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Circular sisRNA identification and characterisation

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ABSTRACT

Stable Intronic Sequence RNA (sisRNA) is a relatively new class of non-coding RNA. Found in many organisms, these sisRNA produced from their host genes are generally involved in regulatory roles, controlling gene expression at multiple levels through active involvement in regulatory feedback loops. Large scale identification of sisRNA via genome-wide RNA sequencing has been difficult, largely in part due to its low abundance. Done on its own, RNA sequencing often yields a large mass of information that is ironically uninformative; the potential sisRNA reads being masked by other highly abundant RNA species like ribosomal RNA and messenger RNA. In this review, we present a practical workflow for the enrichment of circular sisRNA through the use of transcriptionally quiescent systems, rRNA-depletion, and RNase R treatment prior to deep sequencing. This workflow allows circular sisRNA to be reliably detected. We also present various methods to experimentally validate the circularity and stability of the circular sisRNA identified, as well as a few methods for further functional characterisation.

1. Introduction

The traditional view of RNA as dominantly linear molecules is a thing of the past. The increasing number of studies involving the discovery and biological significance of novel circular RNA (circRNA) has added a new dimension to how we view RNA. 1976 saw the first circRNA ever reported; a pathogenic plant viroid was discovered as being a single-stranded RNA molecule covalently closed in a circle [1]. circRNA was then later found to exist in mammals as well, where the *Sry* RNA molecule was found and experimentally verified in mice testes in 1993 [2]. CircRNA have now been found extensively in a variety of organisms, ranging from unicellular eukaryotes to plants and humans [3,4], most of which are listed in a variety of publicly available databases [5]. As a new player in the field of non-coding RNA (ncRNA), circRNA have been found in recent years to perform various regulatory functions, including, but not limited to, regulating transcription and splicing, acting as microRNA (miRNA) sponges, and stabilising messenger RNA (mRNA) [6]. Although circRNA are generally considered to be a class of ncRNA, it is of interest to note that some have actually been found to produce small peptides via IRES elements [7–9]. Expansion into the biomedical field have found circRNA to be increasingly implicated in various pathologies like muscular dystrophy [7], cardiomyopathy [10], cancer [11–13], and in the regulation of innate immunity and autoimmune diseases [14–17]; it is no wonder interest in the field is mounting as it opens up the possibility of circRNA as a new form of disease biomarker [18,19].

CircRNA are molecules with a covalently closed loop or circular structure. Unlike their linear counterparts, circRNA have no exposed ends and therefore no 5′-3′ polarity, nor their associated 5′ caps and 3′ polyadenylated tails. Depending on the method of biogenesis, circRNA can be composed entirely of either exonic or intronic sequences, or a combination of both. The most straightforward pathway in producing a circRNA is through canonical splicing, where the spliced-out intron is removed in the form of a lariat and trimmed at the 3′ end to form a fully circular RNA molecule. Other possible biogenesis pathways proposed are through a mechanism called back-splicing; exon skipping which produces exonic circRNA [20], and direct back-splicing [21] which relies on intronic complementary sequences (ICSs). In addition, RNA-binding proteins (RBPs) like the RNA-editing enzyme ADAR [22], the alternative splicing factor Quaking, and various serine-arginine proteins help regulate circRNA biogenesis [6,23–25]. For the purposes of this review, we are solely interested in the subset of circRNA which contain intronic sequences, also known as circular stable intronic sequence RNA (circular sisRNA).

SisRNA is a fairly new class of ncRNA that, as its name suggests, contain intronic sequences that until recently, many thought to be genomic junk. Formally characterised first in *Xenopus tropicalis* [26], sisRNA are defined as having intronic sequences that originate from the coding strands of their host genes, and are stable RNA that may contain

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exonic sequences, 5′ caps, and 3′ poly(A) tails [27]. Found in organisms ranging from viruses, to eukaryotes from yeast to humans [27], sisRNA are reported to play a variety of molecular functions in regulating cellular processes such as acting as protein or miRNA decoys [28–30] or in regulating expression feedback loops [31–34]. Importantly, they also function in regulating germline stem cell maintenance and primordial germ cell homeostasis [30,33,35,36].

SisRNA can also exist in the circular form as well. Lariat sisRNA have been detected in organisms such as frogs and in other vertebrates like humans, chickens and mice. Specific examples include *sisR-4* in *Drosophila* [32], *ci-ankrd52* [37], circPAIP2 & circEIF3J in humans [38], *lariat41 & 42* in *Arabidopsis* [29], and sisR-*XPO7* and sisR-*tnikb* in chickens and frogs respectively [39]. Similar to what is described above, circular sisRNA can be generated from multiple pathways through different splicing mechanisms such as canonical splicing [31] and back-splicing [38] (Table 1). In addition to splicing-dependent mechanisms, multiple sisRNA have been found to be generated independently of splicing through transcription from the cognate intronic promoter [40]. Interestingly, there has also been reports of circular sisRNA being generated with the use of the tRNA splicing machinery; tRNA introns spliced out are directly ligated to form tRNA intronic circular RNA (tricRNA) [41]. Depending on the mechanism, the resulting sisRNA is either produced as a lariat or circular molecule, the former undergoing 3′ trimming to fully circularise at its 2′-5′ phosphodiester bond branch point. Be it lariat or circular, the detection method described here for circular sisRNA does not differ; both are enriched through the proposed RNase R-centric workflow to be described in the coming sections.

RNase R is currently viewed as the best 3′ to 5′ exoribonuclease for the methodical digestion of eukaryotic linear RNA [42,43]. First identified and characterised in *Escherichia coli* [44], RNase R is a very efficient processive 3′ to 5′ exoribonuclease that degrades linear RNA species such as mRNA and ribosomal RNA (rRNA). It is found to be effective even on RNA with secondary structures [45] due to its dual RNase and Helicase activity [46]. Circular RNA are resistant to degradation by RNase R owing to their lack of free ends, providing us with a method to segregate these RNA species from the total RNA fraction.

In this review, we first introduce an experimental workflow emphasizing the use of RNase R treatment in identifying circular sisRNA in *Drosophila melanogaster* via deep sequencing – from library preparation to data analysis (Fig. 1). Next, we suggest various methods that can be used to validate of the ncRNA of interest, both its stability and circular nature. Finally, we will go into some useful experiments that can be used to perform functional characterisation of the sisRNA, including cellular localisation using biochemical techniques, knockdown and overexpression strategies.

Table 1
List of circular sisRNA and their biogenesis pathways.

Organism/ Source	Name	Type of sisRNA	Biogenesis	Ref
Arabidopsis thaliana	*lariat41*	Intronic lariat	Canonical splicing	[29]
	lariat42	Intronic lariat		
Drosophila melanogaster	*sisR-4*	Intronic lariat	Canonical splicing	[32]
	tric31905	tricRNA	tRNA splicing	[68]
Homo sapiens	*ci-ankrd52*	Intronic circular	Canonical splicing	[37]
	circEIF3J	Exon-Intronic circRNA	Back-splicing	[38]
	circPAIP2	Exon-Intronic circRNA	Back-splicing	
	DDX5 intron circle	Intronic circular	Recircularization from canonical splicing	[75]

2. Library construction

2.1. Obtaining the RNA

As the name implies, a defining feature of sisRNA is its stability. Short of assaying for the stability of each and every intron with reads in the deep sequencing results from total RNA, the starting material used should be able to filter or differentiate between transient and unstable species like pre-mRNA or splicing intronic by-products, and stable RNA such as mRNA or various other ncRNA species.

During canonical splicing, spliced introns are rapidly degraded in a matter of seconds [47] while still in the nucleus. The timeframe of this rapid degradation process provides the basis for the idea of RNA-storing systems being ideal for the mining of sisRNA. This concept is well defined in the first formal characterisation of sisRNA in the Germinal Vesicles (GV) of *Xenopus tropicalis* mature oocytes, where the pool of maternally deposited RNA is implied to be extremely stable all through fertilisation and up to the blastula stage of embryogenesis when zygotic transcription finally initiates [26]. Naturally occurring transcriptionally quiescent systems like the nucleus-less mature red blood cells (RBCs) is also another possibility in the context of mammalian systems [39]. The lack of contamination from nascent transcripts and the second-to-minute lifespans of spliced introns make it so that any intronic sequence detected in these systems can be regarded as stable.

Drosophila, similar to *X. tropicalis*, provides a system containing mature and stable RNA without any contamination from nascent transcripts. The nucleus of mature *Drosophila* unfertilised eggs and 0–2-hour embryos is transcriptionally quiescent, and all RNA species detected originate from the maternal nurse cells in a process known as “dumping” prior to oocyte maturation. Essentially, the RNA detected in these systems have been stable throughout oocyte fertilisation, and up to 2 h post-fertilisation when zygotic transcription initiates.

RNA integrity is central to the entire workflow presented here. It is crucial to ensure that all reagents and equipment used are free of any RNase. Any RNA degradation has the potential to skew the final results, which compounds after every step in the workflow.

- Degraded RNA samples will not have its rRNA pool depleted efficiently. Only the rRNA fragments able to hybridise to the rRNA probes are depleted; other rRNA fragments will remain in the depleted sample
- Any endoribonuclease contamination will linearise circular RNA, resulting in their degradation during the subsequent RNase R treatment
- Larger circular RNA molecules will be more susceptible to endonuclease cleavage and physical sheer stress; this results in a bias towards detecting smaller circular RNA

2.1.1. Unfertilised Egg/0-2hr embryo collection

For the collection of 0-2hr embryos, first feed female flies with wet yeast paste for 1–2 days to stimulate oogenesis. Mate the fed females with males at 25 °C in bottles capped with an inverted apple juice agar plate with more wet yeast paste spread in a ridged fashion atop its surface for egg laying. Keep the flies in the dark overnight as it encourages egg laying. Female virgins were used instead with no mating for the collection of unfertilised eggs. The eggs laid on the apple juice agar will need to be washed down of any residual yeast paste and agarose with distilled water, and air dried for subsequent RNA extraction. Ensure that all impurities are removed to preserve RNA quality.

The recipe for the apple juice agar plate is as follows:

- Water 225 ml
- Sucrose 16.5 g
- Agarose 10.5 g
- Apple Juice 75 ml

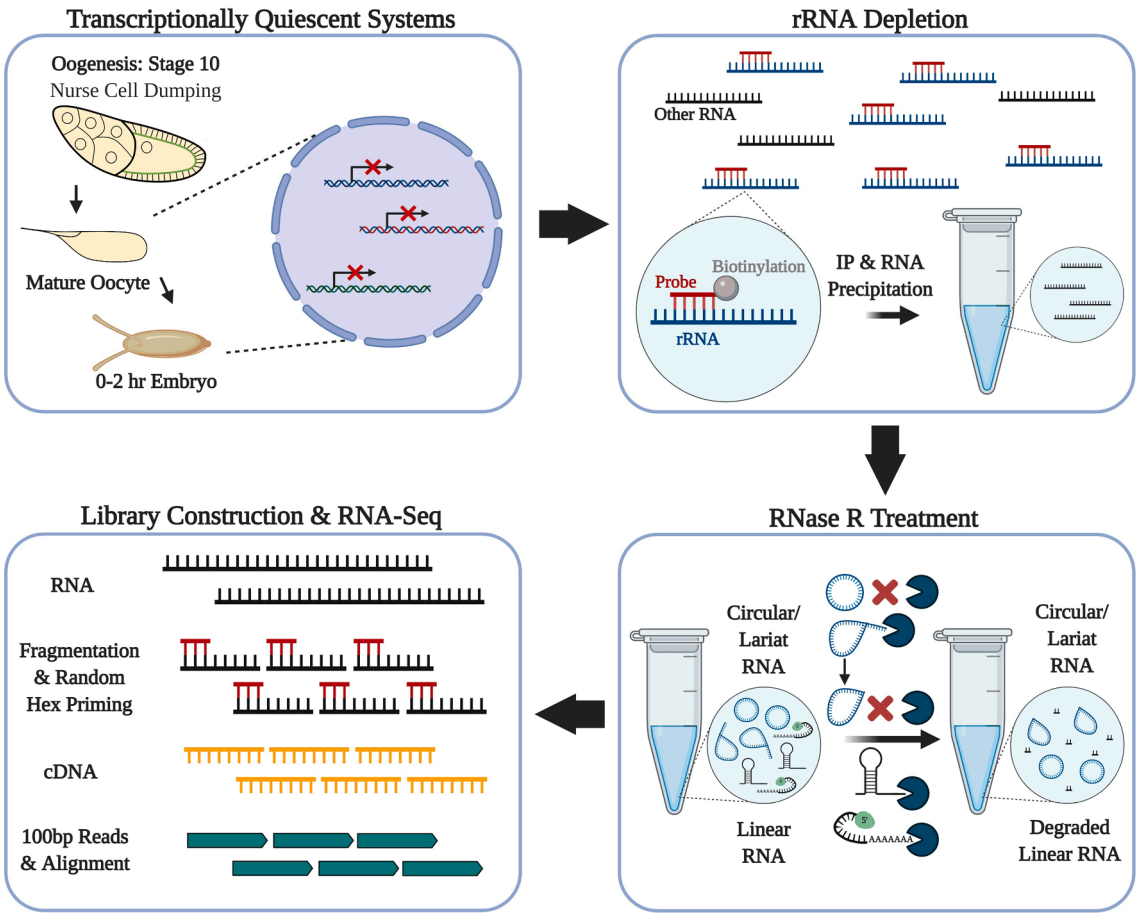


Fig. 1. A schematic overview of the RNase R-centric workflow for reliably detecting circular sisRNA. Starting from isolating RNA from a transcriptionally quiescent system, rRNA depletion followed by RNase R treatment are performed to enrich for circular sisRNA in library construction before deep sequencing is done.

- 100% Ethanol 7.5 ml
- 100% Acetic Acid 3.75 ml

Fully dissolve the sucrose and agarose by heating before adding the ethanol and acetic acid. The apple juice used can be obtained from any generic brand.

2.1.2. RNA extraction

Homogenise the collected eggs or embryos with a plastic pestle in 1.5 ml Eppendorf tubes in TRIzol (Ambion; Thermo Fisher Scientific). RNA purification can be performed as per the protocol from the Direct-zol RNA mini-prep kit (Zymo Research); elute with sterile RNase-free water at the end. We recommend checking the integrity of the total RNA sample by visualising on 1–2% agarose gel before proceeding on to the next steps.

2.2. rRNA depletion & clean-up

It is important to be conscious of the composition of various RNA species in an RNA sample. The composition of total RNA fractions is dominated by the extremely abundant rRNA species, making up at least 90% of the total RNA volume. Compounded with sisRNA being a relatively low abundant RNA species [26,31], it is then imperative that any undesired 'noise' be removed from the RNA sample for the deep sequencing library construction to ensure an efficient read of the target RNA subset.

We recommend a starting material of ~5 μg total RNA, quantified with the Nanodrop 2000 spectrophotometer, to begin with for rRNA depletion. Our original protocol featured the use of the now discontinued Ribo-Zero magnetic kit (Epicentre) which uses the hybridisation and capture method. In place of that, we recommend the use of protocols that use biotinylated antisense probes complementary to the major rRNA species for rRNA depletion. These probes can be hybridised to rRNA via complementary base pairing to produce RNA: DNA hybrids that are then removed via pull down using streptavidin beads [48]. A newly developed rRNA depletion workflow is known as Ribo-Pop (Ribosomal RNA depletion for popular use), where contrary to the lowered specificity of commercially available kits, the probes used in this workflow are tailor made to each specific organism [49]. Using a pool of 15 antisense probes, the developers have already created specific sets of probes for several organisms including *Drosophila*. A computational pipeline for probe sequence selection is also available for those with an interest in other organisms instead. Finally, we suggest isopropanol precipitation as the preferred purification/clean-up method, which is known for optimally retaining small RNA species for recovery among which some sisRNA may be classed under. RNA precipitation is performed as follows:

3 M Sodium Acetate and the nucleic acid co-precipitant Linear Acrylamide (5 mg/ml; Ambion) are added at 10 μl and 2 μl respectively to 100 μl of RNA. Finally, 100 μl of 100% Isopropanol is added to the mix in a 1:1 ratio of the total volume, followed by incubation at −20 °C for 30 min or longer. Centrifuge samples at max speed (~14,000g) for another 30 min at 4 °C. Discard the supernatant, taking care to not dislodge the very small RNA pellet. Wash the pellet with ice cold 70% ethanol and centrifuge for 10 min at 4 °C. Discard the supernatant and air dry the pellet, making sure to remove all traces of residual ethanol. Resuspend in ~8 μl of RNase-free sterile water for subsequent RNase R treatment.

Further fluorometric characterisation of the depleted sample is also strongly encouraged with a Bioanalyzer 2100 (Agilent Technologies) using the Pico chip, to ensure efficient rRNA depletion and optimal sample quality for subsequent steps. Examples of the expected electropherograms are provided, showing how a sample should look like before (Fig. 2A) and after (Fig. 2B) depletion, as well as an example showing degradation in a sample (Fig. 2C).

2.3. RNase R treatment

The prior use of a transcriptionally quiescent system eliminates most unstable RNA species from our sample pool, and subsequently performing rRNA depletion then leaves behind only the stable RNA species such as mRNA and various ncRNA. The final step to enrich for circular sisRNA would now be to exclude all linear RNA from the sample through RNase R treatment.

Some may question why RNase R treatment is not used instead of, or prior to rRNA depletion treatment since rRNA is also a linear RNA species, and RNase R treatment therefore can in principle also help to deplete the sample of rRNA. This is due to the sheer abundance of rRNA in any given total RNA sample; a large amount of enzyme needs to be used, and even then, the risk of an inefficient reaction is high due to the over saturation of enzyme activity. Furthermore, the efficiency of RNase R under normal conditions is estimated to sit at around 80% in standard protocols, leaving more than 20% of highly expressed linear RNAs undigested [43], further illustrating the need for rRNA depletion before attempting RNase R treatment.

In addition, if one wishes to increase the enrichment for circular sisRNA, an optional step before RNase R treatment can be added to address the issue of the 20% undigested RNase R resistant linear RNA which generally consist of RNA with highly structured 3′ ends and GC rich regions. The addition of this step before RNase R treatment is also especially useful in preventing false positives when verifying the circularity of individual candidates. A tested method for efficient degradation of these RNA involves poly(A) tailing at the 3′ end as an optional step to increase the efficiency of the subsequent RNase R treatment using a buffer containing LiCl. The addition of the 3′ poly(A) tail creates access for the RNase R which requires a 3′ overhang to begin its degradation process, and Li+ in the buffer destabilises the secondary structure formed at GC-rich regions, preventing RNase R from stalling [43].

The RNase R protocol is as follows:

Add 10× RNase R reaction buffer (Epicentre) to the previously rRNA-depleted sample to a total volume 10 μl. Incubate the samples at 65 °C for 5 min to denature the RNA. Cool the samples on ice for 2 min before adding the following and incubating at 37 °C overnight:

- 1 μl RNasin (40 U/μl; Promega)
- RNase R + only; 1 μl RNase R (20 U/μl; Epicentre)
- RNase R- only; 1 μl RNase-free sterile water

Perform RNA precipitation as described above to purify the samples. Dissolve the pellet in 15 μl of RNase-free sterile water.

Although our workflow emphasizes the use of a transcriptionally

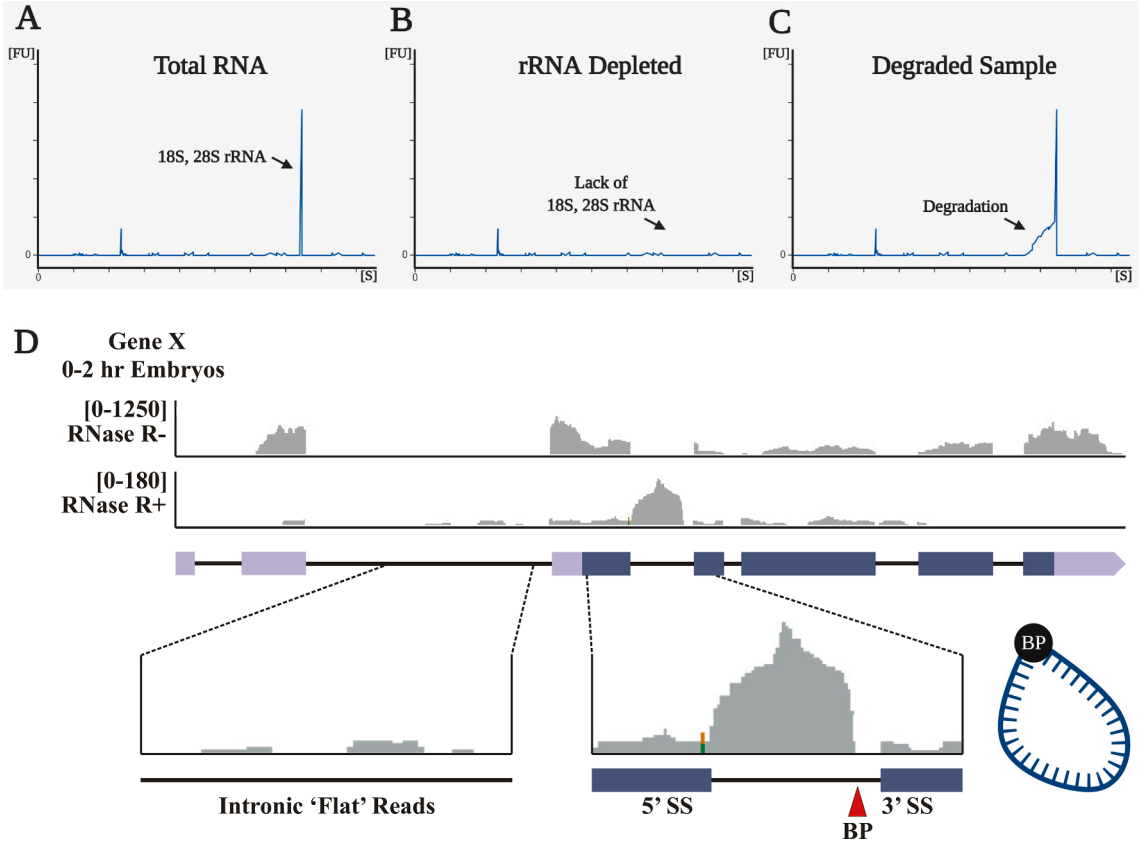


Fig. 2. Result interpretation within the workflow. Examples of representative electropherograms from the fluorometric characterisation of (A) total RNA fractions, (B) rRNA-depleted samples, and (C) degraded RNA samples. Regions of interest on the electropherograms are as indicated. (D) Deep sequencing results of gene X from 0 to 2 hr embryos as presented on the IGV. The light blue boxes represent the UTR regions, while the dark blue boxes represent exons. The introns are indicated by the black lines interspersed between them. The region of interest in the RNase R-treated sample (bottom-right panel) containing the definable peak indicating the candidate sisRNA is highlighted with the branchpoint (BP) and 3′ and 5′ splice sites marked. In contrast, a region of 'flat' intronic reads is also highlighted as an example of intronic reads not from a circular sisRNA (bottom-left panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quiescent system for the isolation of circular sisRNA, work has been ongoing in the effort to mine for such sisRNA in transcriptionally active systems instead. The transcriptionally quiescent systems described above occur under very specific conditions in nature; naturally, expression biases from these specialised systems like the GV of *X. tropicalis* and RBCs will occur and be quite apparent. As sisRNA is increasingly studied in-depth, looking for sisRNA function in general will very likely call for identifying them in more common somatic settings, and in so, RNase R treatment in itself will be key for identifying circular sisRNA in these systems. However, with sisRNA having the nature it does, only the circular – not linear – forms of sisRNA in both the cytoplasm and nucleus can be reliably detected using RNase R treatment.

3. Deep sequencing data acquisition & analysis

3.1. RNA-Seq data acquisition

The generation of the cDNA library is done by following the protocol provided in the Illumina TruSeq RNA sample preparation guide; RNA fragmentation is followed by random hexamer priming for the reverse transcription to create cDNA fragments ~200–500 nt in length. 100-bp single-end sequencing is our method of choice using the Illumina HiSeq 2000 sequencer. The obtained reads are then aligned to the *D. melanogaster* genome using TopHat and Bowtie sequence alignment programs [50,51].

3.2. Post-processing & analysis

Sequence alignment visualisation of the RNase R-treated sample is done using the Integrative Genomics Viewer (IGV) [52], from which we manually screen for potential circular sisRNA that present as definable peaks in the IGV, not as a region of flat read counts (Fig. 2D). The difference between the peak-like appearance of a circular or lariat-forming region and the flat appearance of an intron-retained region or a pre-mRNA read can be attributed to two reasons. The first involves the stoichiometry between multiple introns from the same host gene. Intronic reads from pre-mRNA generally have all their introns present in a 1:1 ratio since a pre-mRNA can only produce one of each intron per transcript. A discernible peak implies an increase in the ratio, indicating that particular intron is present in higher abundance than its counterparts, suggesting a more stable state. The second reason relates to specific characteristics of how sequencing reads are aligned at the middle versus the end of an RNA molecule. The apex of the peak typically indicates the middle of the RNA molecule where reads are easily aligned. The declining ends indicate the ends or the circular junction/lariat branch as read counts begin to drop off due to limits in read alignments. Finally, lariat and circular RNA can generally be distinguished through lariat sisRNA having a further characteristic of having the 5′ end of the peak map near the 5′ splice site and the 3′ end map near the branch point of the lariat (Fig. 2D) [32].

As just mentioned, we do not utilise any bioinformatic tools to pick out our circular sisRNA, opting instead for a manual screen. However, should one require automated screening because of large datasets for instance, we can suggest targeting the inverted reads that are indicative of the circular junction/ lariat branch point like Talhouarne and Gall have done [53]. Alternatively, a computational system called CIRCexplorer2 developed by Chen's group has been shown to effectively pick out circRNAs from genome-wide screens, specifically identifying reads that map to the back-spliced junction (BSJ) sites that are unique to circRNA [54]. However, it should be noted that methods using inverted reads to identify circRNA can potentially lead to the underestimation of lariat-derived circRNAs due to enzyme stalling at lariat branch points during reverse transcription (elaborated in Section 4.1).

4. Validation/Supporting methods

Results from the above give putative circular sisRNA. Both the transcriptionally quiescent system and RNase R treatment provide reasonable evidence for the stability and circular nature of the identified sisRNA, but validation is still strongly recommended. In this section, we detail a few possible experiments to confirm the high stability and circular nature of the sisRNA detected. These experiments will also be of particular use in validating sisRNA from a cDNA library built from transcriptionally active systems.

4.1. Rt-PCR

Although the use of RNase R should result in the degradation of the linear pool of RNA, there are linear exceptions due to the efficiency of the enzyme, and specific substrate incompatibility such as RNA that form double-stranded RNA with extremely short 3′ overhangs [55]. In order to validate the candidate's circularity, the presence of the sisRNA needs to be detected using a feature that is unique to its circular form. One such way is the use of outward pointing primers that target the branch point or circular junction region (Fig. 3A) by Reverse Transcription PCR (RT-PCR).

RNA isolated can be reverse transcribed using a reverse transcriptase primed with random hexamers. However, it should be noted that studies have found that most reverse transcriptases are prone to impediment by the 2′-5′ phosphodiester linkage of a branch point [56,57] which is more of a problem for circRNA with a lariat origin. The Superscript II (Invitrogen) or Episcript (Epicentre) are thus recommended since these enzymes are less likely to stall at the branch point of lariat sisRNA [53]. The expected results visualised on an agarose gel should show a band detected at the appropriate size, with no corresponding band in the genomic DNA control due to the outward orientation of the primers (Fig. 3B).

4.2. Point of circularity

Building on the validation of the circularity of the sisRNA as described above, the same outward pointing primers used for the RT-PCR can be used to amplify the branch point or circular junction region (Fig. 3C) for Sanger sequencing. The characterisation of this region provides concrete evidence of a circular molecule. Due to the circularisation, this highlighted region does not align wholesale to the host genome in the 5′ to 3′ orientation. As illustrated by Fig. 3C, the highlighted region cannot align directly to the host genome past the branchpoint as the downstream sequence actually originates from an upstream genomic region. This will indicate where the circularisation occurs exactly.

In addition to confirming the circular nature of the sisRNA, the region being unique to the sisRNA will be an important characteristic that can be utilised in subsequent experiments where sisRNA-specific detection is needed. This is especially so when we consider the origin of sisRNA; their biogenesis from the intronic regions of the coding strand of their host gene makes it easy to erroneously detect pre-mRNA instead of just the sisRNA specifically. To this end, detection probes for various experiments such as Fluorescence *in situ* Hybridisation (FISH) or northern blots are recommended to be designed within this region spanning across the branch point or circular junction to reliably detect for the sisRNA specifically.

Although it should be noted that while theoretically possible, detecting sisRNA in the *Drosophila* system is not without its difficulties in the context of the aforementioned techniques. For instance, sisRNA is a low abundance species, which makes the traditional FISH techniques unsuitable for reliably picking out sisRNA. Newer, more costlier developments like single molecule FISH (smFISH) techniques that use signal amplification such as RNAscope (ACDBio) would be more appropriate. Furthermore, *Drosophila* researchers typically use whole

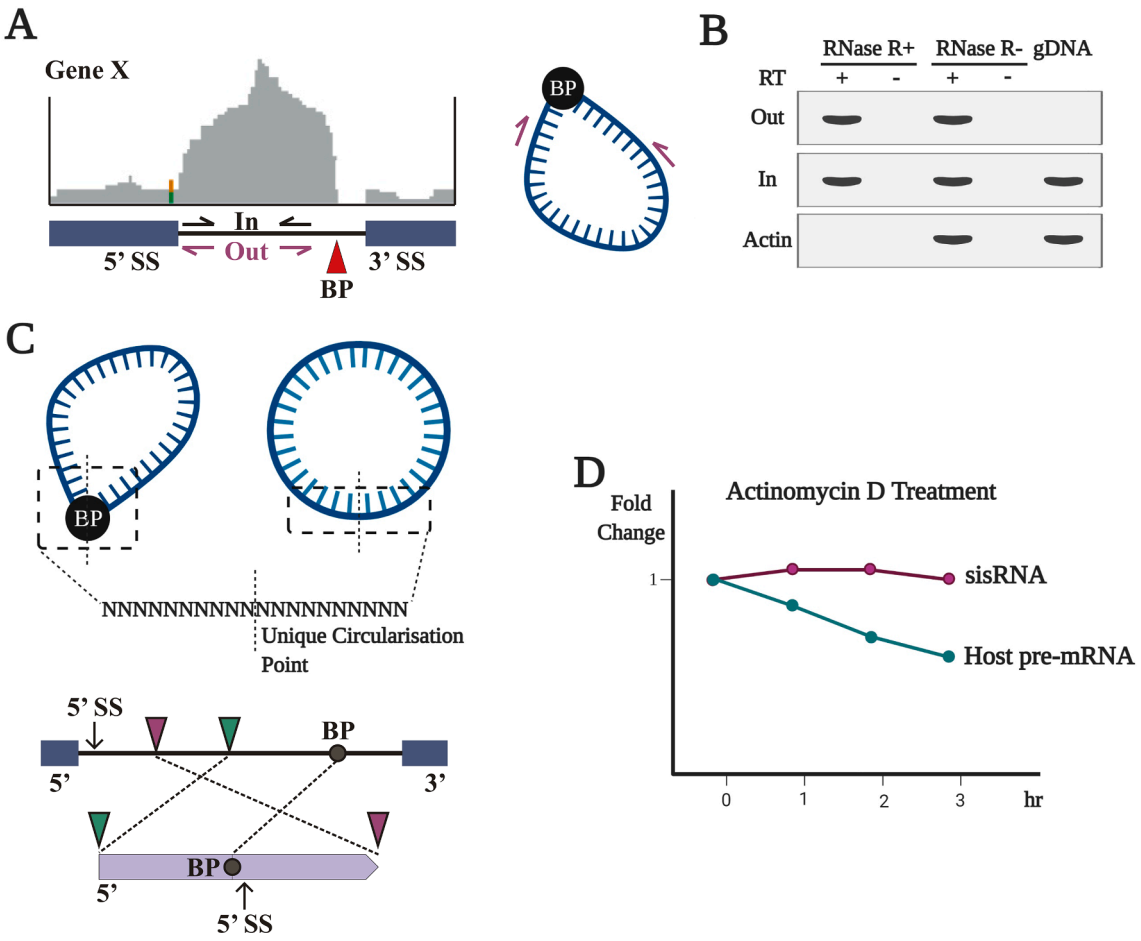


Fig. 3. Validation of the candidate circular sisRNA. Confirming circularity through RT-PCR. (A) A visual schematic of the inward and outward primers used in the subsequent RT-PCR after RNase R treatment. (B) Representative predicted results visualised on a 1–2% agarose gel with genomic DNA and primers against *actin5C* as the negative controls for RNase R treatment. (C) Confirming circularity by cloning and sequencing the branch point and circularisation junction. Bottom panel illustrates how the boxed region does not align directly to the host genome. (D) Actinomycin D-mediated transcription inhibition assay for the candidate sisRNA and host pre-mRNA, presented as *actin*-normalised fold change over time measured by qPCR.

mount tissue as imaging samples, making the sample preparation process for FISH or other techniques that much more complicated.

4.3. Stability

The use of transcriptionally quiescent systems will—in principle—remove unstable RNA species from the pool detected during deep sequencing. However, as in with most systems in nature, there are exceptions to the rule. Although circRNA are generally more stable than other RNA species by virtue of their molecular structure, there are instances where the circularity of certain circRNA are merely transient states, and can still be detected via RNase R treatment despite being so. This is best illustrated by reports that use RNase R treatment as a method to enrich for intron lariats generated as by-products of canonical splicing to study global splicing profiling [58]. Performing a transcription inhibition assay with actinomycin D is therefore encouraged to confirm the stability of the putative sisRNA relative to unstable pre-mRNA (Fig. 3D).

Actinomycin D treatment is performed as follows:

Tissues like *Drosophila* ovaries are incubated for 30 min with 20 μg/ml actinomycin D in Grace's medium with constant rocking at room temperature. 30 min is enough for transcription to cease, but additional lead time is required for the depletion of all existing nascent transcripts. We recommend a duration of no more than 3 hrs for tissue systems owing to tissue death, and a sampling of RNA to be taken at 1 hr intervals for the checking of transcript levels via qPCR.

Actinomycin D—or its alternative α-amanitin—can also be used in place of transcriptionally quiescent systems for deep sequencing library generation. As previously mentioned, studying sisRNA in typical somatic cells could provide a more comprehensive picture than merely being restricted within possibly biased specialised transcriptionally quiescent systems. Actinomycin D treatment with lead time to deplete nascent transcripts is required to increase the confidence of sisRNA being identified; no more than 3 hrs for tissues and 24–36 hrs for those utilising cell cultures. This also subverts the issue of only being able to detect circular sisRNA by relying on just RNase R treatment to deplete all linear RNA – including the unstable species – in transcriptionally active systems.

We also recommend supplementing these experiments with 5-ethynyluridine labelling before proceeding with deep sequencing, especially when performing the transcription inhibition assay on a particular system for the first time. This provides confirmation that the system indeed stops transcription within a set period after actinomycin D treatment; detection using a fluorescent dye like Alexa Fluor 488 would indicate if transcription is still active or not, depending on whether the 5-ethynyluridine is incorporated into nascent transcripts as indicated by its fluorescent signal.

5. Functional analysis

After validating the potential circular sisRNA in terms of its stability and circularity, further experiments can be carried out to characterise the identified sisRNA in greater detail and depth. In this section, we

detail a few techniques for determining the subcellular localisation of sisRNA, as well as genetic tools specific to *Drosophila* that can serve to knockdown or overexpress circular sisRNA.

The advantages of using *Drosophila* as model to study sisRNA is not just restricted to the presence of a transcriptionally quiescent system that makes it easy to pick out stable RNA species. *Drosophila* has been an effective model system for genetics for more than a century. Its relatively short generation time together with its comprehensively sequenced and compact genome makes the *Drosophila* a great model to manipulate genetically. As such, a great deal of genetic tools have been developed, ranging from chromosome balancers, the GAL4-UAS expression system, and tools for RNAi and clonal analysis. A comprehensive review has also been published which will aid newcomers in fly work immensely [59].

5.1. Nuclear-cytoplasmic fractionation

Subcellular localisation often plays a part in determining a RNA's function or physical properties, owing to the particular subcellular compartment's characteristics. For intronic sequences in particular, there are degradation pathways in both the nuclear and cytoplasmic compartments that can target introns. The nucleus has the canonical degradation of spliced out intron lariats by the debranching enzyme Dbr that removes the 2′-5′ phosphosdiester branch point to linearise the lariat before further degradation, while the cytoplasm has the Nonsense Mediated Decay (NMD) pathway that specifically degrades transcripts with premature stop codons (PTCs). It is not too surprising then when it is found that most sisRNA localised in the cytoplasmic compartment are predominantly lariat or circular in structure in the Xenopus oocyte [53]. However, there are exceptions of course, and their nuclear localisation can be strongly attributed to their molecular function found in some circular sisRNA that enhance their host gene transcription either through enhancer activation [32] or interaction with RNA polymerase II [37,38]. Nuclear-cytoplasmic fractionation therefore can provide one with useful insights into the possible functions of sisRNA.

Drosophila embryos need to be dechlorinated in 50% bleach for 2 min and homogenized in cold lysis buffer (100 mM potassium acetate, 0.1% Triton X-100, 50 mM Hepes, pH 7.4, 2 mM magnesium acetate, 10% glycerol, 1 mM DTT, and 1X complete mini EDTA-free protease inhibit cocktail, Roche). The lysate then is centrifuged at 1300*g* for 10 min at 4 °C, keeping the supernatant as the cytoplasmic fraction (Fig. 4A). Homogenise the remaining crude nuclear pellet again in cold lysis buffer and centrifuge once more at 1300*g* for 10 min at 4 °C. The nuclear pellet will now be kept as the nuclear fraction. RNA from both fractions can then be extracted and dissolved in equal volumes of water, using equal volumes of each for subsequent RT-PCR experiments for sisRNA detection using the specific outward pointing primers described in section 4.1. The agarose gel in Fig. 4A shows an example of a nuclear circular sisRNA, with rRNA (18S and 28S) and the U85 small Cajal body-specific RNA (scaRNA) serving as the cytoplasmic and nuclear markers respectively.

5.2. Designing sisRNA-specific shRNA

Drosophila is a well-established model organism with various genetic tools readily available. One such tool is the use of artificial microRNA called short hairpin RNA (shRNA) to induce effective silencing via the RNA interference (RNAi) pathway. Vectors previously created for the expression of the shRNA contains the backbone of miR-1, within which the passenger and complementary microRNA are encoded [60]. The sisRNA specific targeting sequence as the passenger and guide strands can be cloned into those plasmids, which express the specific shRNA as a hairpin with a perfect duplex structure that is highly efficient for AGO2 loading in the RNAi pathway (Fig. 4B). A choice between the plasmids Valium20 and Valium22 depends on whether the targeted RNAi effect is desired in the somatic or germline cells respectively. Transgenic flies can then be generated by microinjection using phiC31 integrase-mediated insertion into 25C7 landing sites [61].

There are a few concerns one might bring up in terms of sisRNA-targeting RNAi in flies, such as its efficacy in the nucleus where some sisRNA are known to reside, as well as the issue of differentiating

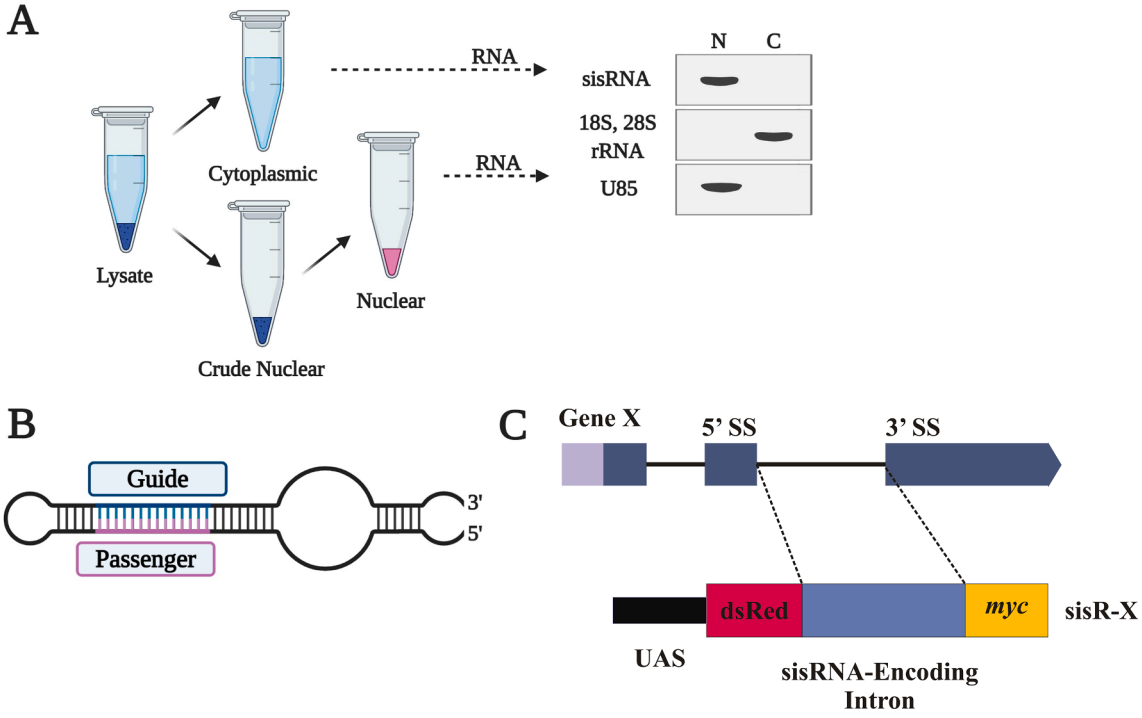


Fig. 4. An overview of various methods for circular sisRNA functional characterisation. (A) A visual schematic of the Nuclear-Cytoplasmic fractionation protocol using *Drosophila* 14-24hr embryos. Results are visualised on 1–2% agarose gel. (B) shRNA construct design with indicated positions of guide and passenger sequences for *Drosophila* RNAi. (C) Circular sisRNA expression construct with the specific intronic region of interest from gene X.

between the sisRNA and pre-mRNA of its host gene since they both share identical intronic sequences. Regarding the first concern, there have already been instances where RNAi has been shown to be working within the fly nucleus, with evidence showing that AGO2 does indeed have function within the nucleus as well in controlling gene transcription [62]. This is also not a concern in mitotic cells, where the breakdown of the nuclear envelope grants the cytoplasmic RNAi machinery access to nuclear RNA. Regarding the second concern, since this review focuses specifically on circular sisRNA, targeting the circularisation junction or branch point region as described in section 4.2 should provide enough specificity to reduce the chances of the shRNA targeting its host gene's pre-mRNA. Furthermore, pre-mRNA are mostly transient due to co-transcriptional splicing, hence making them less likely to be targeted by the RNAi machinery as compared to the more stable and abundant pool of sisRNA. Nevertheless, users should always confirm that their RNAi constructs do not target the pre-mRNA.

Of course, the *Drosophila* does not have monopoly over sisRNA. Therefore, for researchers using other model organisms such as human cell lines, we refer them to the works of the Ling-Ling Chen group. They have made great use of antisense oligos (ASOs) [38] as well as CRISPR-cas13 to effectively knockdown circRNAs [63]. The CRISPR-cas13 system is particularly useful; capable of effectively distinguishing the target exon-intronic circRNA from its host gene pre-mRNA and mRNA products. The Ling-Ling Chen group has even expanded the application of the CRISPR-cas13 system to live cell RNA imaging [64,65].

5.3. Overexpressing circular sisRNA

Circular sisRNA biogenesis has been seen so far to rely on splicing-dependent mechanisms. In order to perform experiments that require the induced or artificial expression of said circular sisRNA, it is imperative that the expression vector is able to ensure circularisation of the transgenic sisRNA sequence. To mimic biogenesis of the sisRNA via a splicing-dependent mechanism, we have previously utilised the UAS-dsRed-intron-myc plasmid [66] (Fig. 4C). Cloning of the sisRNA intronic sequence via PCR and its insertion into the plasmid at the AscI–NotI restriction sites will position the intron of interest between two exons; dsRed and myc. Upon generation of transgenic flies by microinjection using P-element-mediated insertion [67], splicing between the 5′ splice site of dsRed and the 3′ splice site at myc should produce a lariat product, mimicking the sisRNA's biogenesis *in vivo* [32].

There are also tricRNA vectors available to express stable, high-copy, circular RNA in human or animal cells. This technique takes advantage of the tRNA splicing mechanism that produces tricRNA as previously described in the introduction. The Gregory Matera group has created the aforementioned vector where the sequence of interest is cloned between two exonic sequences present in mature tRNA. Driven by an RNA polymerase III promoter site, this expression vector boasts enhanced stability for the circRNA produced, as well as a more robust expression pattern *in vivo* as compared to traditional RNA polymerase II driven systems [68,69].

For users interested in overexpressing sisRNA in the frog system, we refer them to the work of the Joseph Gall group which details the injection of sisRNA-expressing plasmids into *Xenopus* oocytes [39]. In human cell culture systems, the Ling-Ling Chen group has designed overexpression plasmids for exon–intron circRNA which included insertions corresponding to the circularised RNA. These expression plasmids contain essential consensus motifs at the 5′ splice site and branchpoint site to ensure circRNA circularisation [37,38]. More of these plasmids have been developed recently to generate circRNA *in vitro* using features of self-splicing introns [70], split ligation mechanism [71], as well as the endogenous splicing machinery itself [72,73].

6. Concluding remarks

Despite the ever-increasing pool of information and tools, the field of circRNA is not without its challenges and difficulties. Due to its young age, the field still has some issues and uncertainties to iron out. One such area includes methods in identifying circular sisRNA reads from genome-wide sequencing data. While the stability and intronic nature of circular sisRNA leaves no room for ambiguity, the uncertainty most likely comes from the diverse circRNA biogenesis pathways (some examples are listed in Table 1), and how each pathway manifests through a distinct characteristic feature in sequencing data. As such, those intending to create an exhaustive list of circular sisRNA from a particular organism must be aware of such diversity and should carefully consider selection techniques to be employed prior to RNA sequencing to avoid unintentional bias against or for a particular type of circular sisRNA.

Another limited area involves the intronic nature of the sisRNA itself. As sisRNA is produced from its host gene, the two entities are very closely intertwined. As such, difficulties arise from attempts to modify the sisRNA expression levels or sequence since it has the potential to alter the expression level or function of the host gene as well. This makes having the appropriate controls and understanding the region of interest very important to properly knocking out sisRNA without affecting host gene expression. One such example would be where attempts to delete the intronic region where the sisRNA is produced from results in the removal of splicing-dependent and regulatory motifs like the branch site and 5′ and 3′ splice sites. This attenuation of splicing at the sisRNA-producing intron will therefore affect the host gene's ability to remove that intron to produce the correct mRNA for functional protein production.

Still, studies on circRNA are growing in number and popularity, given its many implications in a variety of regulatory processes. sisRNA however, is still relatively unknown; studies are small in number and some groups report sisRNA without naming them as such. Despite this, there is a growing consensus that intronic sequences are no longer merely junk, and functional biological significance has been increasingly attributed to introns such as their involvement in regulating growth and starvation in yeast [57,74]. As the importance of both circRNA and introns are further explored, the methods described in this paper will hopefully serve to further future specialised studies to better understand circular sisRNA.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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