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POINT OF VIEW

Engineering and expressing circular RNAs via tRNA splicing

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ABSTRACT

Circular (circ)RNAs have recently become a subject of great biologic interest. It is now clear that they represent a diverse and abundant class of RNAs with regulated expression and evolutionarily conserved functions. There are several mechanisms by which RNA circularization can occur *in vivo*. Here, we focus on the biogenesis of tRNA intronic circular RNAs (tricRNAs) in archaea and animals, and we detail their use as research tools for orthogonal, directed circRNA expression *in vivo*.

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Background

Recent growth in the study of circular (circ)RNA processing and function has been propelled largely by advances in high-throughput sequencing technology and refined bioinformatic pipelines.[1,2] CircRNAs in eukaryotes were once thought of as rare molecules resulting from aberrant mRNA splicing.[3] It has since become clear that circRNA biogenesis in many eukaryotes proceeds through several diverse and evolutionarily conserved biochemical pathways,[4-7] resulting in an abundant class of RNA molecules with a host of proposed functions.[8,9] CircRNAs have been shown to be remarkably stable in living cells,[10-12] potentially due to their unique structure and lack of free termini on which the exonucleolytic RNA turnover machinery relies. Along with being generally abundant,[2] circRNAs can be differentially expressed in a tissue-dependent manner and can have expression profiles that differ from their parental mRNA,[13] suggesting regulated expression and diversity of function. CircRNAs are enriched in mammalian and *Drosophila* brains,[14-18] are positively correlated with aging,[16] and can be associated with disease risk.[19] The most well-known example of circular RNA function is their ability to act as molecular "sponges," competing for binding of molecules that affect gene regulation. For example, the human circular transcript CDR1as/ciRS-7 contains approximately 70 seed target sequences for microRNA-7 (miR-7) and associates with exosomal Argonaute proteins *in vivo* in a miR-7 dependent manner.[17,18] CDR1as/ciRS-7 represents a striking example of the controversial "competing endogenous RNA" theory, which posits that transcripts can "compete" for binding of miRNAs and other molecules to affect gene expression.[20-22] This transcript has overlapping expression patterns with miR-7 in mouse brains, suggesting a potential *in vivo* regulatory interaction.[17,18] Interestingly, either inhibition of miR-7 or ectopic expression of CDR1as causes midbrain development defects in zebrafish[18], indicating that disruption of this type of regulation could have significant consequence.

In addition to miRNAs, circRNAs can also bind and sequester proteins. In human, mouse, and *Drosophila*, the RNA-binding protein Muscleblind (Mbl) has been shown to promote circularization of several transcripts, including the second exon of the Mbl pre-mRNA itself.[23] In all 3 species, circMbl RNA contains conserved Mbl protein binding sites, and pulldown experiments in *Drosophila* S2 cells revealed an *in vivo* interaction between Mbl protein and circMbl,[23] suggesting a possible feedback mechanism by which Mbl protein and circMbl interact to control splicing of the parent transcript. The relative stability of circRNAs could perhaps contribute to a unique cellular role in initiating and maintaining these types of regulatory interactions.s.

The most common pathway for RNA circularization occurs via a process termed "back-splicing." Back-splicing proceeds via a canonical 2-step transesterification reaction, except that a downstream splice donor sequence becomes ligated to an upstream splice acceptor site, resulting in a non-canonical alternative splicing event (Fig. 1A). Importantly, back-splicing has been shown to compete with forward splicing of certain pre-mRNA transcripts.[23,24] RNA elements that promote juxtaposition of splice sites close to one another in physical space are thought to promote back-splicing. For example, back-splicing of circular transcripts is associated with the presence of repetitive elements (e.g. Alu repeats in humans or different repetitive elements in other species) that flank the circularized region.[2,25,26] Additionally, back-splicing can be facilitated by RNA-binding proteins such as the aforementioned Mbl, QKI

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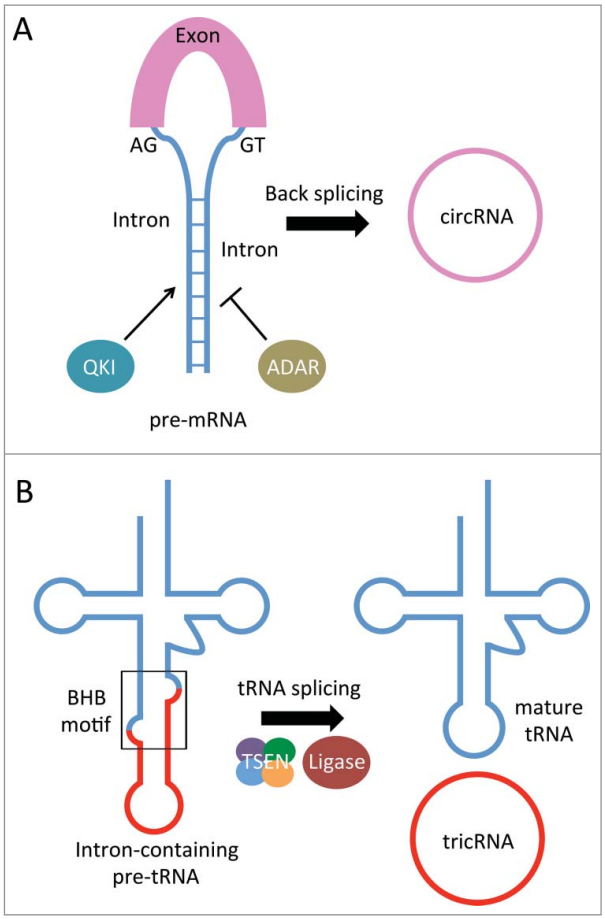

Figure 1. Two *in vivo* RNA circularization pathways. (A) Intron base-pairing and/or RNA-binding proteins facilitate pairing of a downstream splice site and an upstream splice site, bringing them into close proximity. Non-canonical "back-splicing" of these sites results in a circularized exon. (B) The tRNA splicing endonuclease (TSEN) complex cleaves an intron-containing pre-tRNA at the bulge-helix-bulge (BHB) motif. The exon halves are ligated, and the intron termini are also ligated to form a circle.

(quaking), hnRNPs and serine/arginine (SR) proteins.[23,25,27] In the following sections, we detail another conserved mode of RNA circularization occurring in archaea and eukaryotes, in which tRNA introns are spliced to form circles.

Splicing and circularization of tRNA introns in archaea

Despite the absence of the spliceosomal machinery in Bacteria and Archaea, intron-containing genes have been observed in all 3 domains of life.[28] Bacterial introns are of the self-splicing variety (for a review see Ref. [29]). In Archaea, however, introns are fairly common among tRNA and rRNA genes, and their removal is catalyzed by a conserved splicing endonuclease complex.[30,31] Interestingly, archaeal RNA splicing typically produces a circularized intron following its removal from the primary transcript.[32] Splicing of pre-tRNAs is a 2-step process wherein the transcript is first cleaved at defined positions by the archaeal splicing endonuclease complex, which recognizes a bulge-helix-bulge (BHB) sequence motif (Fig. 1B).[33,34] The exon halves are then ligated together to form a mature tRNA, while the intron termini are also joined to form a circle (Fig. 1B). Although the 2 ligation events appear to use the same processing factors, the reactions can occur independently.[32]

Circular RNAs derived from multiple tRNA isodecoder transcripts have been detected,[35] though it is not known if stable circles are made from every tRNA intron. To distinguish these circRNAs from their back-spliced cousins in eukaryotes, we use the term tRNA intronic circular RNAs, or tricRNAs. In the archaeon *Haloferax volcanii*, a tricRNA was detected *in vivo* from $tRNA^{Trp}$ by RT-PCR and Northern blot analysis.[32] In this same study, introns derived from elongator $tRNA^{Met}$ appear to undergo end-joining that would be characteristic of circularization, but tricRNA expression could not be confirmed by blotting. This lack of detectability could be due to differences in overall parental tRNA expression: in *Haloferax volcanii*, there is only one $tRNA^{Trp}$ gene, whereas there are 3 elongator $tRNA^{Met}$ genes, and only one of them contains an intron.[35] Thus, reduced expression of the parent $tRNA^{Met}$ gene might lead to lower amounts of its tricRNA that are detectable only by RT-PCR. In certain archaeal species, tricRNAs are highly expressed and/or are quite stable. For example, a whole-genome sequencing approach in *Sulfolobus solfataricus* revealed that among circRNAs, only those generated from 16S and 23S rRNA were more abundant than tricRNAs.[36] Circles were only detected for 5 of the 19 intron-containing tRNAs, but this is most likely due to an ascertainment bias, as the remaining 14 introns are quite small and would thus require specific methods of library preparation to enrich for them.

Several groups have speculated on the functions of circularized introns in archaea. Circularized rRNA introns have been found to contain putative open reading frames, and it is possible that these encode peptides.[11,12,37] Additionally, a functional C/D box RNA is contained in the $tRNA^{Trp}$ intron of *H. volcanii*, responsible for 2′-*O*-methylation of its parent tRNA, though both circular and linear excised introns are capable of guiding this modification.[38]. Similarly, the $tRNA^{Trp}$ intron of *S. solfataricus* contains regions of sequence complementarity to its parent tRNA, suggesting a role for the excised intron in tRNA modification.[36] Whether tricRNAs from other parental tRNAs and other species also function in RNA modification remains to be determined.

Splicing and circularization of tRNA introns in eukarya

Eukaryotic pre-tRNA cleavage is slightly different from archaeal in that it is performed by a complex of 4 proteins rather than one.[39] The heterotetrameric eukaryotic tRNA splicing endonuclease (TSEN) complex is functionally homologous to the archaeal protein; however, the RNA elements that define the sites of cleavage are different.[34] Many eukaryotic pre-tRNAs indeed contain a BHB-like motif, and the eukaryotic endonuclease complex has been shown to be capable of recognizing and splicing pre-tRNAs with archaeal features,[40] implying conservation of the structural elements underlying substrate recognition by TSEN. However, the currently accepted model of splicing by eukaryotic TSEN relies on recognition of the tRNA body, independent of a BHB, and splicing is dictated by a "ruler" mechanism whereby splice sites are chosen based on distance from the recognized tRNA body.[41,42] Unlike archaeal tRNA introns, which can be found at several positions in the tRNA, eukaryotic introns are nearly always found between the canonical positions 37 and 38 of the mature tRNA, adjacent to the

anticodon.[35] This difference in intron positioning is thought to result from co-evolution of introns and their processing enzymes.[31]

After cleavage by TSEN, tRNA halves are ligated to form a mature tRNA. There are 2 distinct ligation pathways in eukaryotes. The 5′-ligation pathway involves the multifunctional Trl1 protein and is considered to be the predominant one in fungi and plants.[43-45] Also known as the "healing and sealing" pathway, this process incorporates an external phosphate for use as the splice junction phosphate.[34] In vertebrates as well as archaea, tRNAs are ligated by the RtcB enzyme (called HSPC117 in humans) in the 3′-ligation or "direct ligation" pathway, where an external phosphate is not incorporated.[46,47] In RtcB knockdown experiments in *Drosophila*, tricRNA levels decreased significantly, indicating that tricRNA ligation is indeed dependent upon ligase activity of RtcB.[10]

In at least one case, ligation of mRNA splice products by tRNA processing enzymes can also result in intron circularization *in vitro*.[48] The unfolded protein response in metazoans and yeast causes stress signaling, triggering an unconventional splicing of *XBP1* (*HAC1* in yeast) mRNA. The transcript is cleaved by IRE1 and ligated by RtcB in metazoans[49-51] or Trl1 in yeast.[52] *In vitro* reconstitution of these elements resulted in a circularized intron following human *XBP1* splicing, and interestingly a circular intron was produced upon addition of either RtcB or the yeast Trl1 ligase,[48] which has homologues in some animal species. It is unclear whether this circularization occurs *in vivo*, if there are other mRNAs whose splicing is dependent upon tRNA ligase activity, and whether the removed introns have any cellular function.

Sequence conservation could indicate an important functional property for certain tricRNAs. In *Drosophilid* species, many tRNA introns are highly conserved at the sequence level. One tricRNA (tric31905) even displays compensatory mutation and preservation of base-pairing motifs, indicating a conserved secondary structure.[10] Additionally, small RNA sequencing analysis indicates that this circular RNA is further processed into microRNA-sized fragments.[10] It is currently unclear whether these small RNAs have a function in the cell or are merely degradation intermediates. Inspection of sequence and secondary structure of other *Drosophilid* introns does not reveal common sequence motifs or elements, suggesting that processing of tric31905 is not necessarily applicable to all tricRNAs.

Methods of ectopic circRNA expression and detection

To date, few tools exist to direct generation of RNA circles *in vivo*. Several groups have constructed mini-gene vectors that contain complementary sequences flanking the region of interest, designed to facilitate its circularization in a mechanistically similar fashion to endogenous exonic back-splicing.[17,25,53] Effective circularization has been achieved using both endogenous and designed elements, or some combination thereof. Such engineered mini-genes have also shed light on cis-acting sequences that either promote or inhibit circularization.[25,26,53] These ectopic circRNAs are capable of serving as a template for protein translation following inclusion of an internal ribosomal entry site (IRES) in the circularized transcript.[53]

Back-spliced circRNA expression systems are typically driven by an RNA pol II promoter such as the one present in human cytomegalovirus (CMV).[25,53] Ectopic expression of tricRNAs, in contrast, relies on an RNA pol III promoter to drive transcription (Fig. 2A). Robust expression can be achieved with an external pol III promoter, such as the one from the U6 snRNA gene.[10,54] Pol III transcription can exceed that of pol II, and produces more consistent levels of expression across cell types as compared with the CMV promoter.[55,56]

Accurate characterization of circRNA expression can be difficult, however, due to a wide variety of circRNA sizes. Divergent primers are used to specifically amplify circRNA-derived cDNA (Fig. 2A-C). During cDNA preparation, reverse transcriptase (RT) is capable of rolling circle cDNA synthesis on a circular template RNA, resulting in concatemerized cDNA (Fig. 2B-C) that could lead to exaggerated measurement of circRNA levels by sequencing or RT-PCR. Indeed, concatemeric reads containing multiple copies of a tRNA intron were detected in *Drosophila* and *C. elegans* RNA-seq data sets.[10]

For larger circles, concatemerization is less likely due to the limited processivity of reverse transcriptase. However, RT-PCR of a short circRNA can produce concatemers that are many times longer than the original transcript.[54] Thus, circRNA length must be carefully considered when using RT-based methods to quantify transcript abundance, and direct measurement of circRNA abundance by fluorescence or northern blotting may be preferred. We have successfully expressed 'designer' tricRNAs greater than 800 nt in length (Fig. 2D), the primary transcript of which is well over 900 nt. To our knowledge, this is the longest known pol III-based transcription unit in eukaryotes. Hence, pol III processivity should not be a limiting factor for many applications aimed at expression of engineered tricRNAs *in vivo*.

To directly compare the 2 circRNA expression systems, we generated reporter constructs using the Broccoli fluorescent RNA aptamer system (Fig. 3A).[57] Each construct is designed to express an equivalently-sized aptamer-containing circle (see Methods for details). To differentiate between the 2 systems, we refer to the pol II/back-spliced Broccoli reporter as circBroc, and to the tRNA-spliced Broccoli reporter as tricBroc. Following transient transfection in human HeLa and HEK293T cells, RNA was extracted and analyzed using a convenient and powerful in-gel staining assay (Fig. 3B).[54,57,58] RT-PCR (which is more sensitive, but less quantitative) was also performed (Fig. 3C). As shown, the circBroc back-splicing reporter RNA is detectable by RT-PCR but not by in-gel fluorescence, whereas tricBroc was robustly detected by both methods. The in-gel fluorescence assay allows for detection of as little as 100 pg of Broccoli-containing RNA;[58] thus, we can conclude that circBroc is expressed at levels below this detection threshold. In agreement with these data, only tricBroc was detectable by RNA gel blot analysis in 293T cells (Fig. 3D). We note that the size of the circBroc exon used here is smaller than those used in previous circRNA expression vectors (76bp compared with 300–1500 bp),[25,26,53] although it is certainly on par with the size of many endogenous exons (roughly 80% of human exons are shorter than 200 bp).[59] However, back-spliced exons average 690 bp in humans[2] and so it remains unclear whether back-splicing efficiency depends on exon size. Nevertheless, our results show that the pol III-based expression system provides more robust expression of circular RNAs (at least for smaller sized ones) than does the pol II-based system. Thus, the tricRNA expression system described here

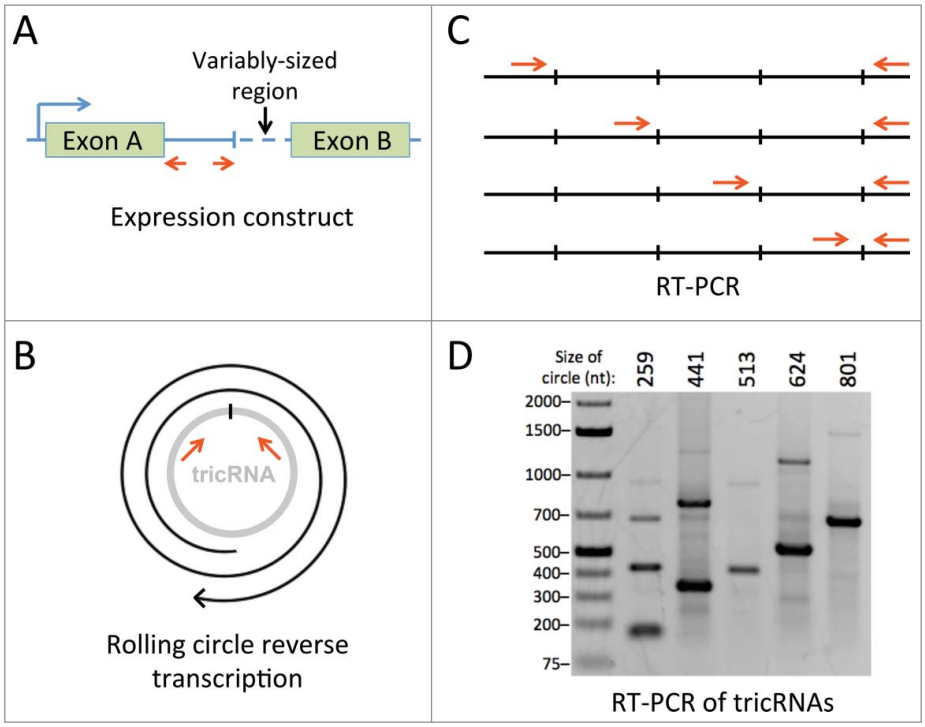

Figure 2. tricRNA expression can be detected by RT-PCR. (A) Schematic of the tricRNA expression construct, showing the PCR primer binding sites (red arrows) in the intron and an external pol III promoter (blue arrow). Exon A and Exon B represent the sequences present in the mature tRNA. The dotted line indicates a variably-sized intronic region. (B) Reverse transcriptase can transcribe around a tricRNA template many times, resulting in a concatameric cDNA with numerous tandem repeats. (C) RT-PCR primers can bind to multiple sites along the cDNA concatamer, resulting in a ladder of potential PCR products. The formula for the sizes of the bands is: [size of the circle] – [distance between the divergent primers] + [size of circle]*n*, where n is the number of tandem repeats. (D) The ladder of PCR products can be seen for circles of several different sizes. In this experiment, the distance between the divergent primers is 105 nt, and the sizes of the circles are listed above the gel. For the 259 nt circle, the formula for the ladder is therefore: 259–105+259*n*. The bands detected on the gel are at 154, 413, 672, and 931 nt.

provides a compact and powerful method for *in vivo* expression of circular RNAs.

Concluding remarks

Despite their conservation across great evolutionary distances, the function of tRNA introns has, for the most part, yet to be elucidated. Many interesting questions remain regarding their role in tRNA biosynthesis (as DNA elements), as well as their fate and function in the cell (as tricRNAs) following removal during processing. As with many non-coding RNAs, characterization of circular RNAs has been greatly aided by the progress of detection technologies and molecular genetic approaches. As detailed here, tricRNA vectors provide an effective means for the orthogonal expression of a wide variety of genetically engineered circRNAs.

Materials and methods

Cloning circBroc reporter constructs

Annealed oligonucleotides were ligated into the pCircGFP plasmid (Zefeng Wang, PICB Shanghai) following digestion with BamHI and SacII to generate a new vector, called pCircBroc. Top oligo: ggtctctttcttccagGAGCGGCCGCGAGACGGTCGGGTCCAGATATTCGTATCTGTCGAGTAGAGTGTGGGCTCGTGGCCGCGGTGTTGAGgtaagtctcgacg, bottom oligo: gatccgtcgagacttacctcaacaccgcggccacgagcccacactctactcgacagatacgaatatctggacccgaccgtctcgcggccgctcctggaagaaagagaccgc. For the top strand oligo, the exon is shown in uppercase letters and the intronic sequence is in lower case. The nucleotides corresponding to Broccoli are underlined.

Cell transfection and RNA isolation

All cells were grown at 37°C with 5% CO2 in DMEM (Gibco) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. One million HEK293T or HeLa cells were plated in T-25 flasks and incubated at 37°C. After 24 hours, cells were transfected with equimolar amounts of either circBroc or tricBroc expression vector using Fugene HD transfection reagent (Promega cat#E2311). After 72 hours, total RNA was isolated from cells using Trizol reagent (Thermo Fisher Scientific cat#15596026).

RT-PCR

Total RNA was treated with Turbo DNase (Thermo Fisher Scientific cat#AM2238) and used to prepare cDNA using SuperScript III (Invitrogen cat#18080051) with random hexamer primers. Divergent primers used for circBroc PCR amplification were as follows: F-GTCGAGTAGAGTGTGGGCTCGT, R-GATACGAATATCTGGACCCGACCGTC.

In-gel fluorescence imaging

Ten micrograms of total RNA from transfected cells was run on a Novex 10% TBE-Urea gel (Thermo Fisher Scientific cat#-EC68572BOX) for 40 minutes at 300V. After washing 3 times with ddH_20, the gel was stained with DFHBI-1T staining

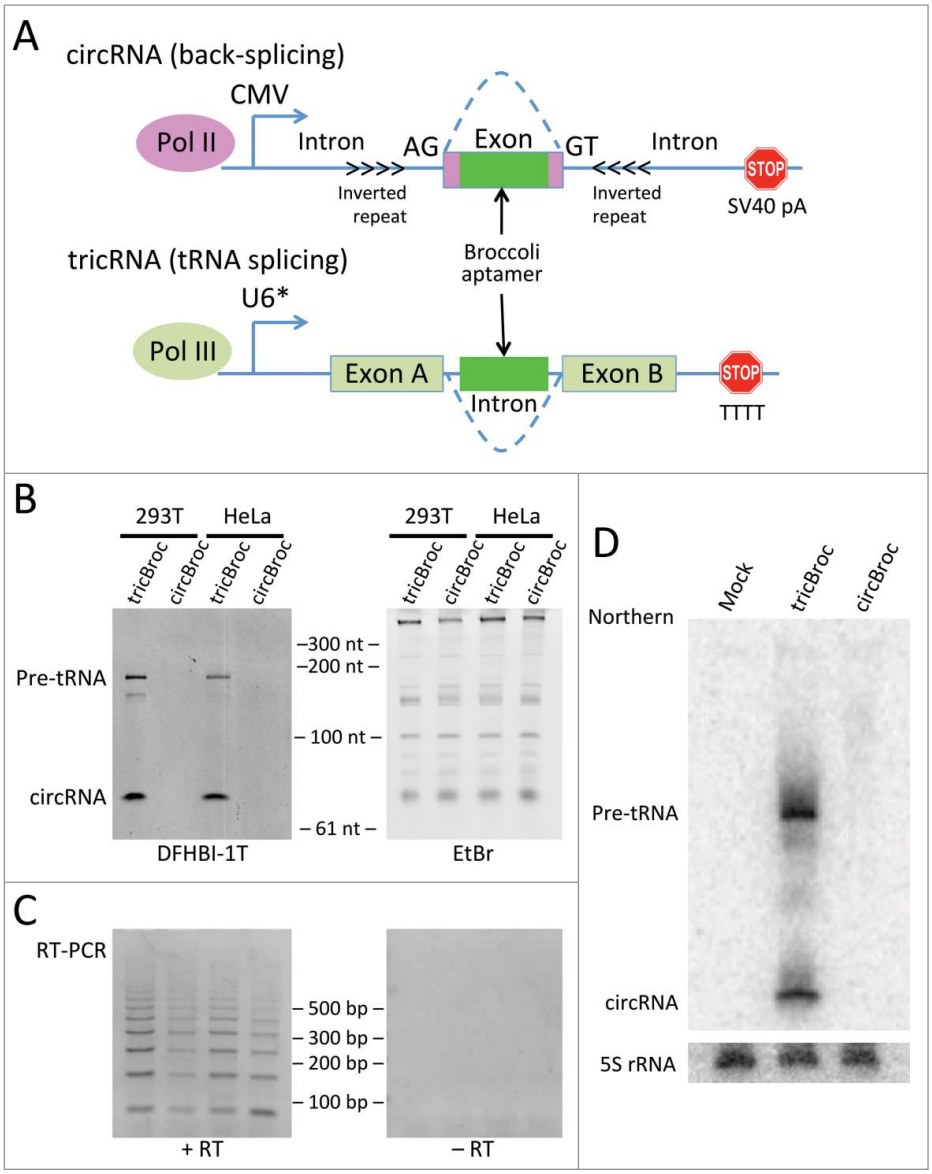

Figure 3. Testing *in vivo* methods of RNA circularization using the Broccoli RNA aptamer as a reporter. (A) Schematic of the 2 constructs, where the bright green box indicates the placement of the Broccoli aptamer sequence. The dotted arcs indicate the splice junctions. The U6* promoter includes the first 27 nucleotides of U6 snRNA in the transcriptional unit. This promotes 5′ capping[60] and enhances stability of the expressed RNA, resulting in a higher yield of tricRNAs.[10] (B) In-gel imaging following transient transfection of the reporter constructs into HeLa and 293T cells. The left hand image is the DFHBI-1T stain, which binds to all Broccoli-containing RNAs. In this image, the top band is the pre-tRNA and the lower band is the circular RNA. The doublet of bands in the first lane is most likely due to transcription beginning from both the external U6 promoter, which has a longer 5′ leader sequence, and the internal tRNA promoter. The right hand image is the ethidium bromide stain of the same gel, which marks total RNA. (C) RT-PCR was performed on cDNA generated from the RNA used in Fig. 3B. Diverging PCR primers were used (similar to Fig. 2A), such that they only generate products of the appropriate size from a circularized template (see also Fig. 2D). In this experiment, the lengths for tricBroc and circBroc are 77 nt and 76 nt, respectively. (D) Northern blot analysis was performed to quantitatively assess circRNA expression in 293T cells.

solution (40 mM HEPES pH 7.4, 100 mM KCl, 1 mM $MgCl_2$, 10 μm DFHBI-1T [Lucerna cat#410–1 mg]) and fluorescence imaged using a GE Typhoon Trio variable mode imager. To visualize total RNA, the gel was then stained with ethidium bromide and fluorescence imaged.

Northern blotting

Ten micrograms of total RNA was run on a 10% TBE-Urea gel for 40 minutes at 300V and transferred to a nylon membrane at 10 mA overnight. The membrane was dried and UV-crosslinked, followed by pre-hybridization in Rapid-hyb Buffer (GE Healthcare Life Sciences cat#RPN1635) at 42°C for 30 minutes. Oligonucleotide probes were radiolabeled using T4 PNK (NEB) and then purified using Illustra Microspin G-50 columns (GE Healthcare Life Sciences cat#27–5330–71). They were then boiled for 5 minutes and cooled on ice for 2 minutes, and then added to the Rapid-hyb Buffer. The membrane was hybridized at 42°C for one hour with rocking, and then washed at ambient temperature twice for 15 minutes with 2X SSC with 0.1% w/v SDS and twice for 15 minutes with 0.1X SSC with 0.1% w/v SDS at 42°C before overnight PhosphorImager screen exposure. The blot was then imaged using a GE Typhoon Trio variable mode imager.

Probe sequences: anti-Broccoli-5′-gagcccacactctactcgacagatacgaatatctggacccgaccgtctc-3′, anti-5S rRNA- 5′-ccctgcttagcttccgagatcagacgagat-3′.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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