Rapid synthesis and functional validation of full-length human dystrophin constructs enabling next-generation gene therapy development for duchenne muscular dystrophy

Perry Demsko[1*], Daniel Lin-Arlow[2*], Victor Yu[1], Andrew Dinh[1], Nica Tofighbakhsh[1], Krishna Ajjarapu[1], Chloe Chen[1], Andrew Huang[1], Martin Huang[1], Varsitaa Srinivasan[1], and Joseph B. Alzagatiti[1*†]

*Shared Authorship; †Corresponding Author
†Primary Contact: joseph.alzagatiti@endofold.bio

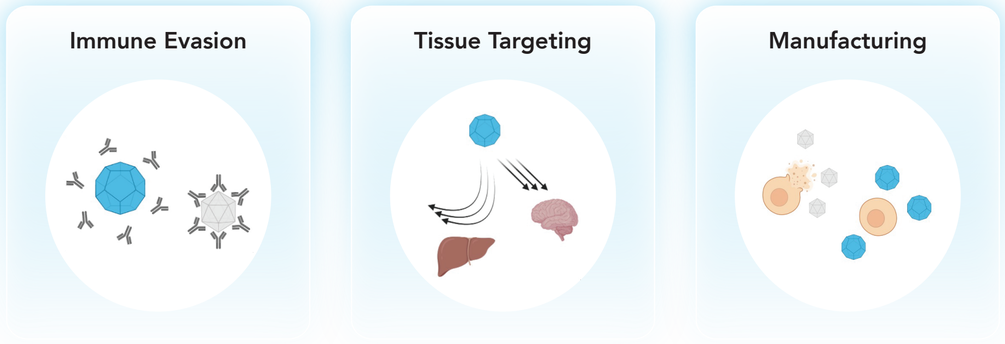
ABSTRACT

Duchenne muscular dystrophy (DMD) is a severe X-linked disorder affecting 1 in 3,500–5,000 males, caused by mutations in the DMD gene that abolish functional dystrophin protein expression. While gene therapy represents a promising therapeutic avenue, clinical development has been constrained by the ~4.7 kb packaging limit of adeno-associated virus (AAV) vectors — far smaller than the ~11–14 kb dystrophin coding sequence. This has forced the field toward truncated micro-dystrophin constructs that omit potentially important functional domains.

Beyond delivery, a less appreciated bottleneck is the difficulty of synthesizing full-length dystrophin constructs: conventional DNA synthesis providers either cannot accommodate sequences of this size and complexity or require extended lead times (>6–8 weeks), severely limiting design iteration. We sought to address these concerns in a proof-of-concept experiment. The preliminary work reports the rapid synthesis of a complex, full-length human dystrophin plasmid for use in next generation, RNA gene therapy applications.

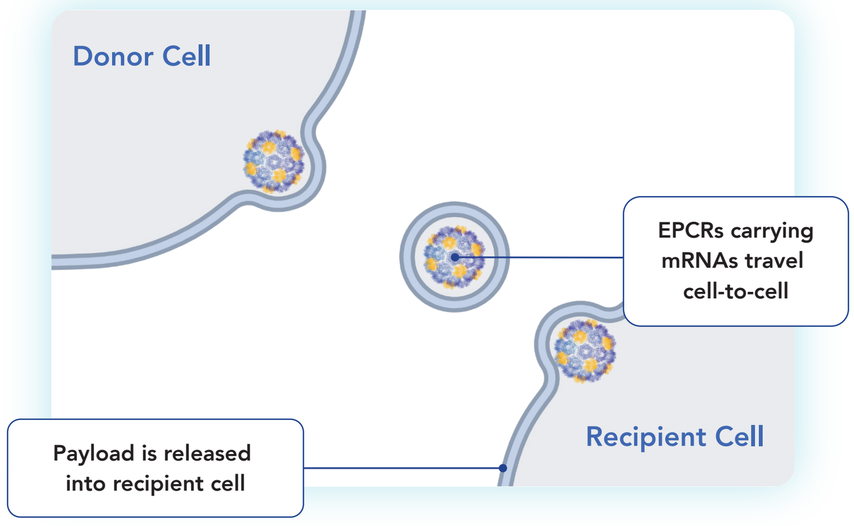
ENDOGENOUS PROTEIN CARRIERS OF RNA

Limitations of Current Gene Delivery Modalities

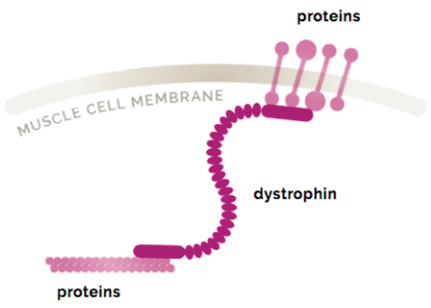


- LNPs and AAVs lack biocompatibility with the human body, leading to high immunogenicity (1,2)
- For LNPs, their widespread deployment has led to a sharp increase in PEG allergies, and the PEG formulation contributes to high immunogenicity; both precluding necessary repeat dosing of RNA-based therapies (1)
- Many gene therapies require extensive R&D for large, complex plasmids for their production instead of plug/play availability

Version Update: Human Proteins Traffic RNAs



Use Case for Complex DNA Synthesis: DMD



Duchenne's Muscular Dystrophy (DMD) is a severe, progressive, X-linked recessive genetic disorder primarily affecting males, caused by mutations in the DMD gene located on the X chromosome (6).

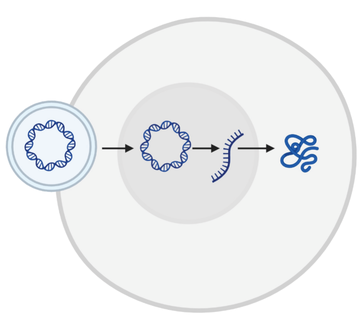
DMD affects 1 in 3500 to 5000 boys worldwide with no definitive cure.

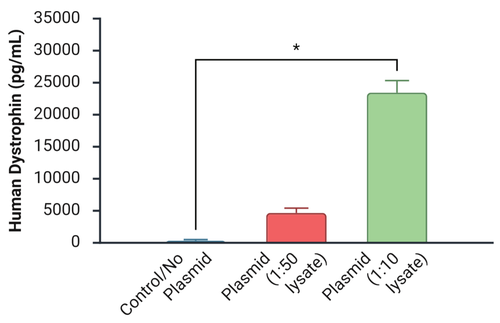
Mutations in the DMD gene lead to the absence or dysfunction of the dystrophin protein, which is essential for muscle stability and integrity.

FIG 1. DESIGN AND HIGH-FIDELITY SYNTHESIS OF CARGO PLASMID FOR FULL-LENGTH HUMAN DYSTROPHIN



Company	ANSA BIOTECHNOLOGIES	TWIST BIOSCIENCE	GenScript Make Research Easy	ThermoFisher
Vector	**Full-length dystrophin with Complex UTRs**	Full-length dystrophin with Complex UTRs	Full-length dystrophin with Complex UTRs	Full-length dystrophin with Complex UTRs
Supports large plasmid	**Yes**	No	Yes	Yes
Supports complexity	**Yes**	No	Yes	High risk
Delivery Time (Quoted)	**~25 business days**	N/A	43 business days	52 business days
Relative Cost	**$$**	N/A	$$$$$$	$$$$$





Using HEK293 human cells and a standard lipofectamine transfection protocol, we determined if the complex, large plasmid made by Ansa Bio expressed high levels of dystrophin protein using an ELISA assay.

We observe concentration dependent human dystrophin expression in naive HEK cells transduced with the plasmid.

FIG 2. EF-D: ENDOFOLD'S DMD GENE THERAPY PARTICLE

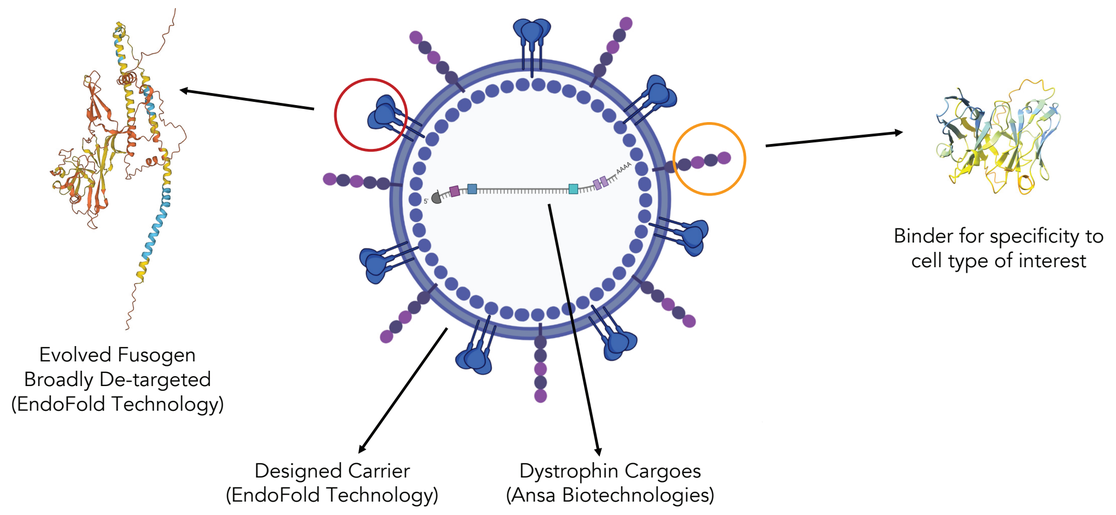
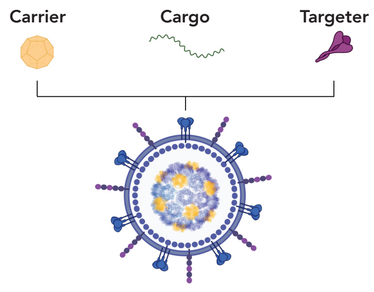
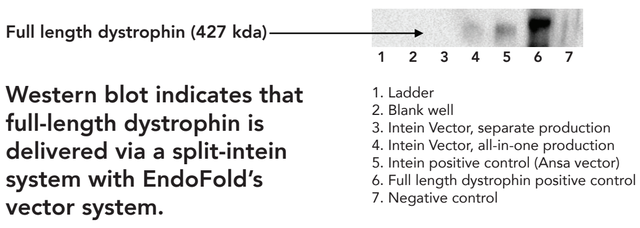


FIG 3. INTEIN HUMAN DYSTROPHIN DELIVERY IN HUMAN CELLS WITH ENDOFOLD PARTICLE



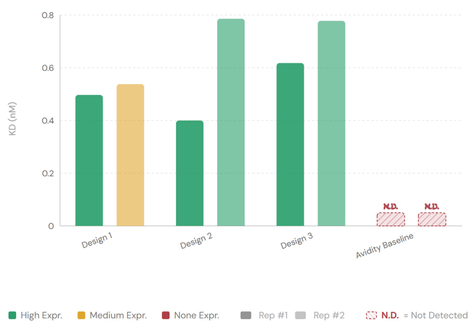
Full-length human dystrophin delivery is possible via RNA expression of a previously optimized three-part, intein system.



Western blot indicates that full-length dystrophin is delivered via a split-intein system with EndoFold's vector system.

1. Ladder
2. Blank well
3. Intein Vector, separate production
4. Intein Vector, all-in-one production
5. Intein positive control (Ansa vector)
6. Full length dystrophin positive control
7. Negative control

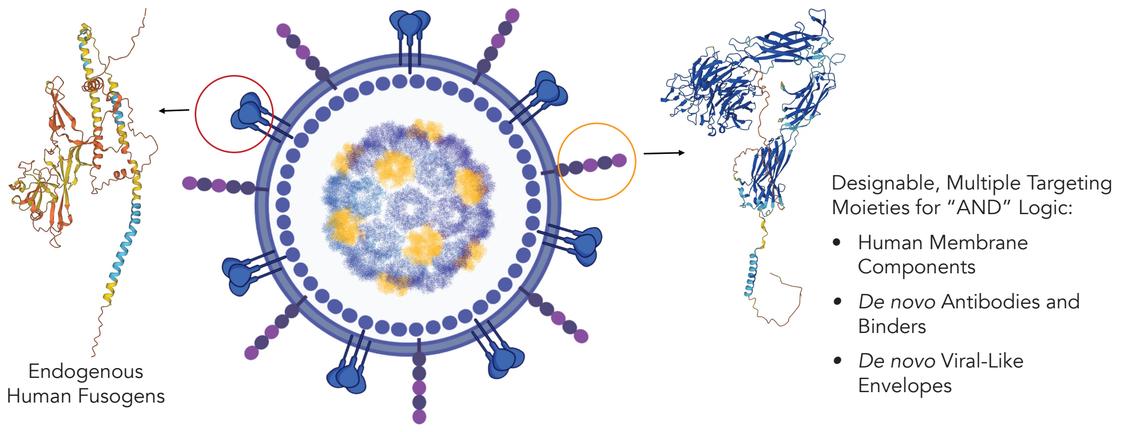
FIG 4. ENGINEERING NEW ANTI-TFR1 SCFVS FOR *IN VIVO* MUSCLE TARGETING



- One of Avidity's anti-TfR1 VH and VL candidates were used to generate a scFv baseline for human TfR1, a well-established target for musculoskeletal targeting.
- EndoFold's ML-based system, EvoTargeter, suggested mutants to improve the baseline scFv lack of expression in sub-optimal situations.

AI-suggested improvements allowed robust expression of the svFvs to human TfR1 in the sub-nanomolar - picomolar region, indicating high binding affinity for TfR1.

CONCLUSION: ENDOFOLD DELIVERS A HUMAN-CENTRIC PROTEIN LIBRARY FOR GENE DELIVERY



We demonstrate that full-length human dystrophin constructs can be rapidly synthesized with high fidelity and express functional protein in human cells. This capability removes a key bottleneck in developing gene therapies for DMD that deliver complete rather than truncated dystrophin, and sets the stage for evaluation in next-generation delivery systems capable of accommodating large genetic payloads. We preliminarily evaluate our particle delivering full length dystrophin via a split intein system against the full length dystrophin synthesized plasmid. Testing our intein vector against the full length plasmid as a control, we validated full length dystrophin reconstitution via western blot. Our novel particles provide a path towards therapy for DMD.

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Affiliations: [1]EndoFold, Inc., Westwood, California; [2]Ansa Biotechnologies, Inc., Emeryville, California

Acknowledgements: We would like to thank the EndoFold protein engineering team for their constant help and support throughout the research process as they are truly a blessing. We also thank multiple individuals who have contributed through helpful conversations regarding the work, including: Drs. Michael Masterman-Smith, Matteo Pellegrini, Leonard Rome, Roger Castells Graells, and William Gelbart.