Inventory

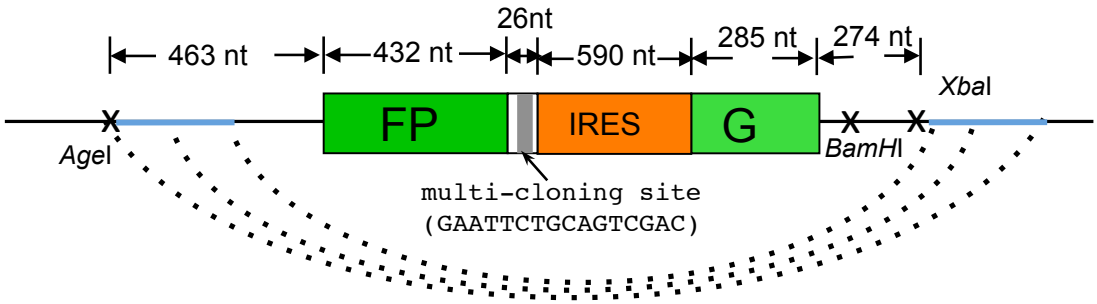
Figure S1. Schematic diagram of engineering circular RNA mini-genes. (Related to Figure 1).

Figure S2. *In vivo* translation directed by unstructured circular mRNA. (Related to Figure 3).

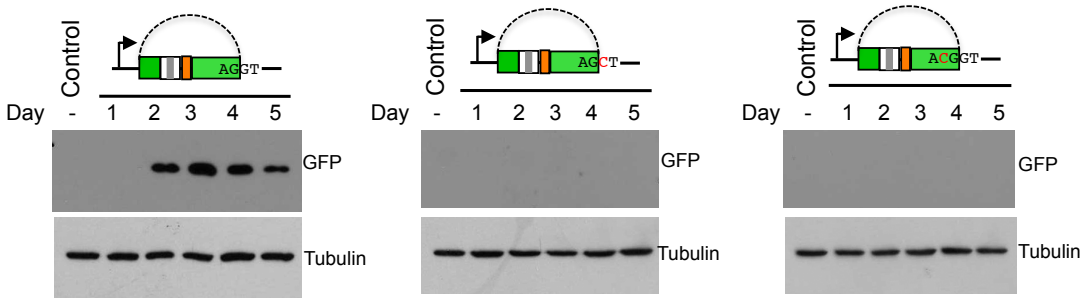
Table S1: List of oligonucleotide sequences used in this study. (Related to Figure 1)

Supplemental Experimental Procedures

Supplemental Data



Supplementary figure S1. Schematic diagram of engineering circular RNA minigenes. The split GFP fragments are inserted in reverse orders in the exon, which is represented by box. The first intron is cloned from the human IGFII mRNA-binding protein. The second intron is either the complementary sequence as the first intron or in tandem fashion as the first intron. The blue lines showed the region that can form a hairpin structure (indicated by dotted lines). The length of each fragment is indicated, and key restriction sites are shown as a cross. The multiple cloning site contains EcoRI-PstI-SalI that can be used to insert regulatory motifs.



Supplementary figure S2. ***In vivo*** **translation directed by unstructured circular mRNA.** Cells were transfected with wild type or mutated minigenes containing unstructured introns, and total proteins were purified at different days after transfection to detect production of GFP.

Supplementary table S1. List of oligonucleotide sequences used in this study.

Primer #	Name	Sequence	Notes
1	IRES-G-Fwd	tcacAGATCTGAATTCTGCAGTCGACGATCCGCC CCTCTCCCTCC	The forward and reverse primer pairs used to clone IRES-G region into pEGFP-C1 vector
2	GFP-E1-R	cacctcgagacttacCTGGACGTAGCCTTCGG	
3	pGZ3-Int2-F	tcacACCGGTGACTGAACATGGAGGAATTG	The primer pair used for cloning Intron-FP region into pEGFP-C1 vector
4	GFP-E2-R-new	cacGGATCCTTACTTGTACAGCTCGTCCATG	
5	xba-intron-fwd	cacTCTAGA AGAGGCCCAATTCAAGgatttgg	The primer pair used for cloning structured intron into the minigene
6	BamHI-intron-rev	cacGGATCC caaataagatgccctcagac	
7	xba-intron-rev	cacTCTAGA AGAGGCCCAATTCAAGgatttgg	The primer pair used for cloning unstructured intron into the minigene
8	BamHI-intron-fwd	cacGGATCC caaataagatgccctcagac	
9	MUT5SS-fwd	CGAAGGCTACGTCCAGCTAAGTCTCGACGGTA C	The primer pair used for introducing mutant 5'ss in the minigene
10	MUT5SS-rev	GTACCGTCGAGACTTAGCTGGACGTAGCCTTC G	
11	INT5SS-fwd	CGAAGGCTACGTCCACGGTAAGTCTCGACGGT AC	The primer pair used for introducing an insertion near 5'ss in the minigene
12	INT5SS-rev	GTACCGTCGAGACTTACCGTGGACGTAGCCTT CG	
13	PolyA40-fwd	TAAGGATCTGAATTC AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAA TGCA GTCGACGATCC	The primer pair used for inserting poly A into the minigene.
14	PolyA40-rev	GGATCGTCGACTGCATTTTTTT	
15	PolyT40-fwd	TAAGGATCTGAATTC TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTT TGCA GTCGACGATCC	The primer pair used for inserting poly A into the minigene
16	PolyT40-rev	GGATCGTCGACTGCA AAAAAAA	
17	Control-circF	AATTC TTACTGTACT G	The primer pair used to insert control SRE into minigene
18	Control-circR	TCGAC AGTACAGTAA G	
19	ESS1-circF	AATTC GTAACGCGGTAAC G	The primer pair used to insert ESS1 SRE into minigene
20	ESS1-circR	TCGAC GTTACCGCGTTAC G	
21	ESS2-circF	AATTC GGTGGTCGTTGGT G	The primer pair used to insert ESS2 SRE into minigene
22	ESS2-circR	TCGAC ACCAACGACCACC G	
23	ESE-circF	AATTC GAACAGGAACAG G	The primer pair used to insert ESE SRE into minigene
24	ESE-circR	TCGAC CTGTTCCTGTTC G	
25	Gexon1f	AGTGCTTCAGCCGCTACCC	The primer pair used for testing circular RNA
26	Gexon3r	GTTGTACTCCAGCTTGTGCC	

27	linear-F	ACG TAA ACG GCC ACA AGT TC	The primer pair used for testing linear RNA
28	Linear-R	CTGAGGGCATCTTATTTGGG	
29	GFP-RT-total-fwd	ACG TAA ACG GCC ACA AGT TC	The primer pair used for testing total RNA
30	GFP-RT-total-rev	AAG TCG TGC TGC TTC ATG TG	

Materials and Methods

Circular RNA reporter constructs

All of the circular RNA reporters were generated from the same backbone construct, pEGFP-C1. Firstly, an IRES-G (IRES and N-terminal GFP sequences) fragment was amplified with a PCR reaction using pIRES2-EGFP as templates with primers (primer 1 and 2) containing *Bgl*II/*Xho*I restriction sites, and subsequently inserted into pEGFP-C1 that was digested with *Bgl*II/*Sal*I. The resulting construct was called pCIRC-IRES-G. Secondly, an intron-FP (intron 12 of IGF2BP1 and C-terminal GFP sequences) fragment was amplified with a PCR reaction using pGZ3 vector {ref} as templates with primers (primer 3 and 4) containing *Age*I/*Bam*HI sites, and then inserted into pCIRC-IRES-G that was cut with *Age*I/*Bgl*II. The resulting construct was named pCIRC-FP-IRES-G. To generate the structured intron circular RNA reporter, the reverse complementary fragment of partial intron 12 of IGF2BP1 was amplified using pGZ3 vector as templates with primers (primer 5 and 6) containing *Xba*I/*Bam*HI sites, and subsequently inserted into pCIRC-FP-IRES-G that was digested with *Bam*HI/*Xba*I. To generate the unstructured intron circular RNA reporter, the part of intron 12 of IGF2BP1 was amplified using pGZ3 vector as templates with primers (primer 7 and 8) containing *Bam*HI/*Xba*I sites, and subsequently inserted into pCIRC-FP-IRES-G that was digested with *Bam*HI/*Xba*I.

To generate the 5′ splice site mutated circular RNA minigenes, the guanine was replaced with cytosine using the mutagenesis approach with primers 9 and 10. To generate another mutated circular RNA minigene with a frame shift of the GFP protein, a single cytosine was introduced in the exon using mutagenesis method with primers 11

and 12. To introduce poly A_{40} or poly T_{40} into the circular RNA minigene, mutagenesis PCRs were applied using QuikChange site-directed mutagenesis tool with primers 13 and 14, or 15 and 16 according to the manufacture's protocol (Agilent Technologies). To determine the effects of splicing regulatory *cis*-elements on the backsplicing of circular RNA, different *cis*-elements were inserted into the *EcoR*I/*Sal*I sites of the circular RNA mini-gene by using primers 17 to 24.

Assay of circular RNA reporters with semi-quantitative RT-PCR

Cells transfected with circular RNA reporters were collected for RNA isolation. Briefly, total RNA were isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions, followed by 1 h DNase I (Invitrogen) treatment at 37 °C and then heat inactivation of DNase I. Total RNA (2 μg) was then reverse-transcribed with SuperScript III (Invitrogen) with random priming, and one-tenth of the RT product was used as the template for PCR amplification (22 cycles of amplification, with trace amount of Cy5-dCTP in addition to non-fluorescent dNTPs). To examine the circular RNAs, primers 25 and 26 were used for PCR. Primers 27 and 28 were applied to detect linear RNAs, and primers 29 and 30 were used to detect the total reporter RNAs. RT-PCR products were separated on 10% PAGE gels, and scanned with a Typhoon 9400 scanner (Amersham Biosciences). The amount of each product was measured with ImageQuant 5.2.

Assay of circular RNA reporters with Realtime PCR

The real-time PCR was performed using the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and a 7500 real-time PCR system (Life Technologies) according to manufacturer's instructions. The expression level of circular RNA, linear RNA was normalized to the endogenous expression of GAPDH.

Western blot

Cells were lysed in lysis buffer containing 50 mM HEPES, 150 mN NaCl (4.38g), 1mM EDTA, 1% (w/v) CHAPS and Sigma protease inhibitor cocktail. Subsequently the cell lysates were boiled in 2X SDS-PAGE loading buffer for 10 min and then resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. The following antibodies were used in this study: GFP (632381) antibody was purchased from Cell Signaling Technology, Bcl-x antibody (610211) was purchased Clontech. Alpha-tubulin (T5168) and FLAG M2 were purchased from Sigma-Aldrich. Bound antibodies were visualized with the ECL kit (GE Healthcare).

Immunofluorescence

293 cells expressing circular RNA mini-gene reporter upon tetracycline induction were seeded onto poly-lysine coated glass coverslips in a 6-well plate, and then added tetracycline to a final concentration of 1 μg/ml. At 48 h after induction, the cells were fixed on the coverslips with 4% formaldehyde in 1X PBS for 20 min at room temperature and washed with 1X PBS three times. Cells were then permeabilized with 0.2% Triton X-100 in 1X PBS for 10 min and washed with 1X PBS three times. The cover slips were mounted with mounting medium (Vector shield's mounting medium with DAPI). Cells

were visualized using an Olympus fluorescence microscope, and photographs were generated using a Kodak digital camera.

Propidium iodide staining and flow cytometry

293T cells were harvested at 48 h after transfection of circular RNA mini-gene and stained for 5 min in a PBS solution containing a final concentration of 2 μg/ml propidium iodide (PI). The GFP positive cells were analyzed with a FACSCalibur fluorescence-activated cell sorter (FACS) using CELLQuest software (Becton Dickinson,NJ).