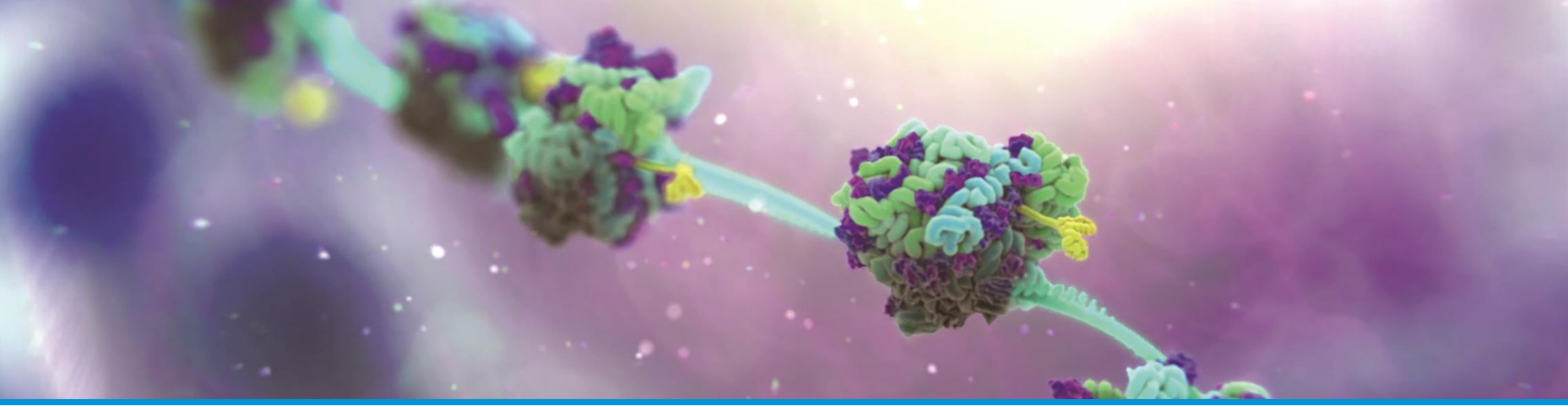

Splint and Ribozyme-Free Enzymatic Synthesis and Purification of Long Circular RNA for in vitro Translation in Human Cells

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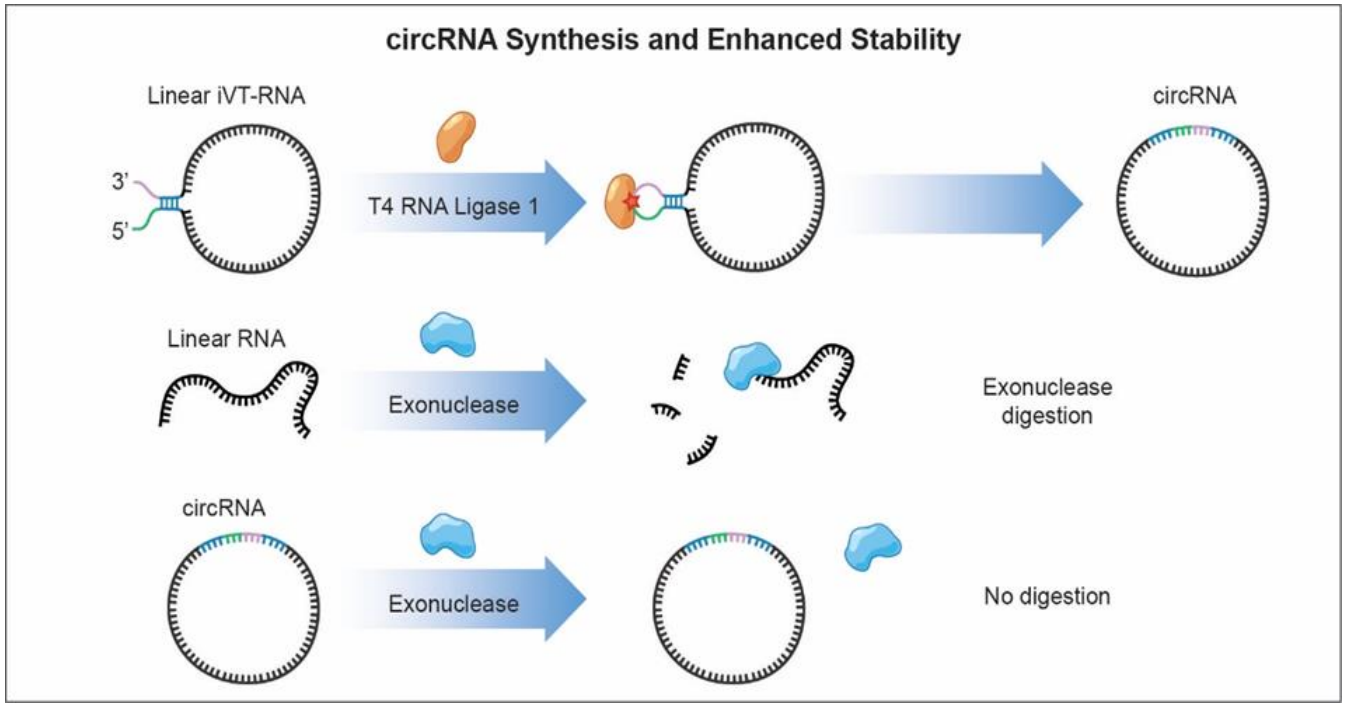
Motivation



- Messenger RNA (mRNA) has many applications in therapeutic development
  - Short half-life in vivo
- Circular RNAs (circRNAs) have enhanced stability
  - Synthesis is more challenging
- Current methods require complex purification
- Efficient homology-mediated method of generating circRNA
  - Simple purification

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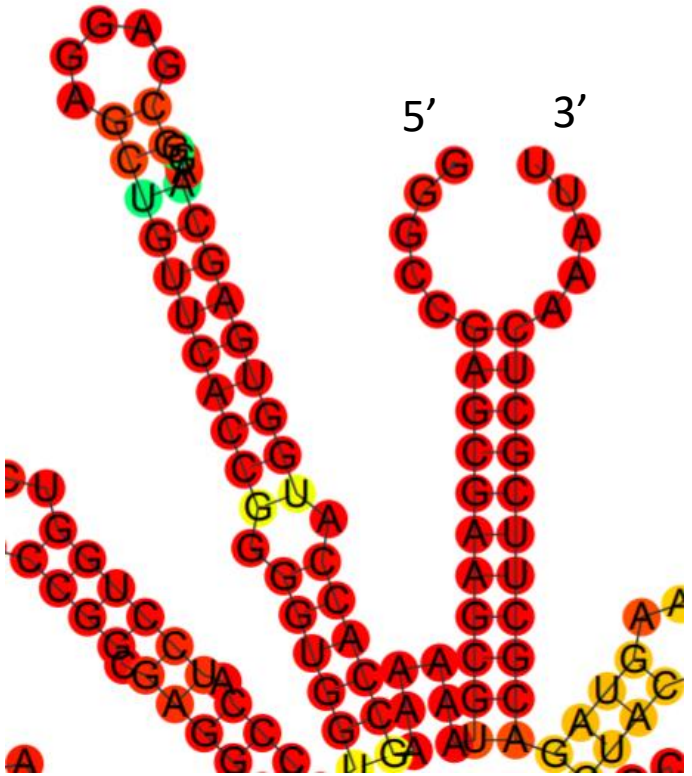
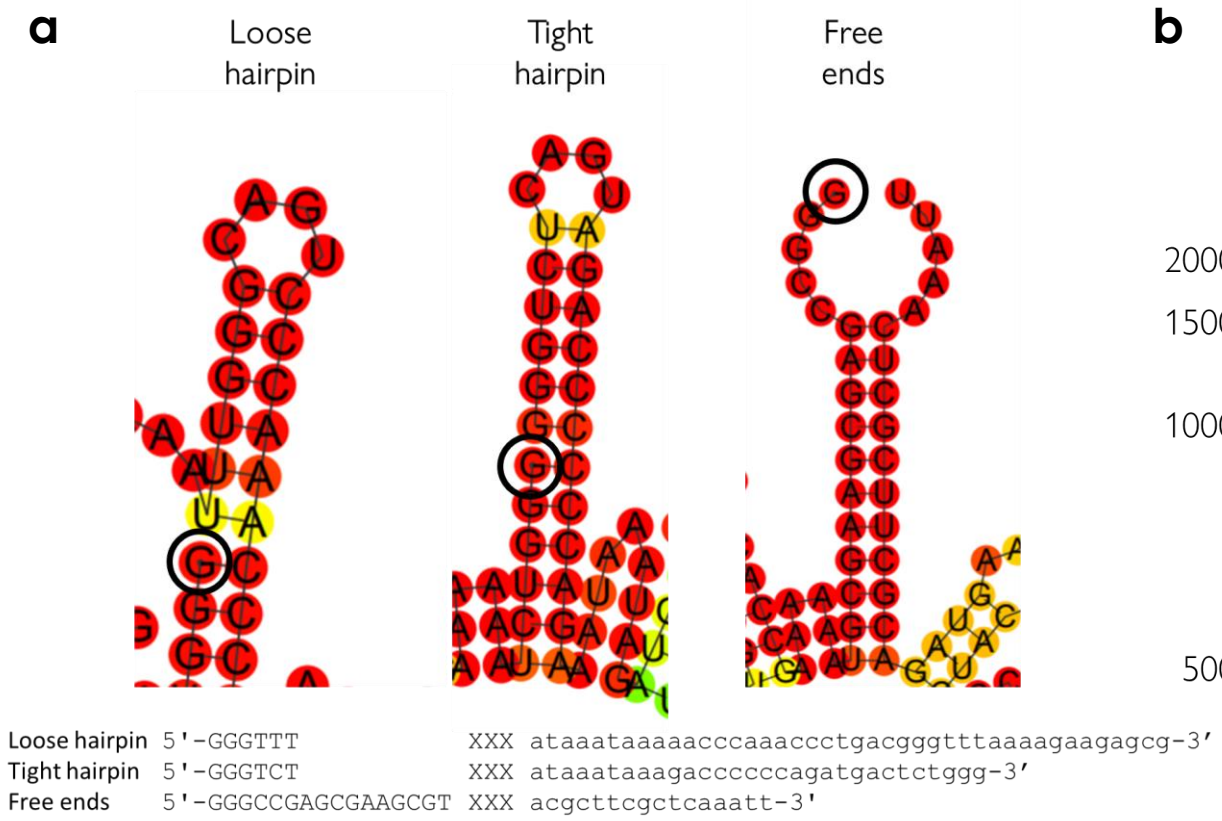
Methods: Synthesis Strategy

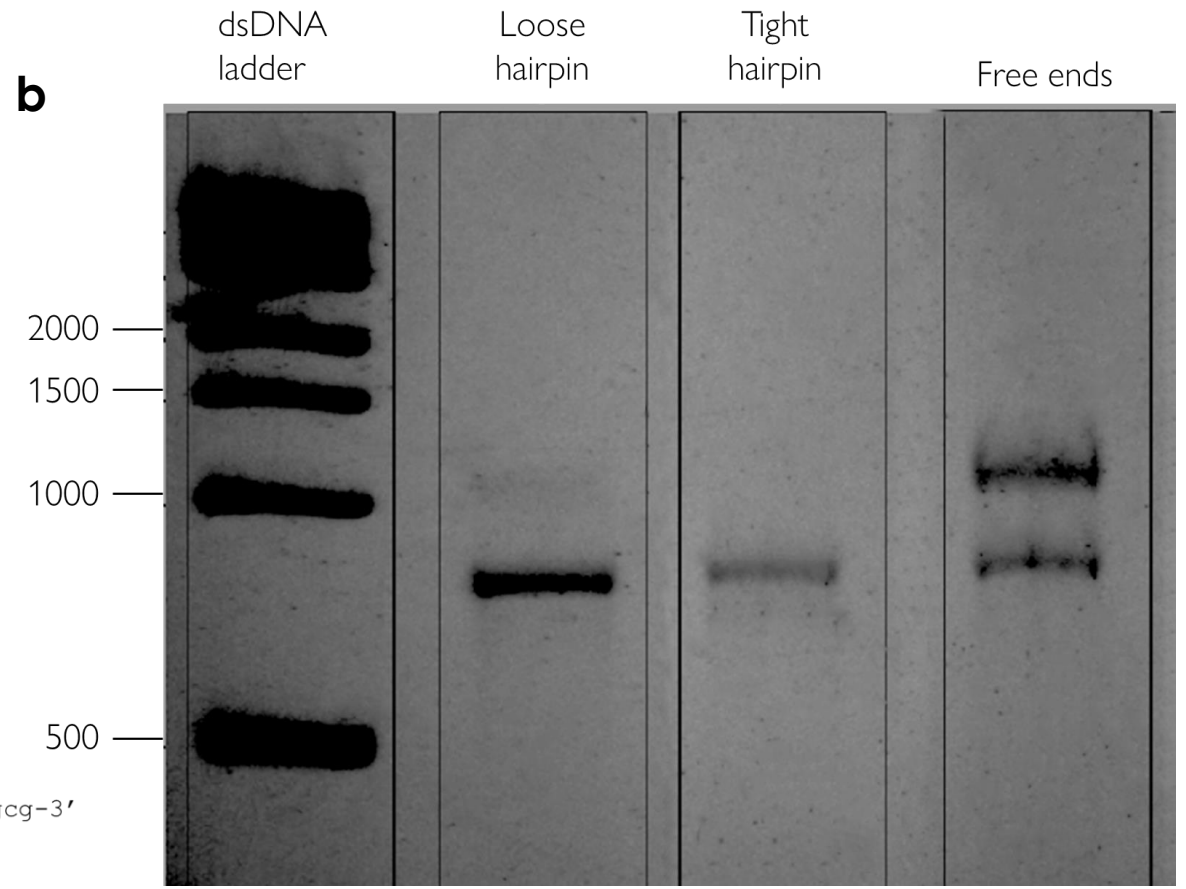
1. In vitro transcription to generate 5' monophosphorylated linear mRNA precursor
   - 20:1 GMP:GTP ratio
2. Anneal hairpin (homology sequence) to bring 5' and 3' ends in close proximity
   - 95°C/2m; cool 1°C/10s to 24°C
3. T4 RNA Ligase 1 treatment overnight
   - Maximize reaction volume to favor intramolecular ligation
4. Phosphatase treatment to digest remaining trinucleotides
   - 1 unit Quick CIP / 1 µg RNA
5. RNase R treatment to digest contaminating linear species
   - 1 unit RNase R / 1 µg RNA



RNA folding image demonstrating hairpin with 5' and 3' ends in close proximity.

Results: 5’ and 3’ Ends of Linear RNA Precursor Must be Relatively Free for Optimal Ligation by T4 RNA Ligase 1





a RNA folding images displaying differential end mobility of three constructs. **b** Effect of 5’ and 3’ end mobility on ligation efficiency. Hairpin was annealed for each RNA construct, and subsequent T4 RNA Ligase 1 treatment overnight was performed. Circular RNA species display higher molecular weight than linear precursor. Lane 1: RNA molecule where 5’ and 3’ ends participate in loose hairpin base pairing. Lane 2: RNA molecule where 5’ and 3’ ends participate in tight hairpin base pairing. Lane 3: 5’ and 3’ ends are free (not involved in base pairing)

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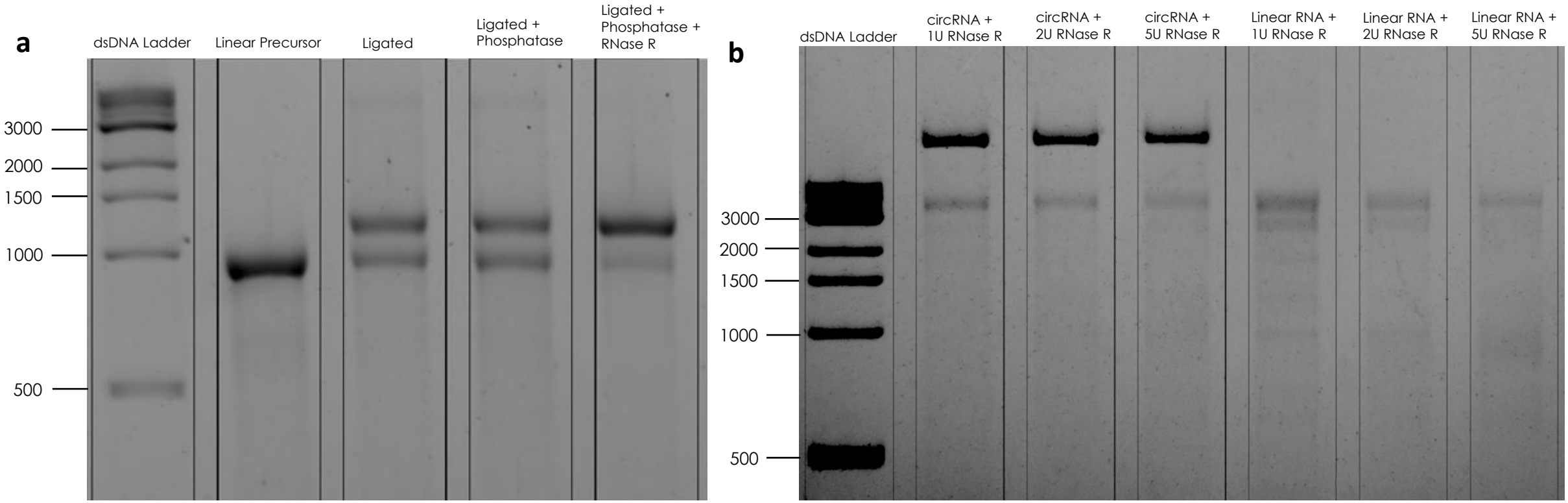
Results: circRNA is Resistant to RNase R Treatment Compared to Linear RNA

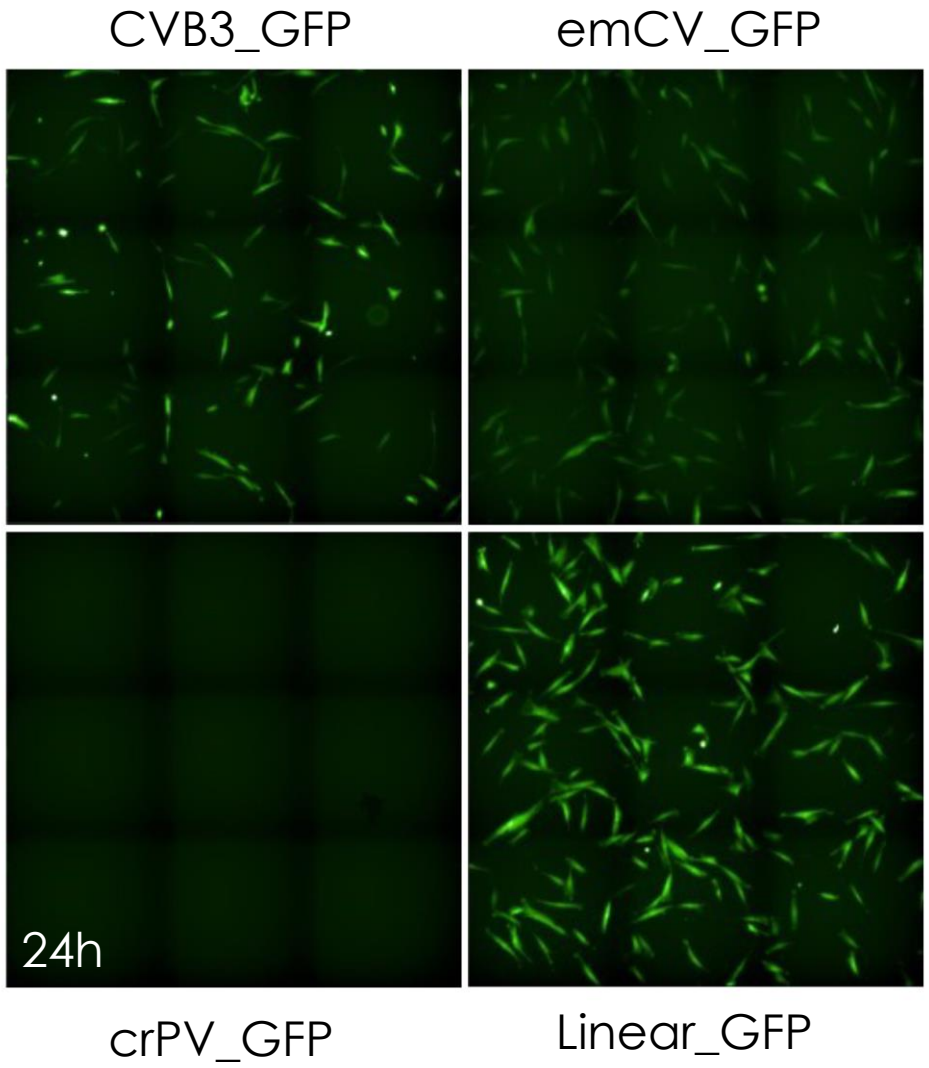


a Sequential processing steps of circRNA. Linear iVT precursor runs at lower molecular weight as demonstrated. Following RNase R Digestion, circular product is enriched, shown in last lane. **b** circRNA displays resistance to increasing amounts of RNase R per µg RNA. Linear RNA is degraded in presence of RNase R.

Results: circRNA is Readily Translated and Expressed in Primary Human Fibroblasts

Confocal microscopy images of GFP Expression 24 hours post electroporation.

120k primary human fibroblasts were electroporated with 0.75µg of circular RNA with different IRES elements and linear RNA with same GFP sequence



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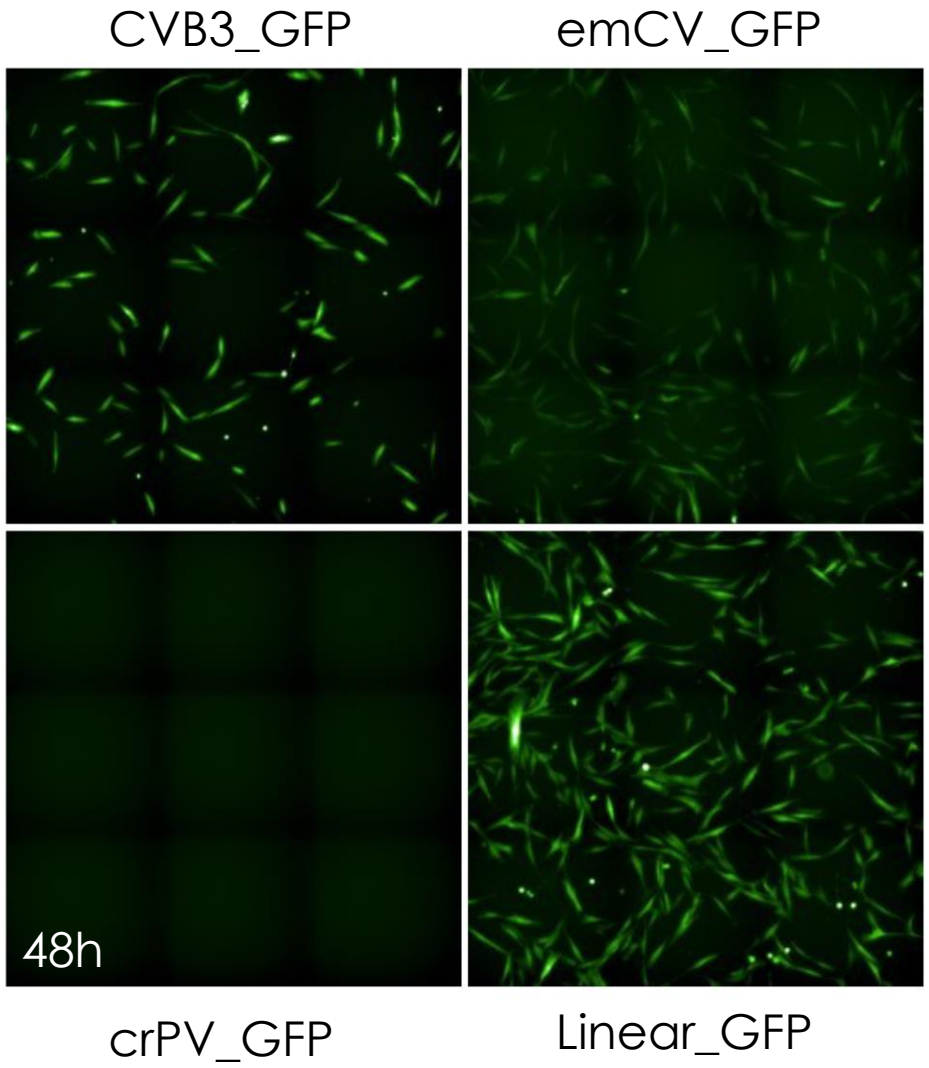
Results: circRNA is Readily Translated and Expressed in Primary Human Fibroblasts

Confocal microscopy images of GFP Expression 48 hours post electroporation.



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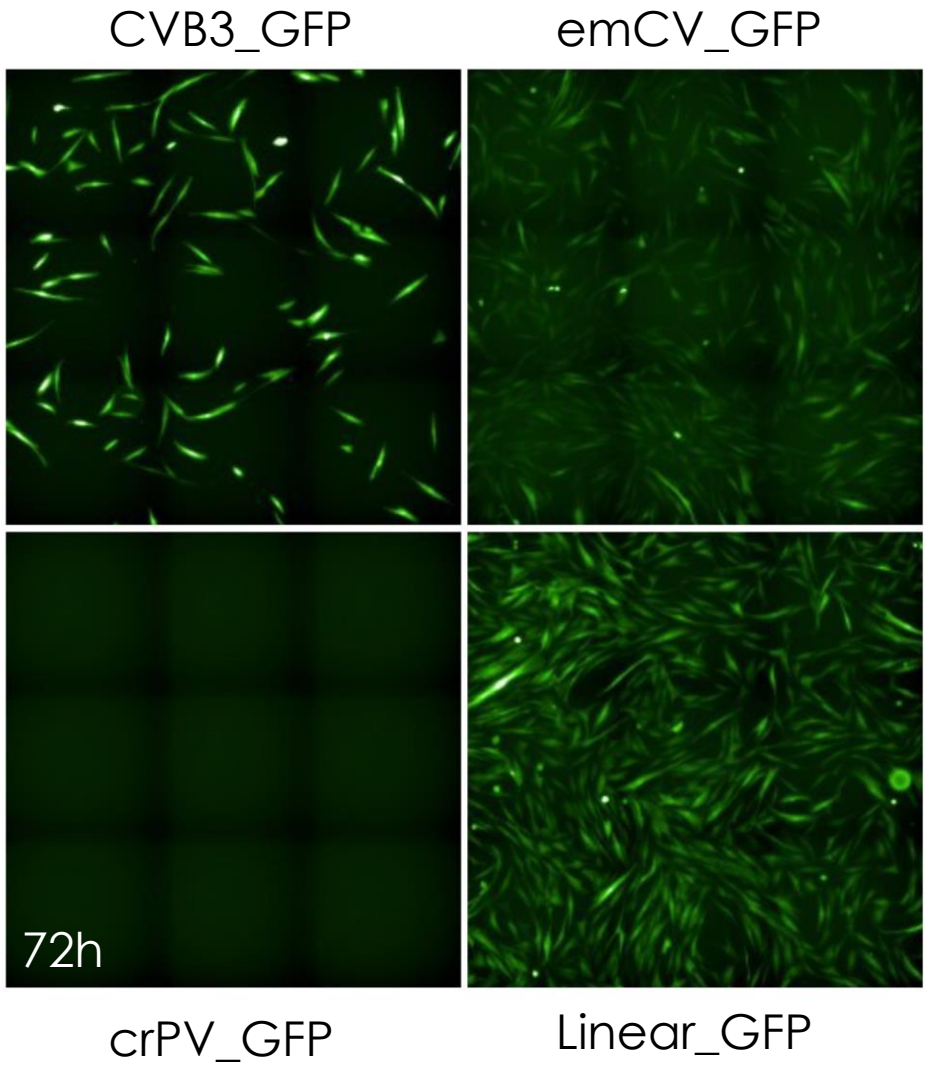
Results: circRNA is Readily Translated and Expressed in Primary Human Fibroblasts

Confocal microscopy images of GFP Expression 72 hours post electroporation.



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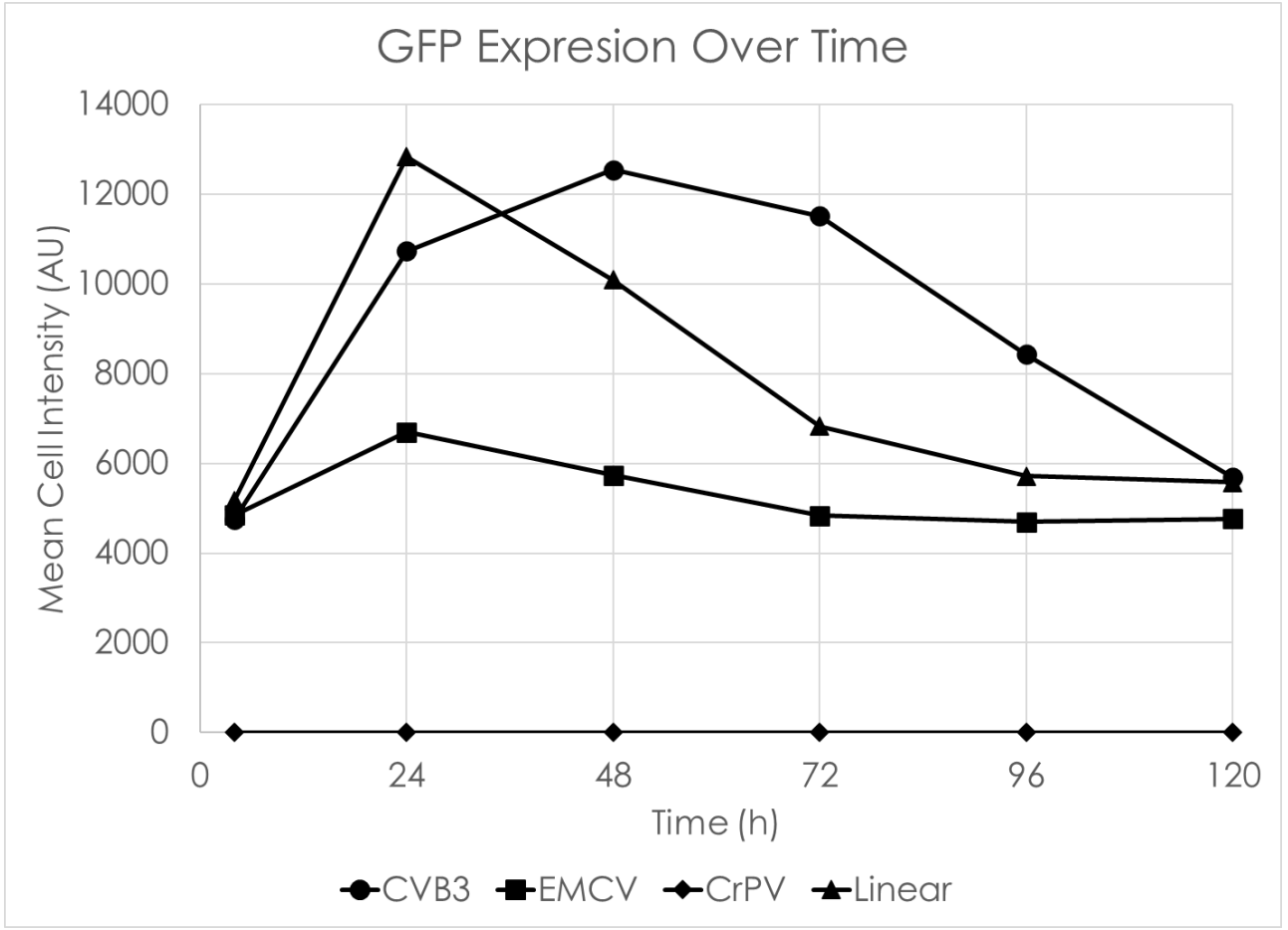
Results: circRNA encoding for GFP with CVB3 IRES has Extended Expression Profile Compared to Linear RNA



Mean cell intensity (GFP) over 5-day time course. As demonstrated by the graph, RNA sequencing encoding CVB3 IRES-GFP displays extended expression profile compared to linear GFP encoding mRNA

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Confidential

Acknowledgments

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