circio

circAde, a circRNA-based system for prolonged and more effective treatment of cancer

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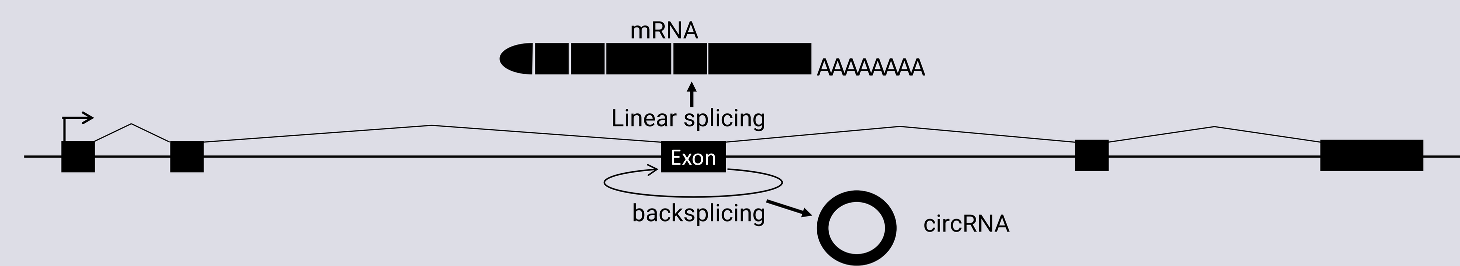
Abstract # 5335
Presented at AACR 2023

Background and aims

Circular RNAs (circRNAs) are naturally occurring transcripts found in all eukaryotes. CircRNAs typically derive from protein-coding exons prone to backsplicing (non-linear splicing in which a upstream splice acceptor attacks a downstream donor, see schematic below). In contrast to mRNAs, circRNAs are highly stable molecules with sustained effects within cells. Understanding the biogenesis and potential of circRNA allows repurposing the stable circRNA features into durable protein-coding templates. Here, we show our development of a circRNA expression platform and our proprietary circAde vector system towards efficient delivery and expression of circRNA *in vivo*.



1. Biogenesis depends on IR

Based on endogenous loci with high-yield circRNA production, the prevalent model for circRNA biogenesis involves flanking inverted repeats (IRs). Here, we show that the IRs are required for biogenesis (**Fig. 1A-C**) and that biogenesis can be enhanced significantly by optimization of the IR sequences (**Fig. 1C**).

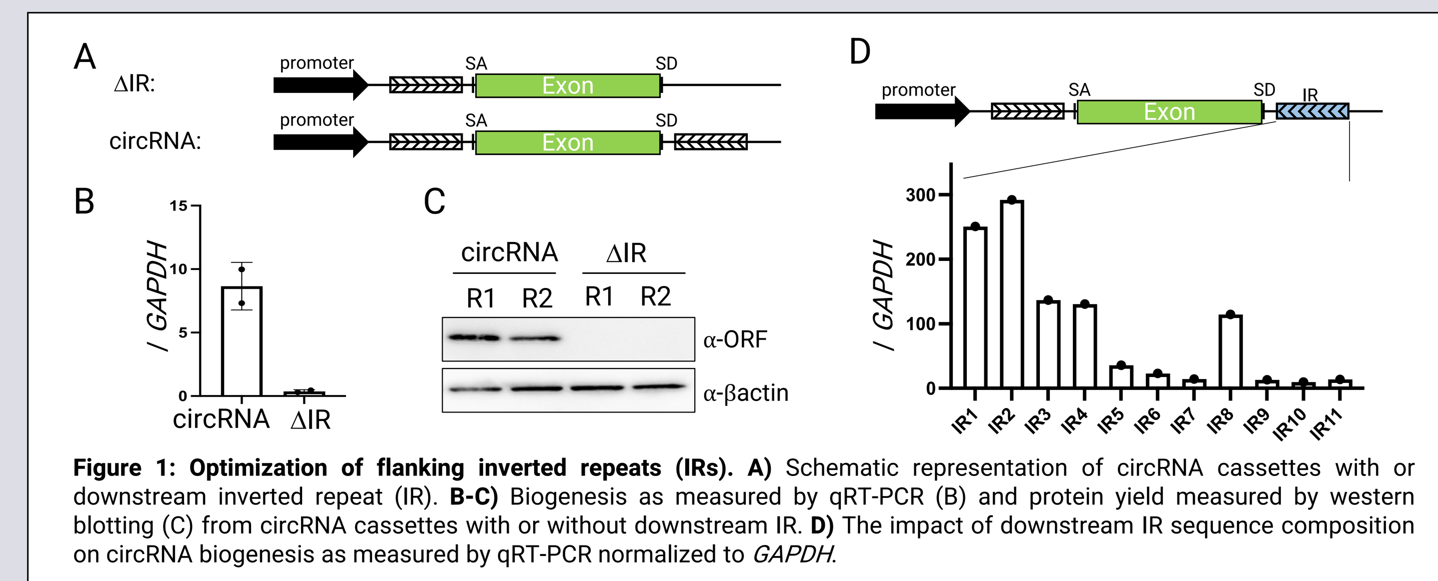


Figure 1: Optimization of flanking inverted repeats (IRs). A) Schematic representation of circRNA cassettes with or downstream inverted repeat (IR). **B-C)** Biogenesis as measured by qRT-PCR (B) and protein yield measured by western blotting (C) from circRNA cassettes with or without downstream IR. **D)** The impact of downstream IR sequence composition on circRNA biogenesis as measured by qRT-PCR normalized to *GAPDH*.

2. Optimization of circRNA cassette

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**).

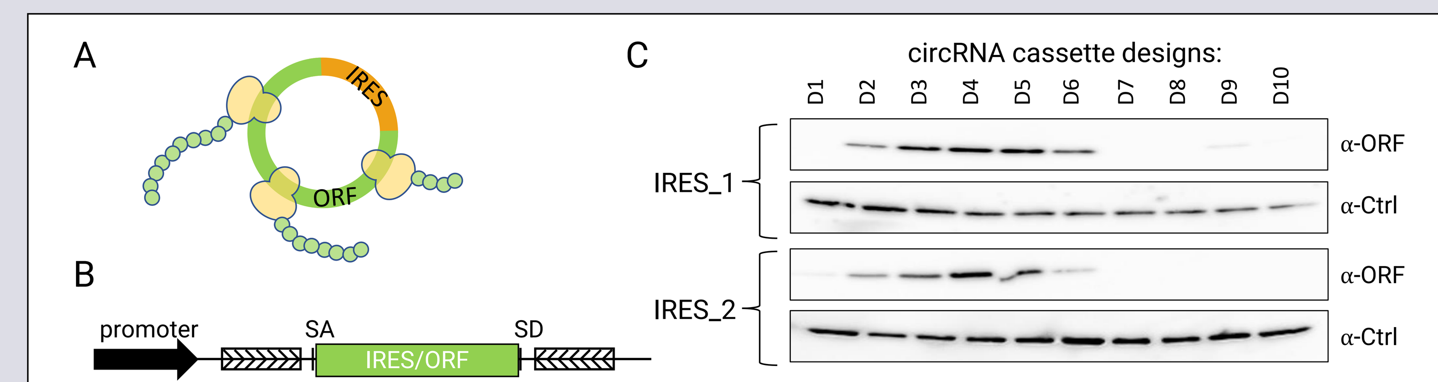
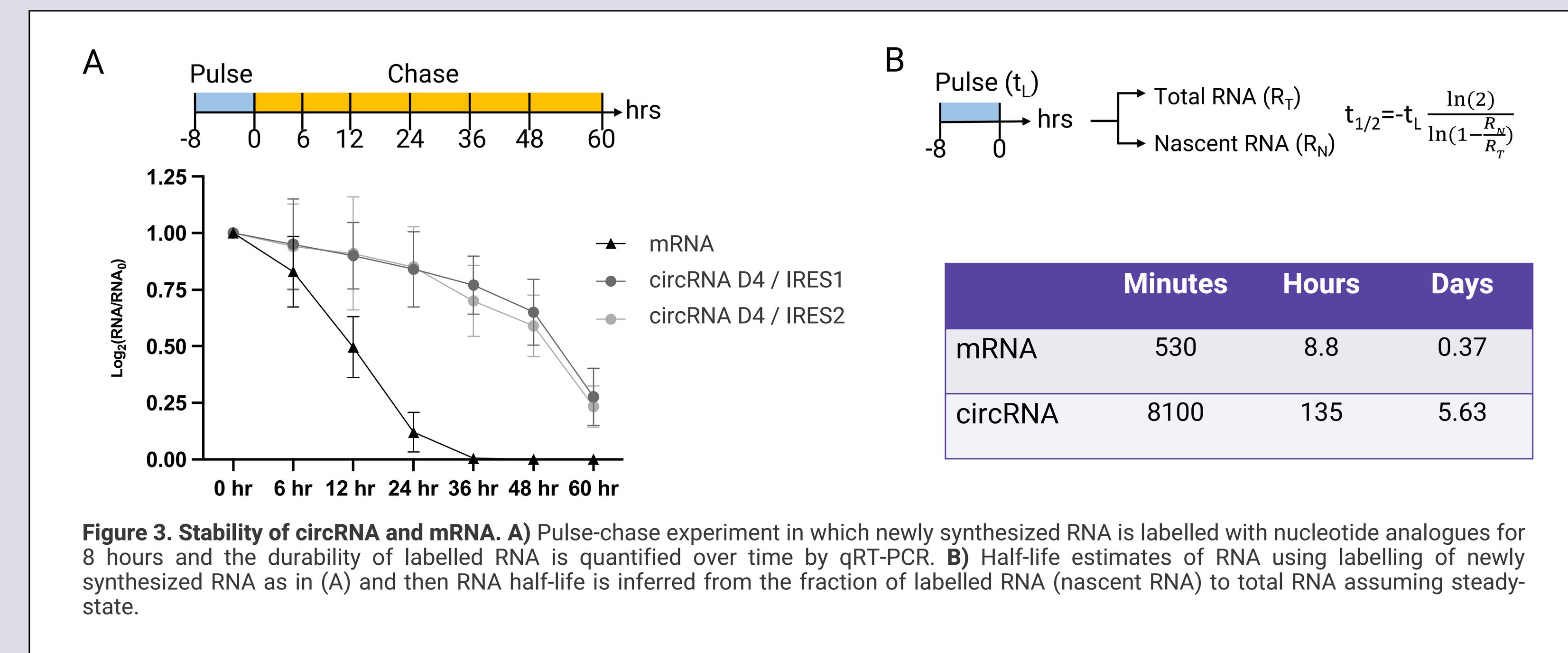


Figure 2. Cassette design impact protein yield. A) CircRNA translation depends on cap-independent translation and requires IRES (Internal Ribosome Entry Site) and ORF (Open Reading Frame) as shown. **B)** Schematic representation of vector cassette used for circRNA expression. **C)** Western blot on lysate from A375 cells transfected with plasmids expressing ten different IRES/ORF designs (D1-D10) using two different IRES elements as shown.

3. Enhanced circRNA stability

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. 3A-B**).



$$t_{1/2} = -t_L \frac{\ln(2)}{\ln(1-\frac{R_N}{R_T})}$$

	Minutes	Hours	Days
mRNA	530	8.8	0.37
circRNA	8100	135	5.63

Figure 3. Stability of circRNA and mRNA. A) Pulse-chase experiment in which newly synthesized RNA is labelled with nucleotide analogues for 8 hours and the durability of labelled RNA is quantified over time by qRT-PCR. **B)** Half-life estimates of RNA using labelling of newly synthesized RNA as in (A) and then RNA half-life is inferred from the fraction of labelled RNA (nascent RNA) to total RNA assuming steady-state.

Conclusion

Data supports:

(1) circRNA dependency on IR composition
(2) Importance of cassette design for protein-coding circRNA
(3) Durable stability of circRNA
(4) Superior protein yield compared to mRNA
(5) circRNA accumulation over time
(6) Effective inhibition of miRNA activity
(7) Successful high-yield design for circAde expression

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 warranted.

Future directions

In addition to further optimization of circRNA performance and building in additional functionalities, we are currently setting up head-to-head comparison of circRNA and mRNA expression systems *in vivo* within oncology, vaccines and gene replacement.

Acknowledgements

This study was funded by Circio ASA Norway. The authors thank everyone in the company for a great atmosphere and working environment.

4. circRNA outcompetes 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. 4A-B**), despite reduced levels of RNA (**Fig. 4C**), suggesting that cap-independent IRES-mediated translation is more effective than cap-dependent translation.

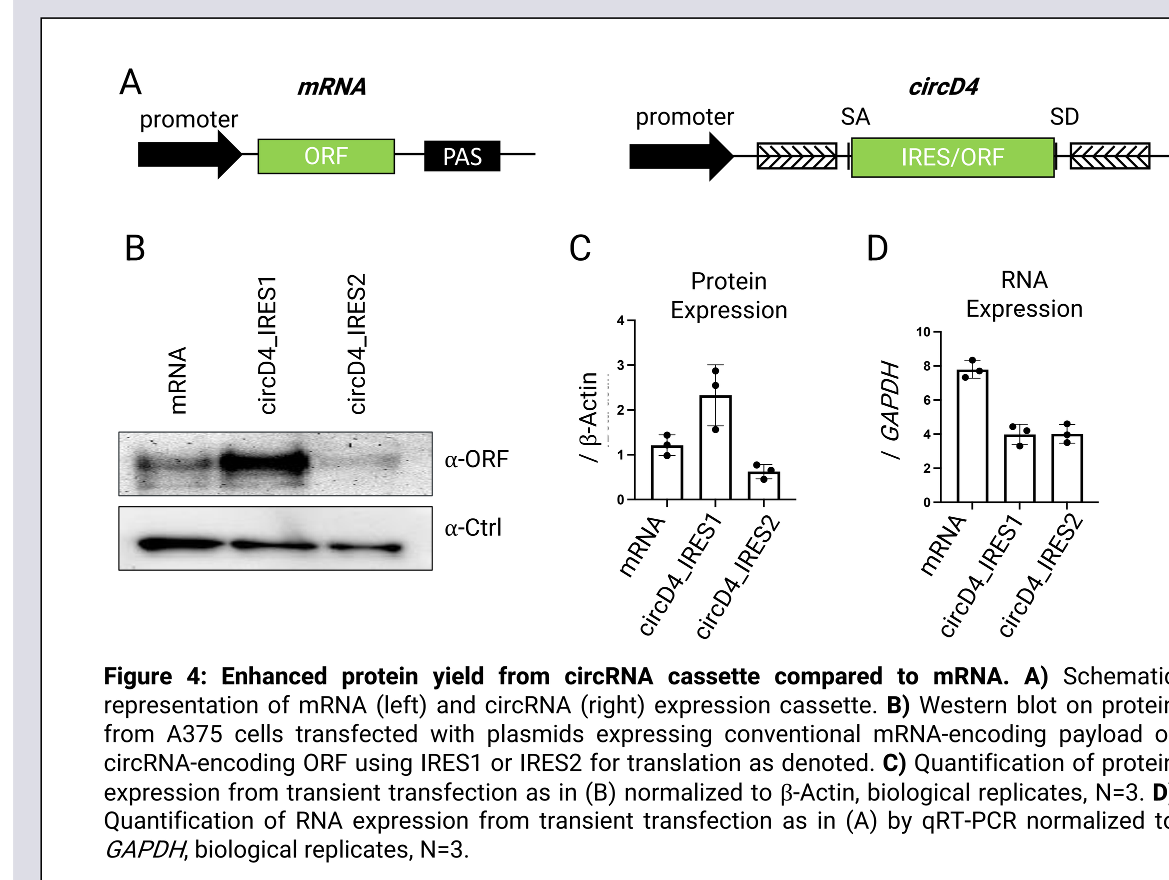


Figure 4: Enhanced protein yield from circRNA cassette compared to mRNA. A) Schematic representation of mRNA (left) and circRNA (right) expression cassette. **B)** Western blot on protein from A375 cells transfected with plasmids expressing conventional mRNA-encoding payload or circRNA-encoding ORF using IRES1 or IRES2 for translation as denoted. **C)** Quantification of protein expression from transient transfection as in (B) normalized to β-Actin, biological replicates, N=3. **D)** Quantification of RNA expression from transient transfection as in (A) by qRT-PCR normalized to *GAPDH*, biological replicates, N=3.

6. Repression of miRNAs

MicroRNAs (miRNAs) are a conserved group of ~22nt regulatory RNAs involved in most if not all cellular pathways. MiRNAs act by targeting and destabilizing mRNAs harboring binding sites in the 3'UTR (**Fig. 6A**). In contrast, circular RNAs are resistant to miRNA-mediated destabilization and thus serves naturally as inhibitors of miRNA activity (**Fig. 6A**). With an effective platform for circRNA biogenesis, we show effective repression of miR-21 by engineering MREs (miRNA response elements) into the circRNA sequence (**Fig. 6B**).

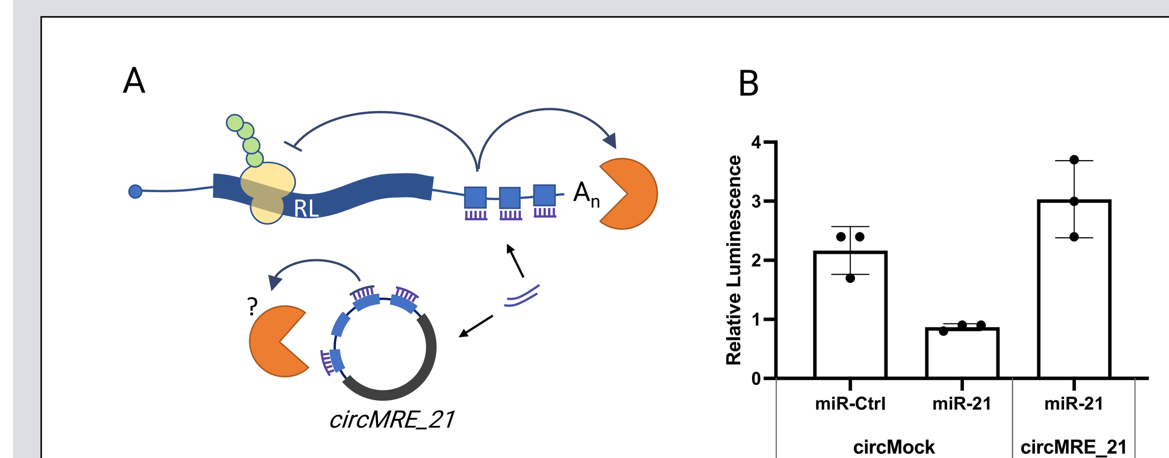


Figure 6. circRNAs effectively inhibit miRNA activity. A) Conceptual representation of miRNA inhibition by circRNA: Conventionally, miRNAs target the 3'UTR in mRNA, repress translation and stimulate mRNA turnover by recruiting exonucleolytic factors. Inserting miRNA response elements (MRE) into the circRNA results in miRNA sequestering and miRNA turnover without any impact on circRNA stability thus leading effectively to alleviation of target gene repression. **B)** Luciferase reporter assay measuring miRNA activity (low luminescence infers high miRNA activity).

5. circRNA level accumulates

The enhanced stability of circRNA (see **Fig. 3**) results in RNA and protein accumulation over time, whereas mRNA levels decline (**Fig. 5A-B**). Despite reduced RNA quantities at the early timepoint (48 hrs, **Fig. 5A**), circRNA-based translation results in elevated protein yield (**Fig. 5B**).

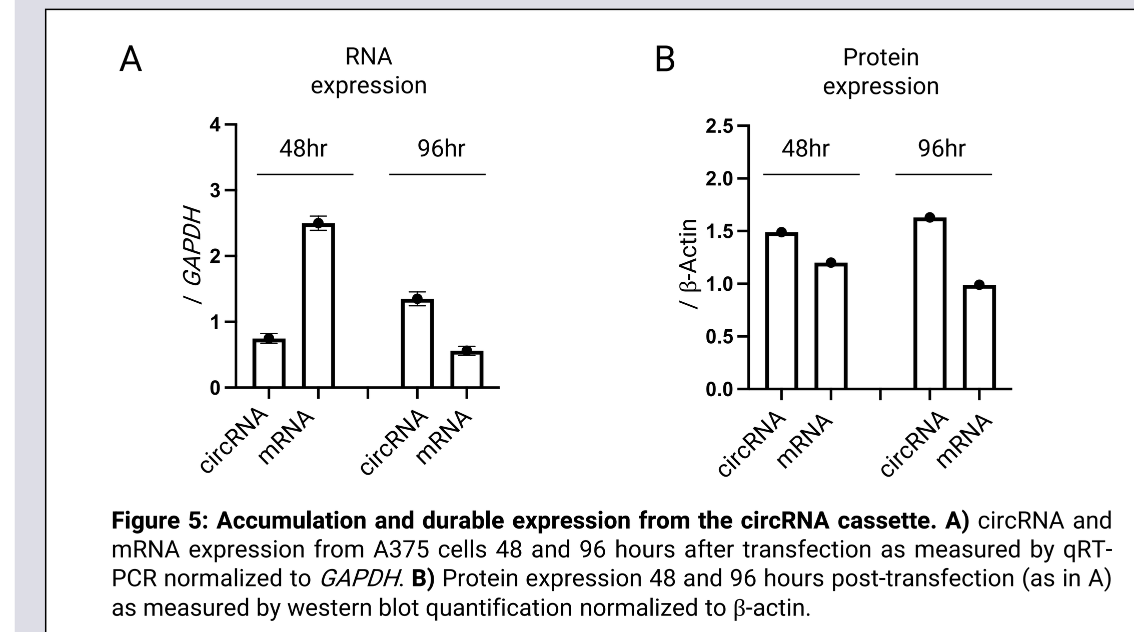


Figure 5: Accumulation and durable expression from the circRNA cassette. A) circRNA and mRNA expression from A375 cells 48 and 96 hours after transfection as measured by qRT-PCR normalized to *GAPDH*. **B)** Protein expression 48 and 96 hours post-transfection (as in A) as measured by western blot quantification normalized to β-actin.

7. circAde vector development

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. 7A**). Dramatic differences in circRNA-based protein expression were observed for the functional circAdes (**Fig. 7B**).

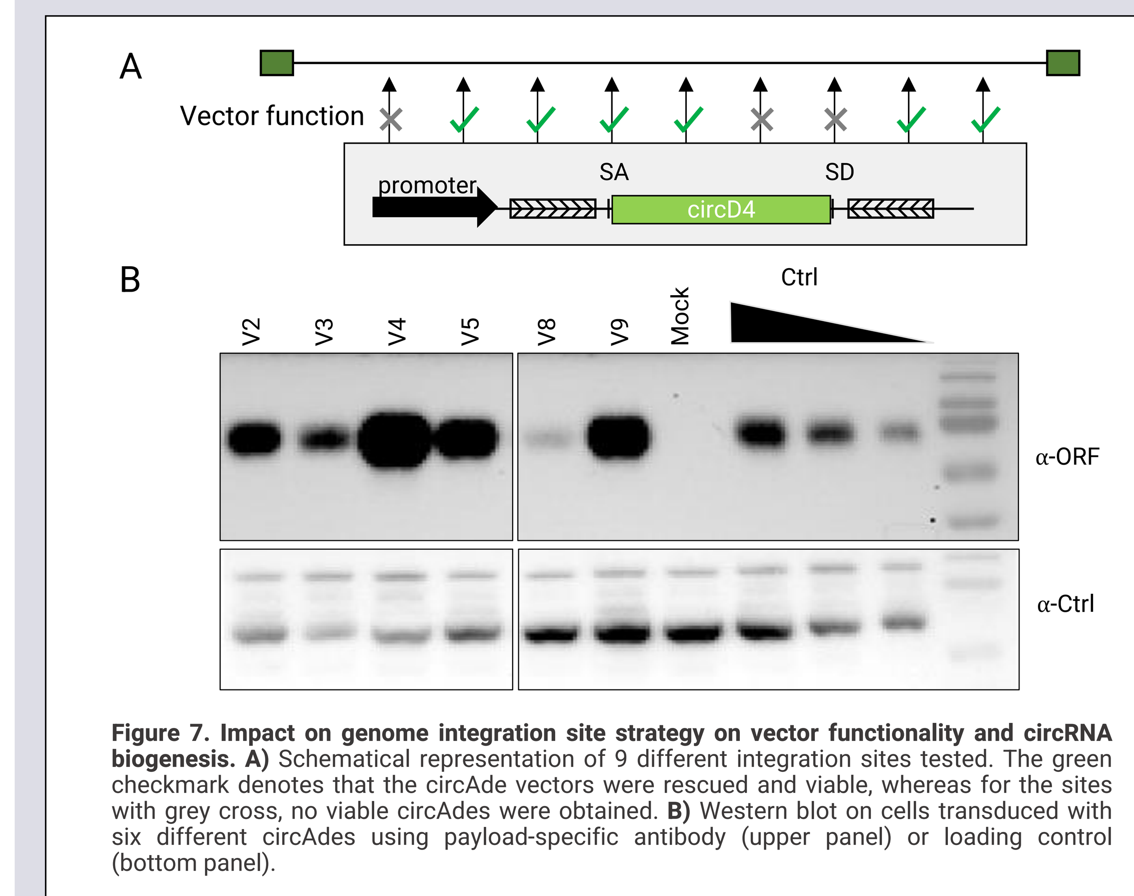


Figure 7. Impact on genome integration site strategy on vector functionality and circRNA biogenesis. A) Schematical representation of 9 different integration sites tested. The green checkmark denotes that the circAde vectors were rescued and viable, whereas for the sites with grey cross, no viable circAdes were obtained. **B)** Western blot on cells transduced with six different circAdes using payload-specific antibody (upper panel) or loading control (bottom panel).

Contact information

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