CircAde, a vector system for spliceosome dependent circRNA biogenesis and prolonged more effective protein expression

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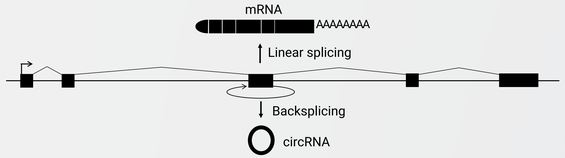
Introduction

Circular RNA (circRNA) constitutes a novel class of endogenously expressed RNA. CircRNAs are generated by a non-linear splicing event in which a upstream splice acceptor attacks a downstream donor, known as backsplicing. In contrast to mRNA, circRNAs are resistant to exonucleolytic decay which results in high intra-cellular stability and persistence. Here, we show our development of a circRNA expression platform and our proprietary circAde vector system towards efficient delivery and expression of protein-encoding circRNA *in vivo.*



1. Choice and composition of IR dramatically impacts circRNA biogenesis

Based on endogenous loci with high-yield circRNA production, the prevalent model for circRNA biogenesis involves flanking inverted repeats (IRs). We show that the IRs are required for biogenesis (**Fig. 1A-C**), and that the choice of IR impacts circRNA yield. Here, bioinformatic analysis of publicly available RNA-Seq datasets, was used to identify highly abundant circRNAs. Several IR's flanking these abundant circRNAs were incorporated into our circRNA cassette to examine their ability to drive backsplicing (**Fig. 1E-G**). Moreover, modification of these IR sequences was found to further enhance the biogenesis of circRNAs (**Fig. 1H**).

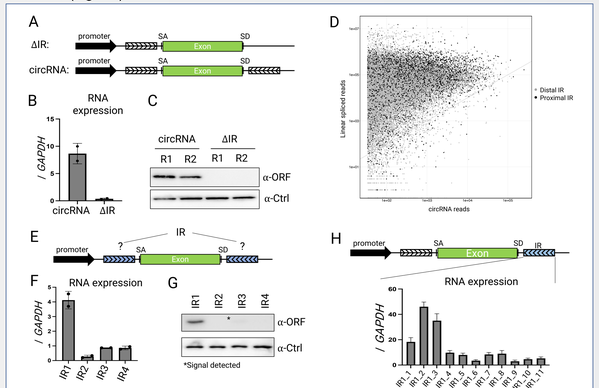


Figure 1: Optimization of flanking inverted repeats (IRs). A) Schematic representation of circRNA cassettes with or downstream inverted repeat (IR). **B-C)** Biogenesis as measured by qRT-PCR (B) and protein yield measured by western blotting (C) from circRNA cassettes with or without downstream IR. **D)** IRs from highly expressed circRNAs, stratified by distance to backsplicing sites, were identified by bioinformatic analysis of publicly available datasets, where circRNA specific reads were compared linear spliced reads. **E)** Schematic representation of circRNA cassettes with depicting IR position, where different proximal IR elements from highly expressed endogenous circRNAs are incorporated. **F-G)** Biogenesis as measured by qRT-PCR (F) and protein yield assessed by western blotting (G) from circRNA cassettes with different IRs. **H)** The impact of downstream IR sequence composition on circRNA biogenesis as measured by qRT-PCR normalized to *GAPDH.*

2. Importance of cassette design for protein-coding circRNA

Protein translation from circular RNA requires an IRES (internal ribosomal entry site) for cap-independent translation (**Fig. 2A**). Here, testing 10 different designs using 2 different IRES elements (schematically shown in **Fig. 2B**), a dramatic design-dependent effect on biogenesis was observed by protein expression, with high and consistent yield from the circRNA producing D4 and D5 designs (**Fig. 2C**). In addition to affecting circRNA-derived protein translation, choice of IRES significantly affects circular RNA biogenesis (**Fig. 2D-F**). Here, we show that specific IRES-elements capable of initiating high levels of translation negatively impact circRNA biogenesis.

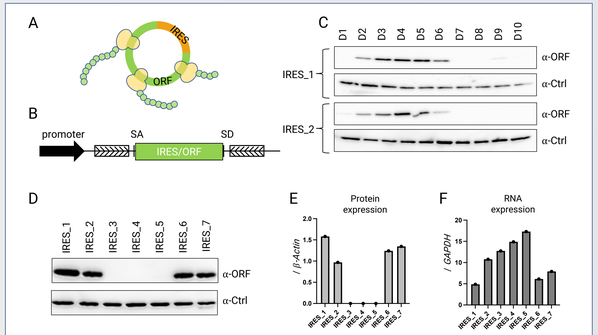


Figure 2: Choice of IRES and IRES/ORF composition impact circRNA expression: A) CircRNA translation depends on cap-independent translation and requires IRES (internal ribosome entry site) and ORF (Open Reading Frame) as shown. **B)** Schematic representation of vector cassette used for circRNA expression. **C)** Protein expression from ten different IRES/ORF designs (D1-D10) using two different IRES elements was assessed by western blot. **D)** Impact of different IRES elements on circRNA derived protein translation assessed by western blot. **E)** Quantification of protein expression from (D) normalized to β-Actin. **F)** The impact of IRES choice on circRNA biogenesis, as measured by qRT-PCR normalized to *GAPDH.*

3. Modifications of flanking regions affect circRNA biogenesis

To further boost circRNA yield from our circRNA cassette, modifications were introduced in the flanking regions of our cassette. Two approaches were examined; modification of upstream intron (U) to facilitate enhanced transcription (**Fig. 3A**) or introduction of downstream (D) elements to stimulate backsplicing (**Fig. 3A**). Here, insertion of upstream introns reduced circRNA yield (**Fig. 3B-C**), while insertion of downstream elements were found to have little impact on circRNA yield (**Fig. 3B-C**).

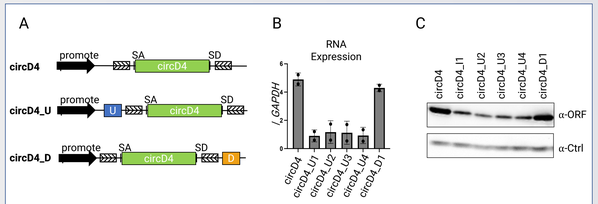


Figure 3: Flanking regions of circRNA cassette impact circRNA biogenesis. A) Schematic of position of modification of upstream intron (U) to facilitate enhanced transcription or introduction of downstream (D) elements to stimulate backsplicing. **B-C)** Biogenesis as measured by qRT-PCR (B) and protein yield measured by western blotting (C) from circRNA cassettes flanked by different upstream intronic elements (U1-4) or downstream elements (D1).

4. IRES-mediated circRNA translation outperforms mRNA

To benchmark the circRNA expression cassette with conventional mRNA-based expression vectors, the D4 circRNA comprising either IRES1 or IRES2 was compared to mRNA expression from the same vector backbone. The D4_IRES1 design showed superior protein yield 48 hours after transfection (**Fig. 4B-C**), despite reduced levels of RNA (**Fig. 4D**), suggesting that cap-independent IRES-mediated translation is more effective than cap-dependent translation.

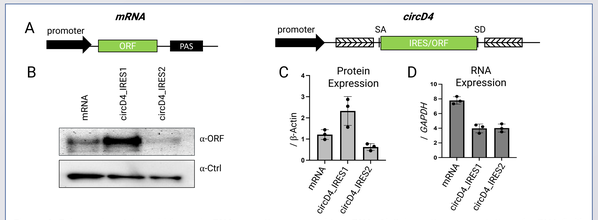
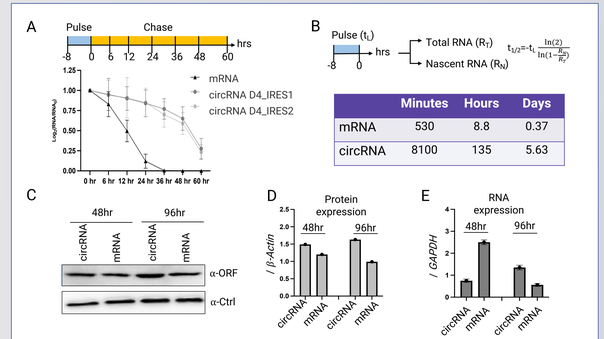


Figure 4: Enhanced protein yield from circRNA cassette compared to mRNA. A) Schematic representation of mRNA (left) and circRNA (right) expression cassette. **B-C)** Protein yield measured by western blotting (B) and quantified relative to β-Actin (C) from from A375 cells transfected with plasmids expressing conventional mRNA-encoding payload or circRNA-encoding ORF using IRES1 or IRES2 for translation as denoted. **D)** Biogenesis as measured by qRT-PCR normalized to *GAPDH.*

5. Enhanced circRNA stability confers prolonged protein expression

Almost all cellular RNA turnover is facilitated by exonucleolytic decay. Circular RNAs are devoid of 5' and 3' ends and thereby resistant to exonucleases. Consequently, high stability and long half-lives are observed for circRNA compared to mRNA (**Fig. 5A-B**). To benchmark the circRNA expression cassette with conventional mRNA-based expression vectors, the D4 circRNA comprising either IRES1 or IRES2 was compared to mRNA expression from the same backbone. The inherent stability of circRNA results in RNA and protein accumulation over time, whereas mRNA levels decline rapidly (**Fig. 5C-E**), suggesting that the circRNA-based vectors result in prolonged expression.



	Minutes	Hours	Days
mRNA	530	8.8	0.37
circRNA	8100	135	5.63

Figure 5: Superior circRNA stability facilitates circRNA accumulation and prolonged protein expression. A) Newly synthesized RNA was labelled with nucleotide analogues for 8 hours and the durability of labelled RNA was quantified over time by qRT-PCR. **B)** Half-life RNA estimates were inferred from the nascent fraction (newly synthesised labelled RNA as in(A)) relative to total RNA assuming steady-state. **C-D)** Protein yield measured by western blotting (C) and quantified relative to β-Actin (D) at indicated timepoints post-transfection. **E)** Quantification by qRT-PCR of RNA levels relative to *GAPDH* at indicated timepoints post-transfection.

6. High yield circAde expression is dependent on the positioning of the circRNA cassette

Effective expressing of circular RNA from proprietary circAde vectors depends highly on site of integration. From nine initial genome designs, only six circAdes were functional (**Fig. 6A**). Dramatic differences in circRNA-based protein expression were observed for the functional circAdes (**Fig. 6B**).

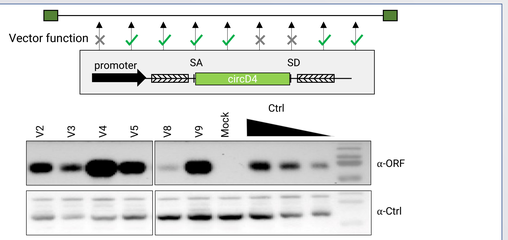


Figure 6: Impact on genome integration site strategy on vector functionality and circRNA biogenesis. A) Schematic representation of 9 different integration sites tested. The green checkmark denotes that the circAde vectors were rescued and viable, whereas for the sites with grey cross, no viable circAdes were obtained. **B)** Western blot on cells transduced with six different circAdes using payload-specific antibody (upper panel) or loading control (bottom panel).

Conclusions

1) Choice and composition of IR is crucial for high yield circRNA biogenesis.
2) IRES/ORF positioning is essential for generation of protein-coding circRNAs.
3) Upstream intron modifications negatively impact circRNA biogenesis.
4) IRES-mediated circRNA translation outperforms mRNA.
5) Superior circRNA stability facilitates accumulation of circRNA and prolonged protein expression.
6) Successful high-yield design for circRNA expression from circAde demonstrated.

The results support further development of the circRNA cassettes and circAde vectors towards therapies where high and prolonged expression of any gene of interest is desired.

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