HSPB8 targeting and modulation – a dual modality approach

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1 INTRODUCTION

Disease background

- HSPB8 Myopathy, also known as Myofibrillar Myopathy type 13 (MFM13) with Rimmed Vacuoles is an ultrarare, incurable genetic disorder marked by muscle wasting and progressive weakness, typically manifesting in young adults between their 20s and 40s.
- This slowly progressing, autosomal dominant condition is caused by mutations in HSPB8 (Heat Shock Protein Family B (small) member 8) gene. These mutations are often frameshift mutations in the carboxy-terminus leading to increased sequence disorder and more prone to protein aggregation. Genetic diagnostics have identified the pathogenic variant c.515delC in HSPB8 as one of these mutations.
- HSPB8 is a chaperone protein widely expressed across several human tissues, with particularly high expression in skeletal and cardiac muscle, as well in the motor nervous system. In muscle, HSPB8 plays a crucial role in chaperone-assisted selective autophagy (CASA), contributing to protein quality control and maintaining proteostasis.
- The pathogenic mechanisms associated with specific HSPB8 mutations include an enhanced HSPB8 oligomerization/aggregation propensity and sequestration of other HSPBs, a dominant negative effect on the WT HSPB8 proteins, and CASA impairment.

Project Summary

This project aims to develop an individualized therapy for a male patient affected by a pathogenic HSPB8 c.515delC frameshift mutation. With no available therapies, our strategy focused on a dual-modality discovery program that includes a drug repurposing screening and an allele-specific antisense oligonucleotide (ASO) screening. Both modalities were evaluated in patient and healthy fibroblasts.

- For the drug repurposing, we used Cell Painting as a primary screening tool to identify compound efficacy based on a multiplexed, high-content image-based morphological profiling assay that identified differential phenotypes between patient-derived and healthy cells under stress conditions. Approximately 4000 compounds were screened, followed by potency determination and hit identification.
- For the ASO approach, we screened allele-selective ASOs in patient cells and identified two lead candidates achieving approximately 72% knockdown of the mutant HSPB8 allele while having minimal effects on the wildtype allele. Preliminary in vitro immunotoxicity studies using human peripheral blood mononuclear cells (PBMCs) further supported a favorable safety profile of these sequences.

2 ASO SCREEN

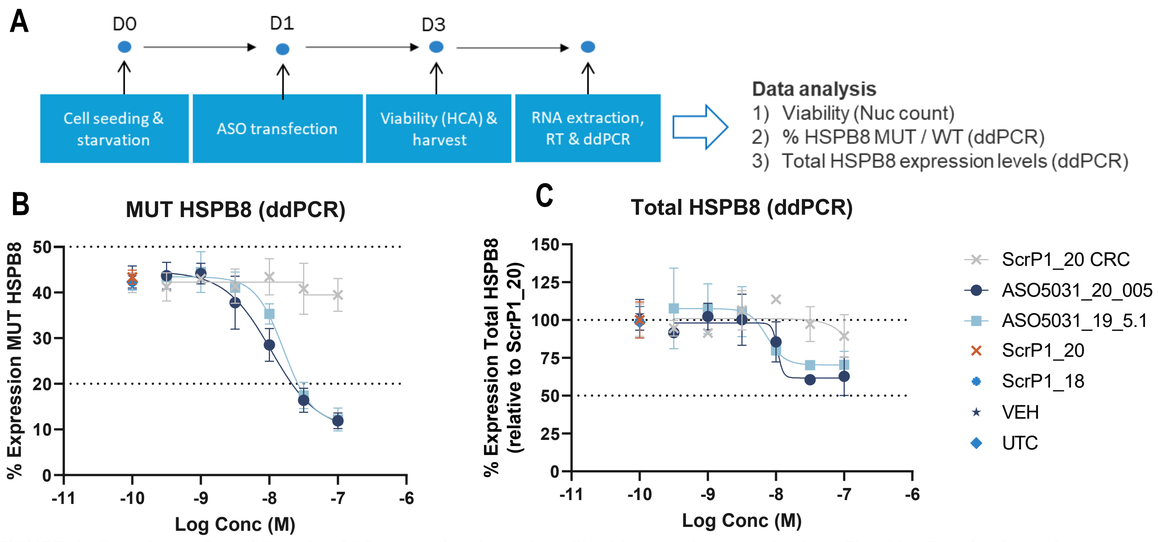
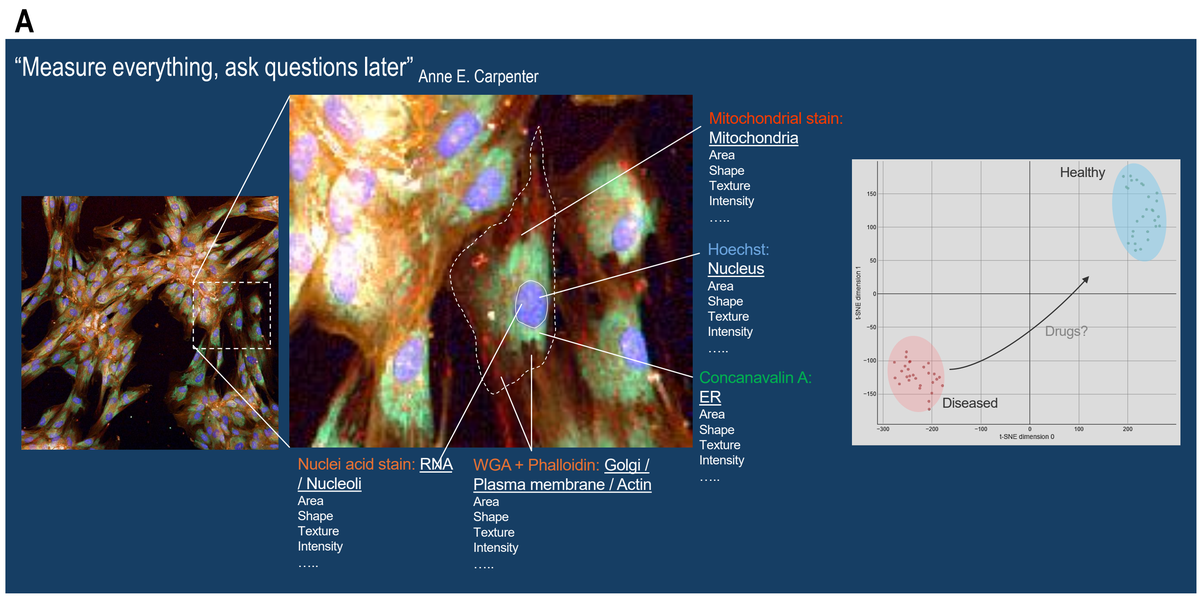


FIGURE 1: Experimental workflow for ASO screening in patient fibroblasts using transfection (Fig. 1A). Results from the potency determination of 2 top ASO hits in 6-point concentration-response curves (100 nM top concentration, half-log dilution steps) showed a 72% reduction in MUT HSPB8 at the top concentration (Fig. 1B). A comparable reduction in the total HSPB8 RNA levels indicate good selectivity (Fig. 1C)

Thirty-four test ASOs (between 21 and 18 nts) were screened in patient fibroblasts for their capacity to reduce MUT HSPB8 RNA expression levels while not affecting WT HSPB8 at 48 hr post-transfection (**Fig. 1A**). Viability was assessed before harvesting via Hoechst staining and High Content-based analysis, with none of the candidates showing any toxicity (data not shown). ASO efficacy and selectivity were measured using a SNP assay and a total assay by Droplet Digital PCR (ddPCR). The top performers 20_005 and 19_5.1 lower MUT HSPB8 from ~43% down to ~12% at 100 nM (this 31% drop means ~72% less MUT HSPB8, **Fig.1B**). These ASOs reduced total HSPB8 expression levels from 100% to 63% and 70.5% at 100 nM (~37% and 29.5% total KD, **Fig.1C**) respectively, indicating high selectivity. This is essential to ensure that enough WT HSPB8 is still present.

3 DRUG REPURPOSING SCREEN USING CELL PAINTING



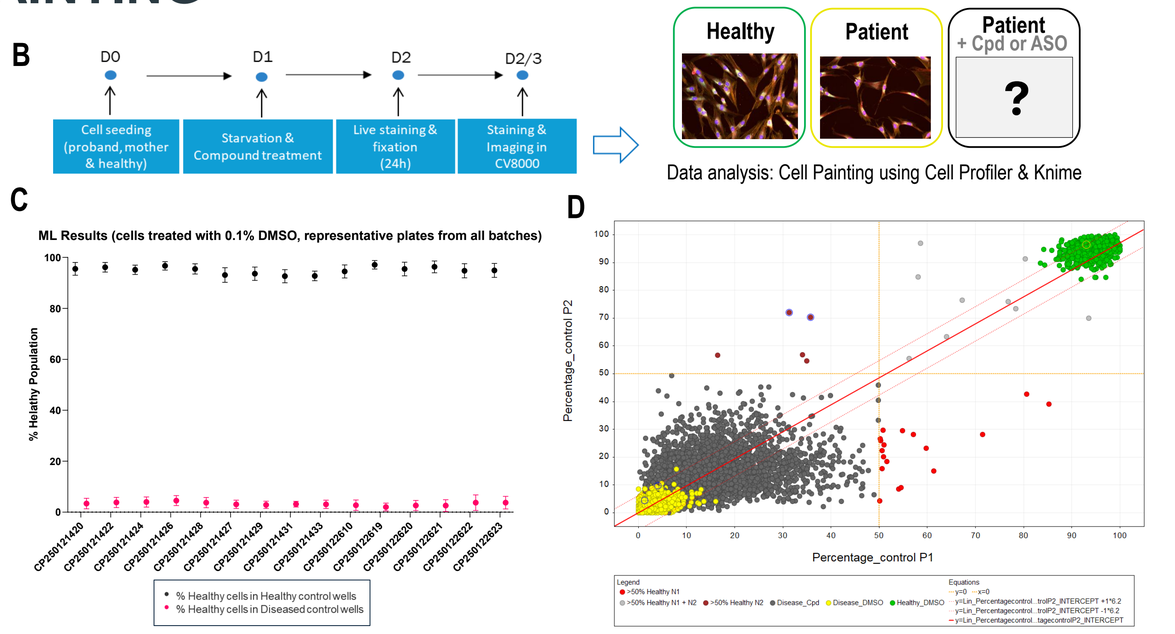


FIGURE 2: Cell Painting assay for phenotypic high-content screening (Fig. 2A). Experimental workflow for compound screening in patient fibroblasts (Fig. 2B). Quality control of the Machine Learning (ML) model performance showing the % cells classified as healthy from patient and healthy DMSO-treated control wells (Fig. 2C). Results from the drug repurposing screen at 10 μM are shown in a correlation plot where each point represents the % healthy cells per well, with replicate 1 on the X-axis and replicate 2 on the Y-axis. The 120 hit compounds selected for validation and potency determination included several different drug classes and >70 targets (Fig. 2E). The two best ASOs were also tested in the CP assay at 100 nM and 31.6 nM for 72h to assess phenotypic changes, but there were no effects in the ML results. However, Principal Component Analysis (PCA) showed morphological differences at 100 nM in dimension 1, mostly related to Mitochondrial features (Fig. 2F).

Cell Painting (CP) assay

- CP is a high-throughput screening approach to assess drug effects through multiplexed staining of multiple cellular compartments (RNA, nucleus, Golgi, cytoskeleton, ER, mitochondria; Revvity).
- Thousands of features per cell are quantified using image analysis (CellProfiler), generating phenotypic fingerprints compared between healthy and diseased cells using machine learning (KNIME) (**Fig. 2A**).
- This platform was applied to a drug repurposing screen of 4,158 compounds at 10 µM and 1 µM in patient-derived and healthy fibroblasts under stress conditions (**Fig. 2B**).

Machine Learning (ML) Model and Performance

- The ML model is trained on 80% of DMSO-treated cells, with 20% held out for validation.
- Each cell is assigned a probability score (0–1); cells ≥0.5 are classified as healthy.
- Model performance is assessed by calculating the percentage of healthy cells per well in both patient and control conditions, showing high predictive confidence (**Fig. 2C**).

Hit Identification and Validation

- Compound-treated patient cells are analyzed with the trained model to identify treatments that shift phenotypes toward a healthy state.
- A ≥50% threshold of healthy cells is used to identify rescuing compounds across replicates (**Fig. 2D**).
- Hit selection includes exclusion of toxic compounds and selection of the top 120 candidates (no clear MoA or chemical clustering observed) (**Fig. 2E**).
- Selected hits were validated in a 10-point dose-response assay to confirm activity and determine potency.

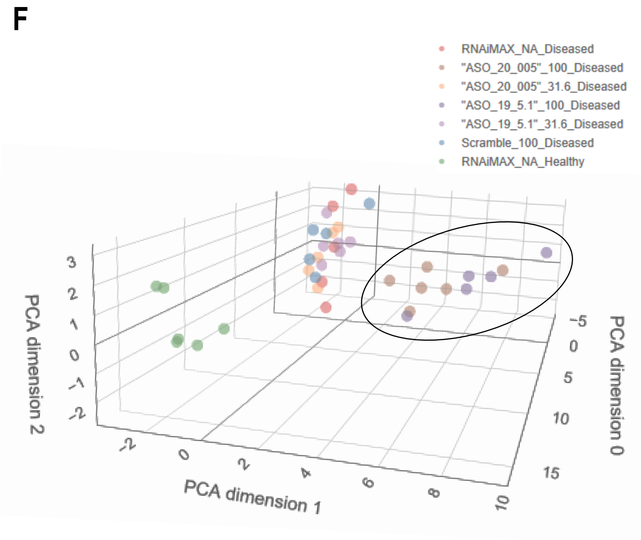
Can the patient phenotype be rescued in the CP assay after knockdown of MUT HSPB8 with an ASO?

- Cells were treated with the selective ASOs 20_005 and 19_5.1 for 72h and analyzed by CP. ML results did not show an ASO-induced phenotype rescue in the patient cells (data not shown), but they showed a change in their morphology detected in the PCA as a separate cluster in a secondary dimension linked to mitochondrial staining (**Fig. 2F, see circle**).

E

Drug category	Compounds per category
Hormones (Analogues)	13
Anti-Bacterial Agents	11
Natural product	9
Various	47
Anti-viral	7
Kinase Inhibitor	7
Anti-inflammatory	3
Corticosteroids	3
Food additive	3
Vasodilating agent	3
Anti-coagulant	2
Anxiolytics Sedatives and Hypnotics	2
Calcium channel blocker	2
Iron Chelating agents	2
Lipids	2
Neurokinin Receptor Antagonists	2
Platelet aggregation Inhibitor	2

F



CONCLUSIONS & NEXT STEPS

- Two therapeutic modalities are being explored in parallel to identify an appropriate treatment for a rare disease called MFM13. We have developed two disease-modifying allele-selective ASOs with good efficacy, selectivity and tolerability in vitro and we have identified a number of FDA-approved small molecule drugs promoting a healthy phenotype in patient fibroblasts in the CP assay.
- Knockdown of MUT HSPB8 did not reverse the phenotype of patient cells compared to the healthy line, but it showed a change in their morphology. More patient and healthy lines would need to be tested to further understand this, but perhaps phenotype rescue does not always correlate with increased similarity to a healthy cell line.
- Next steps include assessing the potential of some of those and ASOs in patient iPSC-derived skeletal muscle cells before going into in vivo studies.

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