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Circular RNA-based AAV heart gene therapy enhances transgene expression levels by up to 40-fold, reduces liver off-targeting, and minimizes cellular stress.

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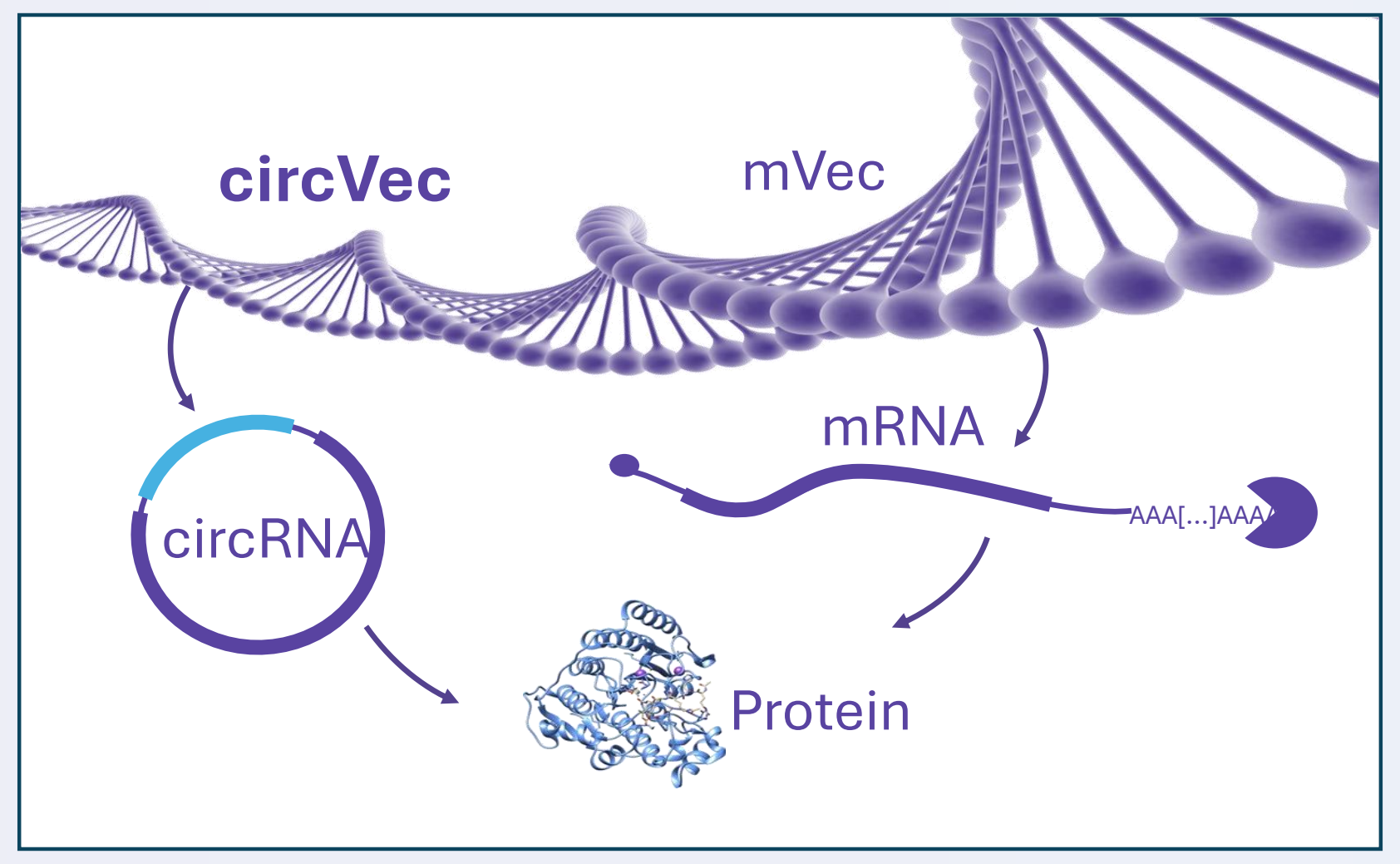
Introduction

Circular RNA (circRNA) is a novel class of endogenously expressed RNA generated by a non-linear splicing event, known as **backsplicing**. circRNAs **are resistant to exonucleolytic decay**, which results in **increased intra-cellular stability** and persistence compared to mRNA. We have developed a circRNA-based expression platform, **circVec**, which utilizes this natural stability advantage to improve vector-based protein expression.



1 Optimizing gene expression from circVec

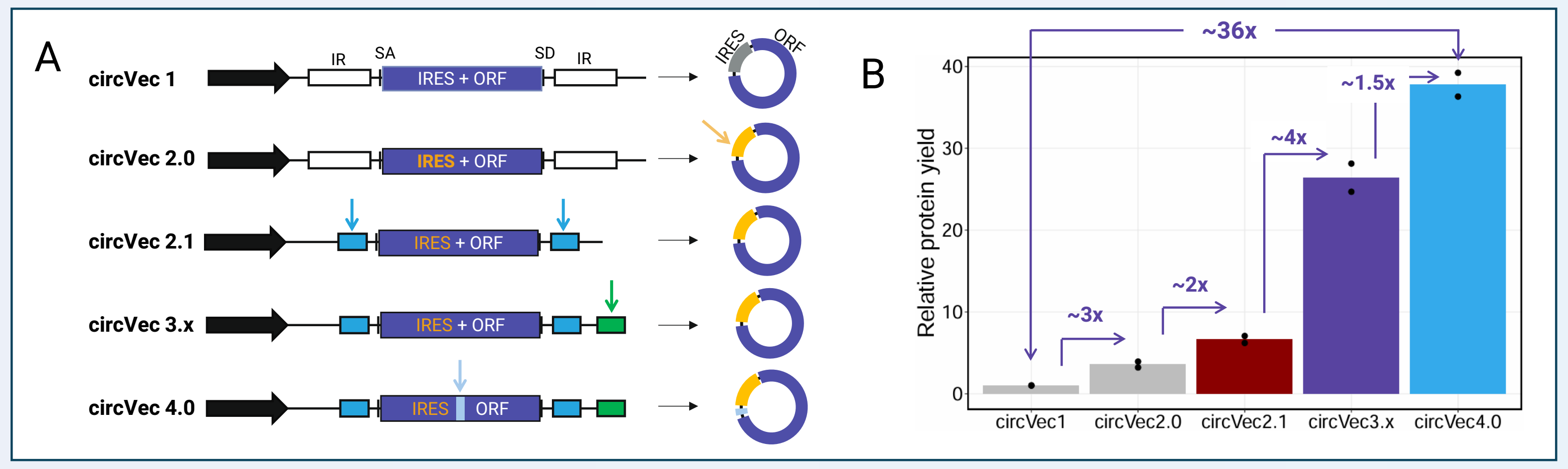


Figure 1: circVec platform evolution. A) Schematic representation of circVec designs. **B)** Relative protein yield from different generations of circVec in HepG2 cells (n=2).

2 circVec increases gene expression in heart by up to 40-folder compared to benchmark AAV9

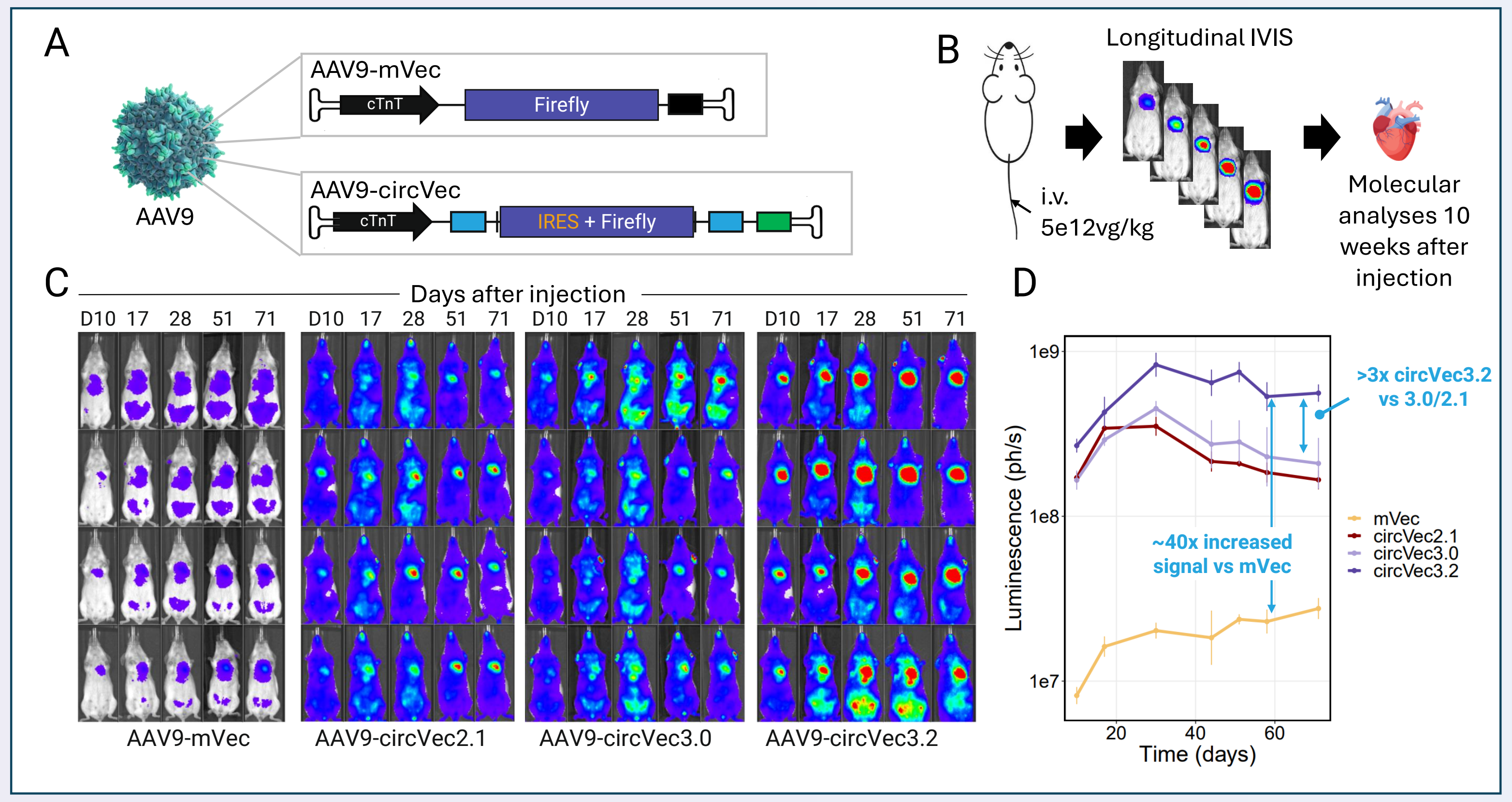


Figure 2: In vivo expression of different cassettes using AAV9 with heart-specific promoter (cTnT). A) Schematic representation of AAV9-mVec and AAV9-circVec, both encoding Firefly luciferase. B) Experimental design: intravenous tail vein injection followed by longitudinal IVIS scans to monitor Firefly luminescence and distribution. Finally, select tissues are subjected to downstream molecular analyses. **C)** Longitudinal IVIS analysis at different timepoints after i.v. injection of different AAV9 vectors (5e12 vg/kg) in female BALB/c mice (n=4). **D)** Quantification of IVIS signal from whole mouse body over time shown as geometric mean + s.e.m.

3 Ex vivo analysis confirms enhanced and highly tissue-specific expression in the heart

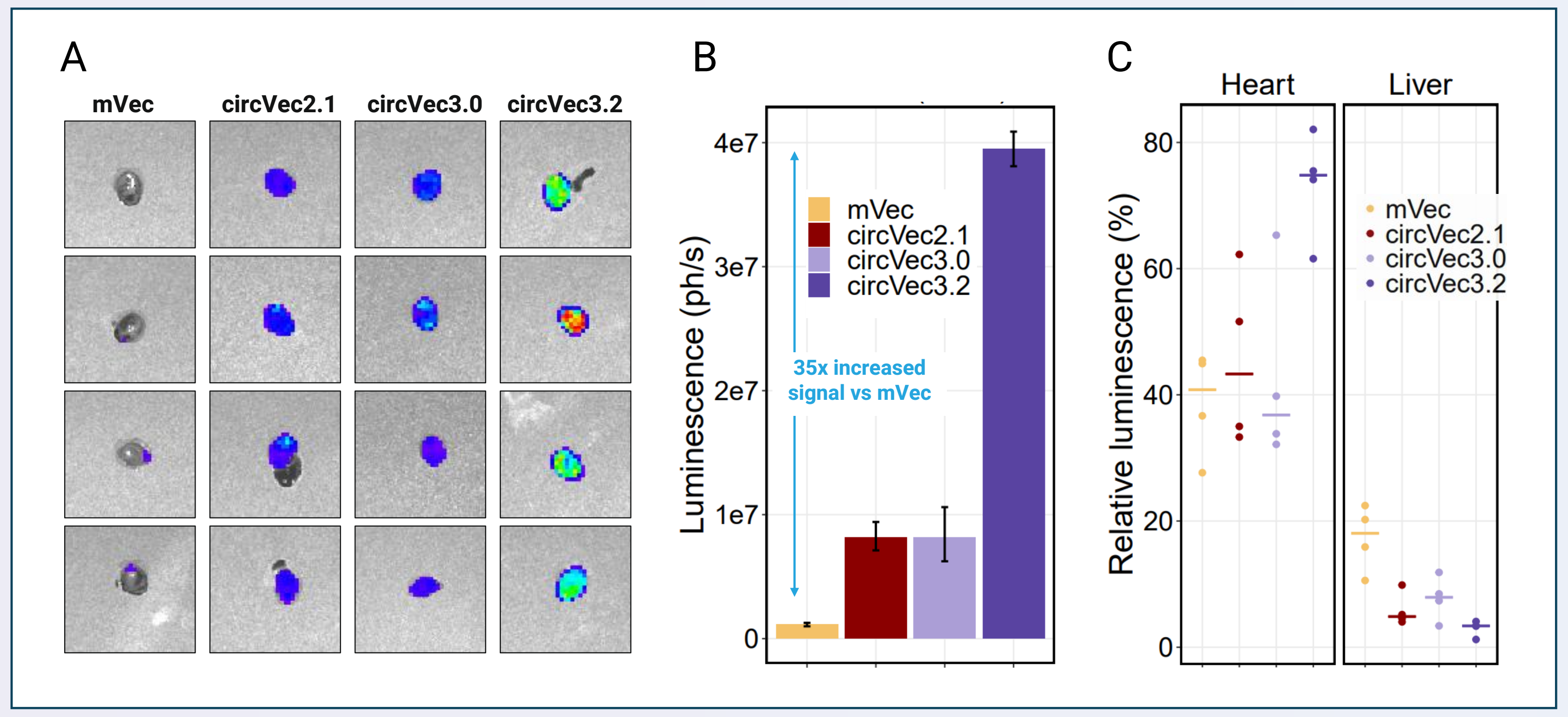


Figure 3: Ex vivo analysis of heart-specific luminescence. A) IVIS scans of the heart organs from the different groups as denoted (n=4). **B)** Quantification of signal from the heart presented as geometric mean ± s.e.m. **C)** Percentage of expression across all tissues analyzed ex vivo (heart, liver, kidney, lung, spleen, intestine, diaphragm, and hindlegs). Here, only heart and liver data is shown for individual mice (points) and the group-wise mean (horizontal line).

4 RNAscope analysis shows 80% circRNA positive cardiomyocytes at 5e12 vg/kg dose

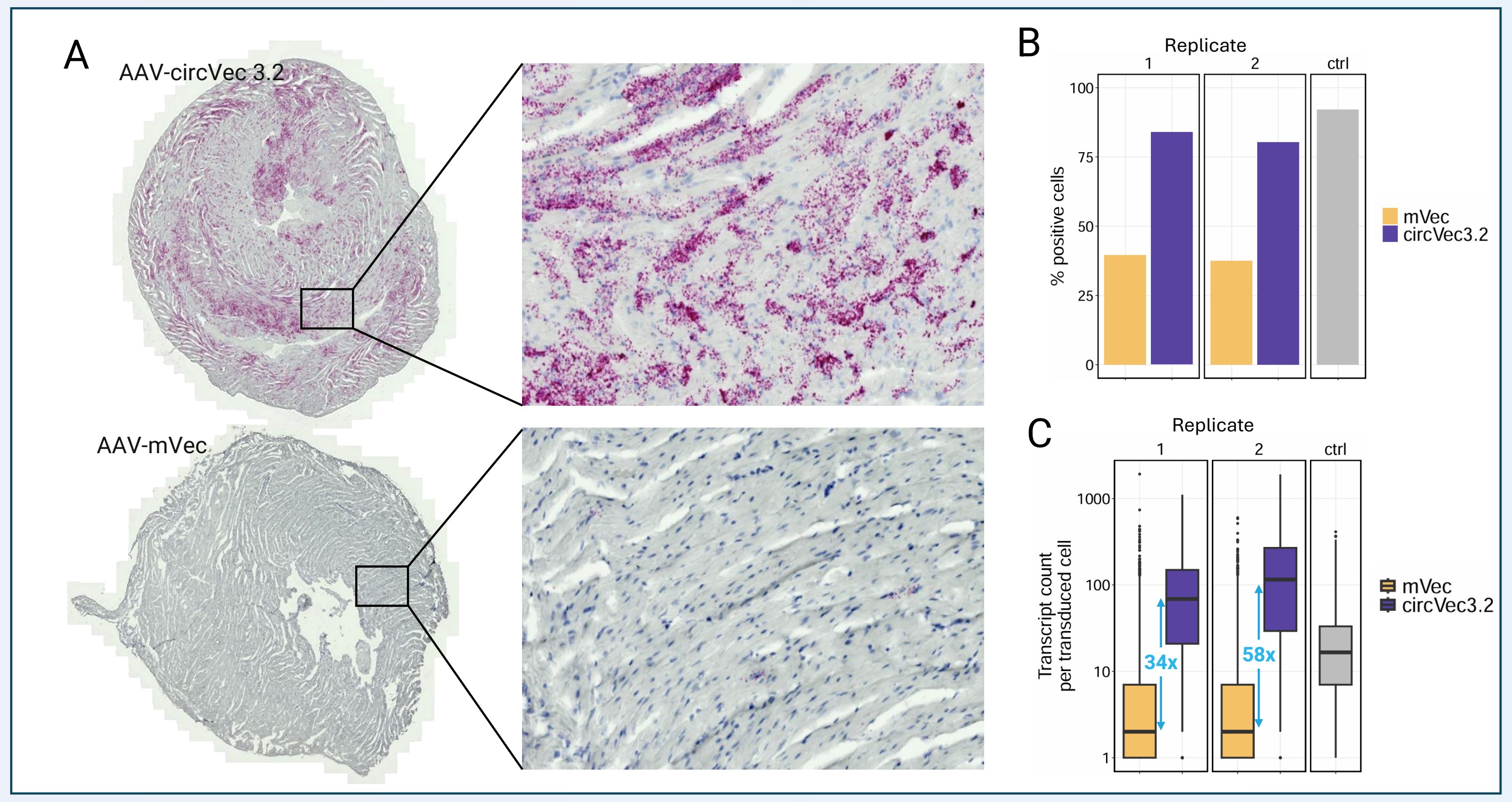


Figure 4: RNAscope analysis on mouse heart. A) Representative heart sections from AAV9-circVec3.2 or AAV9-mVec treated mice (as denoted) probed for Firefly RNA (violet) using RNAscope technology and strained with Dapi (blue). **B)** Using the QuPath software, number of positive cells were extracted. *Ppib* (peptidyl prolyl iosomerase B) is used as the positive control marker (ctrl). **C)** Distribution of Firefly transcript copy-numbers in positive cells showing 34x-58x more RNA from circVec3.2 compared to mVec.

5 Circular RNA accumulation drives enhanced gene expression and reduces cellular stress response

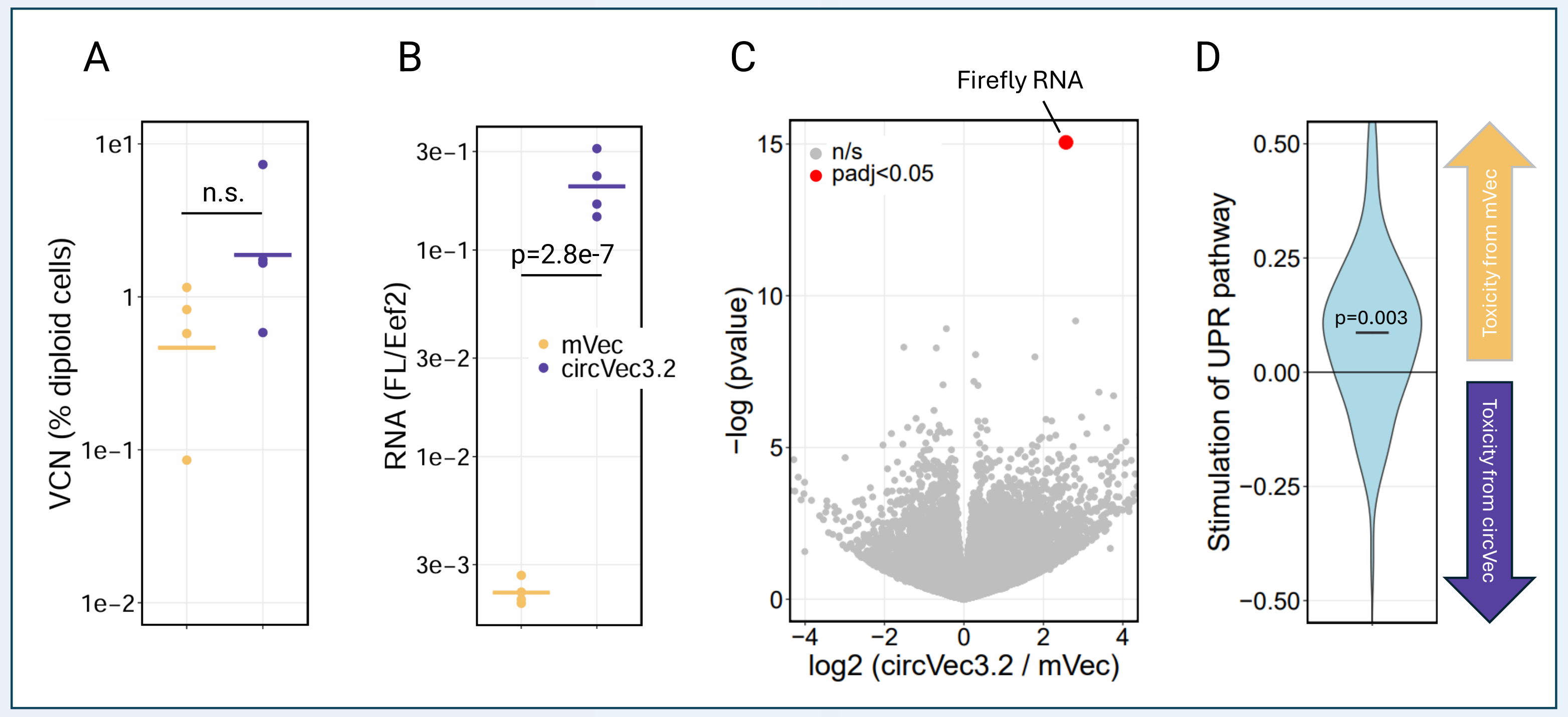


Figure 5: Viral genome and transcriptomic analysis on heart samples. A-B) AAV genome (A) and Firefly RNA (B) quantification on heart tissue 92 days after i.v. injection of 5e12vg/kg AAV9-circVec3.2 or AAV9-mVec, as denoted. T-test on log-transformed values is used to determine significance, n.s.: not significant. **C)** Volcano plot based on whole transcriptomic analysis of heart tissue (n=4/group). Only Firefly RNA shows a significant change in expression (red dot, BH-adjusted p-value (padj) = 0.01). **D)** Unfolded protein response (UPR) genes as a class are significantly upregulated in mVec heart tissue compared to circVec (p=0.003, Wilcoxon rank sum test).

6 circVec enables at least 10x dose reduction compared to conventional AAVs

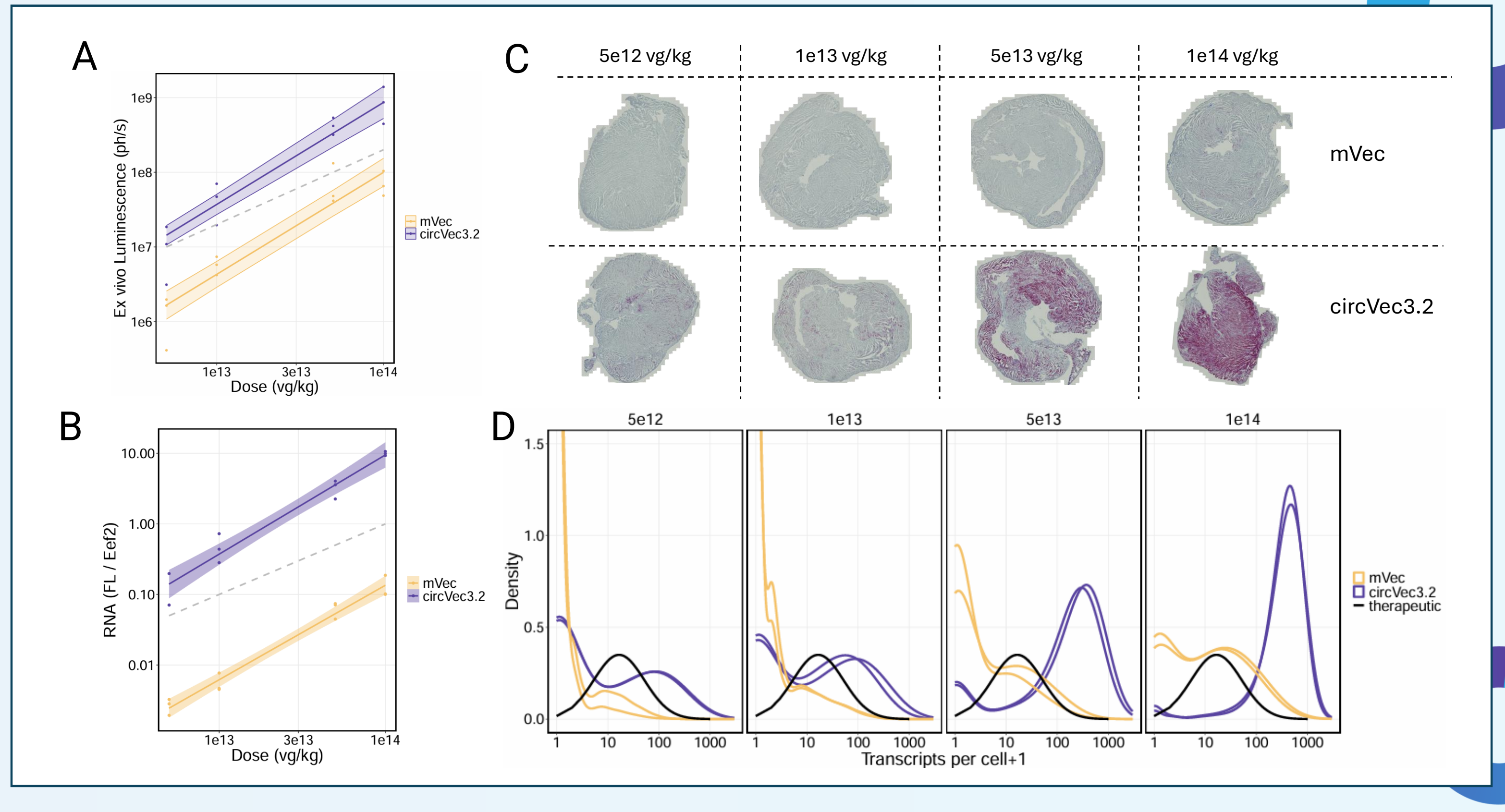


Figure 6: Dose-response relationship for AAV9-circVec and AAV9-mVec. A-B) Super-linear dose response curve on ex vivo luminescence (A) and Firefly RNA quantities (B) from heart 54 days after injection of AAV9-mVec or AAV9-circVec3.2 using 4 different dose levels: 5e12, 1e13, 5e13, and 1e14 vg/kg. Dashed line represents linear dose-response with a slope of 1. **C)** RNAscope images on representative hearts form mice subjected to the different doses of AAV9-mVec or AAV-circVec as denoted. **D)** Impact of dose level on transcript distribution based on RNAscope. Black line represents estimated distribution of a therapeutic relevant mRNA, such as *Pkp2.*

7 circVec 4.0 boosts heart expression by 50%

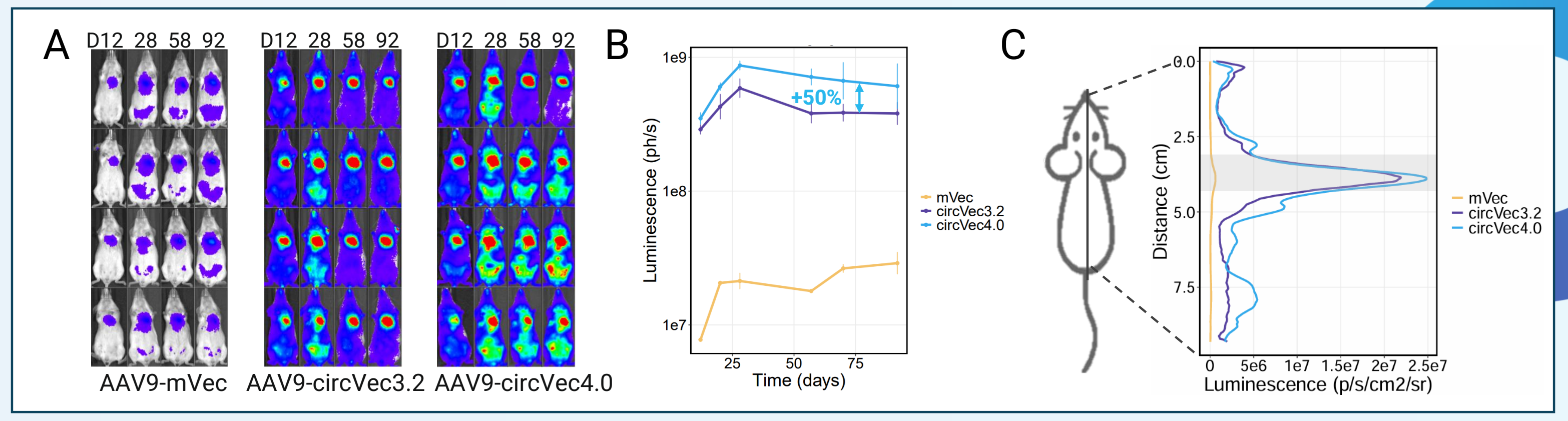


Figure 7: Characterization of AAV9-circVec4.0 expression in heart. A) Longitudinal IVIS analysis (as in 2C). **B)** Quantification of luminescence over time for mVec, circVec3.2 and circVec4.0 as denoted. **C)** signal distribution across the vertical section from head to tail. Shaded area corresponds to heart region.

CONCLUSIONS

- ○ Superior stability and accumulation of circRNA results in up to **40x enhanced protein expression** in heart from **AAV9-circVec** compared to benchmark AAV9
- ○ **Reduced off-target liver expression** observed from AAV9-circVec, likely due to reduced circRNA stability and gene expression in hepatic cells
- ○ RNAscope analysis shows **higher and broader expression** of circRNA compared to mRNA from equivalent vector copy numbers in heart
- ○ **Reduced activation of unfolded protein response (UPR)** pathway, despite increased RNA and protein levels by circVec
- ○ Dose-response exploration shows indicates opportunity for **at least 10x dose reduction** compared to conventional AAV-based heart gene therapy
- ○ In vivo evaluation of the therapeutic potential of AAV9-circVec ongoing in **arrhythmogenic cardiomyopathy (AC)** and **Danon disease.**

Interested in more data?
Join our talk, "New Insights into AAV Genome Biology",
Friday 4.15pm, Westlin Seaport Ballroom AB