A New Approach to Modeling LGMDR1: Pyrvinium-Treated *capn3b* crispant Zebrafish

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Abstract

The lack of an accurate animal model for LGMDR1 is a major obstacle to therapeutic development. While murine models do not replicate the human gene expression profile, zebrafish offers a promising alternative. We generated a *capn3b* mutant zebrafish, which showed minimal phenotypic changes. However, when this model was treated with pyrvinium, a Wnt signaling inhibitor, its gene expression patterns mimic those observed in LGMDR1 patients, reinforcing the role of the Wnt pathway in LGMDR1 pathology. This pharmacologically enhanced model, despite lacking a clear phenotype in young larvae, could serve as a valuable tool for identifying potential therapeutic targets upon further investigation and validation.

INTRODUCTION

Limb-girdle muscular dystrophy R1 calpain 3-related (LGMDR1) is an autosomal recessive muscular dystrophy due to mutations in *CAPN3* that causes progressive degeneration of the proximal muscles. For most patients, onset occurs in the second decade of life and as muscle degeneration progresses; patients require a wheelchair for mobility after around 20 years of disease progression.[1,2]

CAPN3 is a muscle-specific protease[3] whose function in muscle is not completely understood. However, it has been reported that a lack of CAPN3 alters several structures, gene expression and signaling pathways in the skeletal muscle.[4–7]

To advance the development of effective treatments for any disease, a biologically relevant *in vivo* model is essential. However, finding an animal model that perfectly mimics the clinical features of calpainopathies remains elusive.

Previous attempts have used various murine models to approximate the human condition and study the effects of *CAPN3* gene alterations. However, none have fully replicated the human phenotype.[4,8–11] Even though DNA microarray analyses were performed to establish expression profiles in mouse models, unfortunately, these failed to reflect the gene expression patterns seen in human muscle. [5,10,12]

This difficulty is also noticeable in zebrafish, which have two *CAPN3* homologs, *capn3a* and *capn3b*, but only *capn3b* is expressed in muscle. Earlier studies on a *capn3b*-deficient zebrafish model focused on its role in other processes, such as the calpain3 involvement in the nucleolar degradation pathway of p53, liver regeneration, and autoimmune responses.[13–15] More recently, RNA-less and partial deletion *capn3b* mutants were generated using CRISPR/Cas9 mutagenesis. While this model showed no significant muscular dystrophy phenotypes under normal conditions, it was more susceptible to muscle damage under challenging conditions, such as in mechanical stress situations.[16]

Due to the difficulties in generating a suitable animal model that accurately reflects the phenotypic characteristics of the disease, we have developed a pharmacologically enhanced zebrafish model. We generated a *capn3b* crispants and investigated the effects of treatment with pyrvinium, a known inhibitor of the Wnt signaling pathway.[17] Our results show that untreated *capn3b* crispant zebrafish display altered expression of several genes. When this model was treated with pyrvinium, the gene expression deregulation

demonstrated a greater resemblance to the deregulated patterns observed in LGMDR1 patients' muscles. This finding suggests that modulation of the Wnt signaling pathway may represent a promising therapeutic target for LGMDR1.[5]

MATERIALS AND METHODS

Zebrafish Husbandry and Breeding

Adult wild-type AB strain zebrafish were maintained at 28.5 ± 1ºC in a 14 h light/10 h dark cycle in a closed recirculating tank system, according to standard protocols.[18]

Generation of Crispants

Wild-type or Casper one-cell-stage embryos were injected following published protocols [19,20] with a mix containing the Cas9 and sgRNAs targeting the coding sequence of the gene of interest or the Cas9 and a control sgRNA (scramble) (Table S1).

Mutation rates quantification

Genomic DNA was extracted from ten individual embryos at the stage of 24 hpf. The target region was amplified by PCR employing the primers capn3b-fw and capn3b-rev. The percentage of mutated alleles present in the PCR was quantified using online tools upon sequencing of the resulting amplicons.

Treatment

Pyrvinium pamoate salt hydrate was reconstituted in Dimethyl Sulfoxide (DMSO) to a stock concentration of 1mM and diluted in E3 1X medium to a final concentration of 0.400, 0.200, 0.100, 0.050, and 0.025 µM. 100% DMSO was diluted in E3 1X medium to a final concentration of 0.1%. 48 hpf larvae injected with scrambled sgRNA and capn3b sgRNAs were treated with 0.1% DMSO and with each of the Pyrvinium concentrations, they were maintained at 28.5ºC with a 14:10 hour light:dark cycle. The medium was renewed at 72, 96, and 120 hpf.

Morphological Analysis: birefringence

Larvae were anesthetized with tricaine 0.28 mg/mL, and images were captured using a stereomicroscope. After the imaging, larvae were euthanized and the birefringence was quantified as follows in Berger et al 2012. [21]

Analysis of locomotor activity

For the locomotion analysis, the standardized 25 min dark/light cycles paradigm (5 min darkness—5 min light up to 25 min) was used, the employed methodology was based on the protocol described in Locubiche *et al.*, 2024.[20]

Statistical analysis

Statistical analysis is performed using One way ANOVA followed by Sidak's test.

RNA extraction

For RNA extraction, 16 zebrafish larvae were used per condition. The PicoPureTM RNA Isolation Kit (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, EE. UU.) was used, following the manufacturer's instructions for RNA extraction.

RT-qPCR

The High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, EE. UU.) was used for cDNA synthesis, following the manufacturer's instructions.

To study gene expression levels by qRT-PCR, *SYBR Green* primers (Table S2) and *TaqMan* probes, in Gene Expression Array Cards (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, EE. UU.) (Table S3) were used. Genes were selected according to LGMDR1 patients' expression profiling, however since less zebrafish probes are available, additional muscular genes were included. The CFX384 C1000 thermocycler (Bio-Rad Laboratories, Hercules, CA, EE. UU.) and QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, EE. UU.) were used. The data were analysed using the Bio-Rad CFX Maestro programme and the ThermoFisher Connect Platform.

RESULTS

The CRISPR/Cas9 technology allows the efficient targeting of the *capn3b* gene.

To achieve a high rate of somatic mutations in F0 zebrafish larvae, we developed a strategy using two sgRNAs targeting distinct coding regions of the *capn3b* gene. Four different sgRNA combinations were designed and tested, with their cutting efficiencies listed in Table S4. Among these, the combination of guides 2 and 3 produced the highest mutation rates across the greatest number of larvae; almost the totality of their genetic material (97.8%) contained mutations in the *capn3b* exogenous coding sequence (CDS).

The inactivation of the *capn3b* gene and the treatment with pyrvinium do not affect locomotion.

Our first step in the characterization of *capn3b* crispants was to evaluate how gene inactivation, alongside with the treatment with pyrvinium, influenced larval locomotion.

To achieve this, we tracked the swimming behavior of 144 hpf. By monitoring these behavioral responses under different experimental conditions, we aimed to determine how gene knockout and drug exposure interact to affect larval motility.

We observed that all groups (scrambled and *capn3b* crispants) did not show differences in their response to the environmental illumination changes (Figure 1A).

To evaluate how the overall locomotor activity of larvae was affected by the genetic manipulation and the pharmacological treatment, we quantified the total distance moved during the trial by each larva (Table S5). No statistical difference was detected between the different experimental groups suggesting that neither the gene KO nor the drug exposure affected larval behavior (Figure 1B).

Gene expression alteration mimic the expression patterns found in LGMDR1.

After analysing gene expression, we were able to identify that some of the genes deregulated in the muscle of LGMDR1 patients[5] were also slightly altered in the *capn3b* crispants model. Furthermore, this alteration was increased in the *capn3b* crispants model after treatment with 0.4 µM Pyrvinium, making it more similar to the alterations observed in patients.

The genes that showed a notably similar profile in the pyrvinium treated *capn3b* crispants model, compared to LGMDR1 patients, are part of the extracellular matrix (*aspn, col1a1b, col15a1b*), transcription factors (*fos, junbb, jun, jdp2b, trf1e*), cell adhesion (*cd9*) and endocytosis genes (*vldlr*) (Figure 2). It is worth noting that other genes, deregulated in LGMDR1 patients, showed a similar tendency in their expression, but they did not reach the established threshold of 0.5 fold-change value in the pyrvinium treated knockout model (Figure S1).

DISCUSSION

The generated disease model, with respect to its muscular morphology and function, does not resemble the characteristics observed in LGMDR1 patients. However, it is noteworthy that this model is of great interest due to the rapid generation, made possible by CRISPR/Cas9 technology. This technology has been further demonstrated by other studies to generate zebrafish models for different muscular dystrophies, providing valuable insights into disease mechanisms and potential therapies.[22–24]

Effective inhibition of GSK-3β by drugs such as Tideglusib leads to the stabilisation and nuclear translocation of β-catenin, thereby reactivating the Wnt pathway. [25] Previous studies by the group have demonstrated the involvement of the Wnt pathway in patients with LGMDR1 and how, after treatment with Tideglusib in patient cells, the expression of several dysregulated genes and structural proteins is restored.[6] Therefore, the results obtained in the zebrafish model treated with pyrvinium, support the relevance of Wnt pathway regulation in disease progression.

On the one hand, the absence of clinical or functional characteristics similar to those of LGMDR1 patients makes this model not fully validated for drug evaluation. On the other hand, the improved similarity in gene expression patterns achieved with the drug Pyrvinium paves the way to obtain a potential animal model.[17,26]

However, it is important to note that due to animal experimentation limitations, the study only reached the larval stage at 144 hpf. At this early stage, the fish may not yet exhibit a clear phenotype due to their age.[27] Ideally, to better mimic the clinical onset of the disease in patients,[1,2] a more advanced stage should be reached, such as the juvenile stage (approximately 30 days old) or later. Evaluating these fish at a more mature stage would help to determine if we have generated a truly representative disease model. Therefore, we suggest that subsequent studies are required on juvenile and adult models to confirm the possible phenotype.

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Institutional Review Board Statement: For breeding of zebrafish, ethical approval was obtained from Generalitat de Catalunya "Direcció General de Polítiques Ambientals i Medi Natural" under referential procedure number 18-011-ISA- RESOLUCIO 9421: Breeding of zebrafish (*Danio rerio*) for the maintenance of lines and the creation of transgenics intended to study human diseases.

Conflicts of Interest: F.D.S. was employed by ZeClinics SL, a contract research organization providing zebrafish-based services, at the moment of the manuscript preparation.

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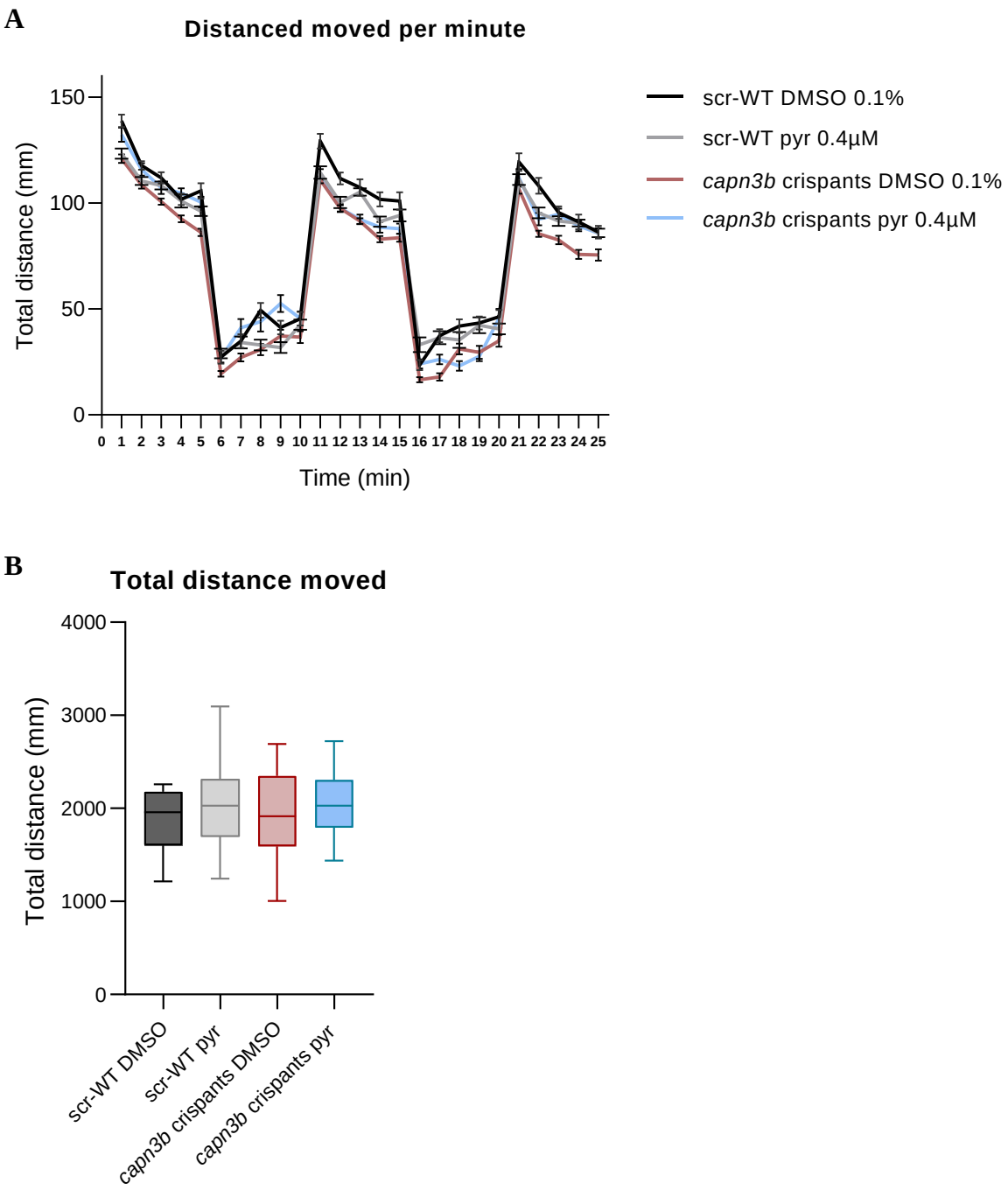
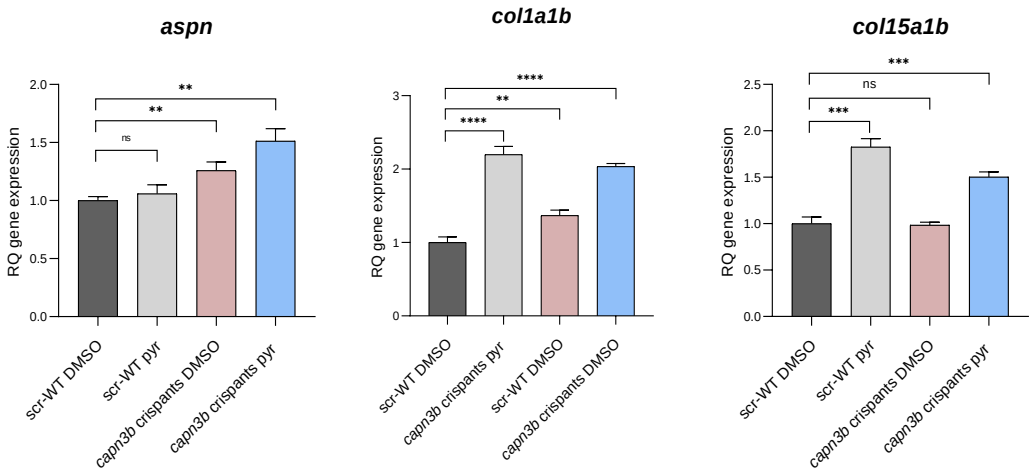
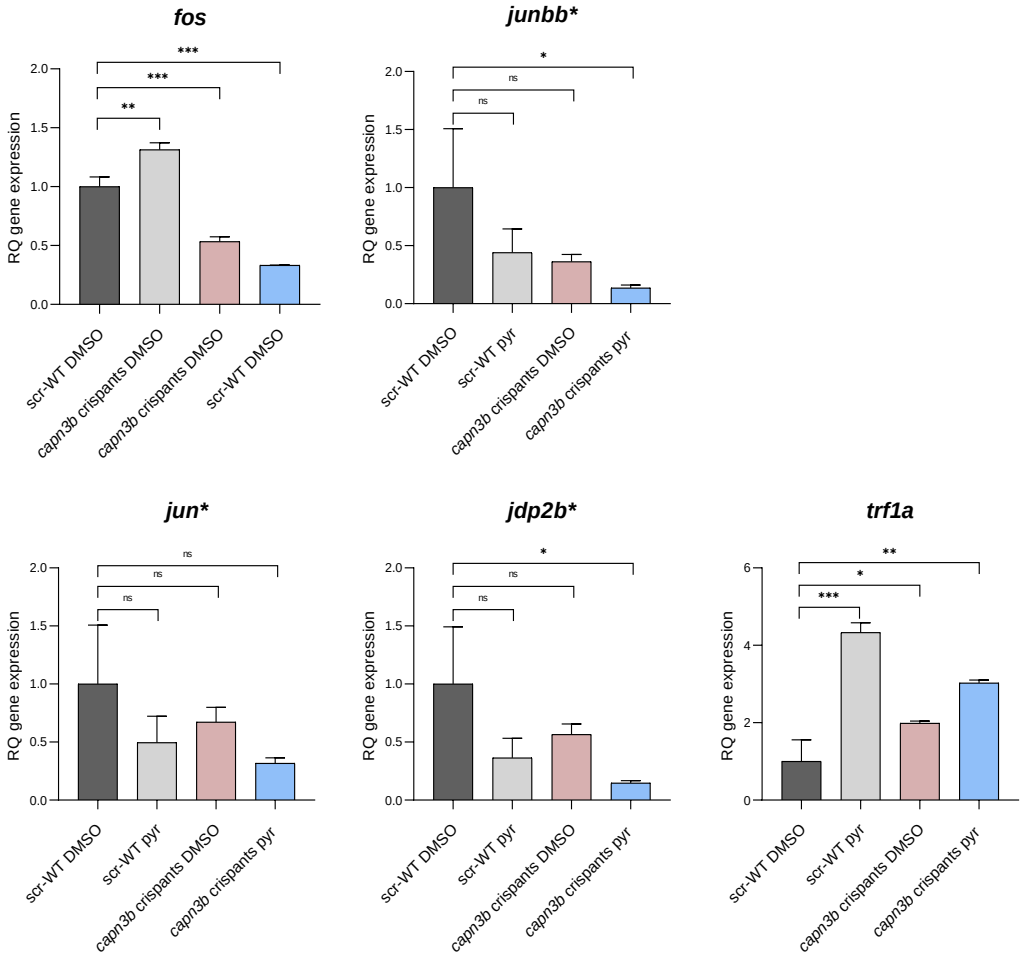


Figure 1. Locomotion Activity Assessment. **(A)** Total distance moved per minute during the dark/light phase. Different experimental conditions are represented in different colors as shown in the legend on the left. Error bars represent the standard error of the mean (SEM). (**B)** Total distance moved during the dark/light transition test. Scrambled is represented in gray, capn3b crispant is represented in red (0.1%DMSO) and the treated with pyrvinium 0.4 µM, in blue. Error bars represent the minimum to maximum values. All comparisons are not significant (One-way ANOVA followed by Sidak's test for multiple comparisons).

A) Extracellular matrix



B) Transcription factors



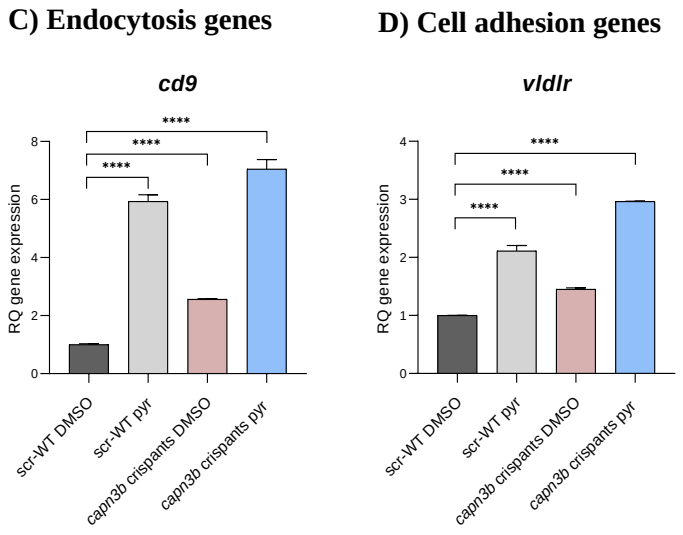


Figure 2. Deregulated genes in *capn3b*-/- model and after treatment with pyrvinium. Data shows the relative expression levels of **A)** Extracellular matrix (*aspn, col1a1b, col15a1b*), **B)** Transcription factors (*fos, junbb, jun, jdp2b, trf1a*), **C)** Endocytosis genes (*cd9*) and **D)** Cell adhesion genes (*vldlr*). Gene expression was analysed in both untreated control and 0.4 µM Pyrvinium-treated *capn3b*-/- zebrafish samples. Fold change threshold ≥1.5 or ≤0.5. Data is normalized to a reference gene, *ef1α*. *TaqMan probes. Error bars represent the minimum to maximum values. Unpaired T-Test was performed to assess statistical significance (*p-value<0.05; **p-value<0.01; ***p-value<0.001 ****p-value<0.0001).