processed upon splicing from the pre-mRNAs?
- Do sisRNAs exert their functions on their own or through interactions with proteins?
- How do sisRNAs exert their functions? Do they have similar structural characteristics? Do they function by base-pairing with their substrates?
- For linear sisRNAs:
- How do they remain stable in the cell?
- Do they associate with proteins that protect them from degradation?
- For circular sisRNAs:
- What prevents them from being debranched and degraded?
- How are they transported out to the cytoplasm?

Although we classify sisRNAs as intronic RNAs that are spliced from its primary transcripts, we should not rule out the possibility that sisRNAs can be independently transcribed from its host gene and subsequently remain stable in the cell. If so, this could suggest that sisRNAs can be generated via two pathways: a splicing-dependent pathway and a splicing-independent pathway. This is another aspect of sisRNA biogenesis that should be addressed to better understand this novel class of ncRNA.

The advent of deep sequencing paved the way for the genome-wide analyses and discoveries of sisRNAs in model organisms such as *Xenopus* and *Drosophila*. However, sisRNAs represent a minor species of RNA in the cell and intronic reads are often regarded as noise or artefacts in RNA-sequencing data. By understanding the underlying chemistry of sisRNAs, it would allow for the global enrichment of sisRNAs prior to deep sequencing. For example, mRNAs can be enriched through poly(A) selection to specifically look at the transcriptome profile of a particular cell or tissue. Similarly, if sisRNAs have some unique characteristics or chemistry, this information could be used to allow for the enrichment and identification of many more undiscovered sisRNAs. This has been shown by the discovery of thousands of circular sisRNAs by RNase R treatment. In addition, if the proteins that

associate to the currently known sisRNAs were identified, these proteins could then be utilized to discover other sisRNAs by RNA immunoprecipitation. If such enrichment was made possible and a large number of sisRNAs were discovered, the next major challenge would be ascertaining the functions of these newly identified sisRNAs in the cell.

It has been shown previously that a majority of the ncRNAs present in mammalian cells originate from the intronic regions of the genome [70]. Several of these intronic transcripts are implicated in cancer [71–73]. Similarly, several cancer-associated susceptibility loci were also found in introns [74, 75]. Studying intronic transcripts such as sisRNAs may possibly provide us with mechanistic insights, which can further improve our understanding on the pathology of cancer.

## Concluding remarks

The discoveries of functionally significant intron-derived RNAs such as snoRNAs, scaRNAs and miRNAs have provided support that introns are more than just mere junk. Instead these ncRNAs play important roles in gene regulation. In this review, we have described another class of intron-derived ncRNAs known as sisRNAs. sisRNAs have been shown or suggested to play various roles in gene regulation. They can regulate their host's genes expression, function as molecular sinks or sponges, and regulate protein translation. Taken together, sisRNAs are a biologically active class of ncRNAs conferring an additional layer of gene regulation.

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