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Drug Repurposing for Neuromuscular Diseases using Zebrafish

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Drug Repurposing for Neuromuscular Diseases using Zebrafish

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Abstract

Duchenne Muscular Dystrophy (DMD) is a rare genetic disorder and the most frequent among muscular diseases. It is due to the deficiency of a protein called dystrophin and is characterized by progressive weakness and degeneration of skeletal muscles. Since no cure for DMD has yet been found, there is an urgent need to identify new therapies. Given that the pathogenic mechanisms of this disease are not fully understood, performing phenotypic drug screens *in vivo* represents a promising strategy for therapeutic discovery. With this aim, we believe that a chemical high-throughput screening strategy will identify potential small molecules that will restore disease-related phenotypes in DMD models, ultimately providing lead candidate compounds for clinical testing. A previous screen by our group has identified a total of 26 chemical compounds that presented a significant improvement of motility in DMD-mutant worm models. This study aims at validating these hit candidates in a zebrafish genetic model of DMD. We first generated a DMD-mutant zebrafish model using the CRISPR/Cas system. Detailed phenotypic characterization of this mutant demonstrated features of the DMD pathology consisting of a reduced survival rate, muscle weakening and motor dysfunction manifested by swimming defects and abnormal organization of muscle fibers. We next screened the 26 candidate small molecules for their ability to rescue the DMD-like phenotype in this model. None of these 26 candidate compounds presented a statistically therapeutic impact on the pathology's characteristics of DMD in our zebrafish model. Additional studies aimed at ameliorating the drug screening methods and phenotype readouts in our DMD models are needed in order to use this powerful approach to identify potential therapeutic targets for DMD in humans.

Keywords: Duchenne muscular dystrophy, neuromuscular disease, dystrophin, zebrafish, phenotypic drug screening.

Résumé

La dystrophie musculaire de Duchenne (DMD) est une maladie génétique rare et la plus fréquente des maladies musculaires. Elle est due à la déficience d'une protéine appelée dystrophine et se caractérise par une faiblesse progressive et une dégénérescence des muscles squelettiques. Come qu'aucun remède contre la DMD n'a encore été trouvé, il est urgent d'identifier de nouvelles thérapies. Étant donné les mécanismes pathogènes de cette maladie ne sont pas entièrement compris, la réalisation de dépistages phénotypiques de médicaments *in vivo* représente une stratégie prometteuse pour la découverte thérapeutique. Dans ce but, nous croyons qu'une stratégie de dépistage chimique à haut débit permettra d'identifier de petites molécules potentielles qui restaureront les phénotypes liés à la maladie dans les modèles de DMD, fournissant finalement des candidats pour les essais cliniques. Un criblage précédent par notre groupe a permis d'identifier un total de 26 molécules chimiques qui présentaient une amélioration significative de la motilité dans les modèles de vers mutants DMD. Cette étude vise à valider ces candidats dans un modèle génétique de poisson zèbre de la DMD. Nous avons d'abord créé un modèle de poisson zèbre mutant DMD à l'aide du système CRISPR/Cas. Une caractérisation phénotypique détaillée de ce mutant a démontré des caractéristiques de la pathologie de la DMD consistant en un taux de survie réduit, un affaiblissement musculaire et un dysfonctionnement moteur manifestés par des défauts de natation et une organisation anormale des fibres musculaires. Nous avons ensuite examiné les 26 molécules candidates pour leur capacité à sauver le phénotype de type DMD dans ce modèle. Aucun de ces 26 candidats n'a présenté d'impact statistiquement thérapeutique sur les caractéristiques pathologiques de la DMD dans notre modèle de poisson zèbre. D'autres études visant à améliorer les méthodes de dépistage des médicaments et les lectures du phénotype dans nos modèles de DMD sont nécessaires afin d'utiliser cette approche puissante pour identifier les cibles thérapeutiques potentielles pour la DMD chez les humains.

Mots clés : Dystrophie musculaire de Duchenne, maladie neuromusculaire, dystrophine, poisson zèbre, dépistage phénotypique des médicaments.

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List of abbreviations

ABD: actin-binding domain

ALS: amyotrophic lateral sclerosis

AO: antisense oligonucleotide

ASOs: antisense oligonucleotides

AVVs: adeno-associated virus

BMD: Becker muscular dystrophy

CNFs: centrally nucleated fibers

CNS: central nervous system

CR: cysteine-rich domain

CRDs: complex repetitive discharges

CT: C-terminal domain

DAPC: *dystrophin*-associated protein complex

DAMPs: damage-associated molecular patterns

DG: dystroglycan

DGC: Dystrophin-glycoprotein complex

DPF: days post-fertilization

DMD: Duchenne muscular disease

EMG: electromyography

GRMD: golden retriever muscular dystrophy

HT DMD: heterozygous DMD

HGF: hepatocyte growth factor

HRM: High Resolution Melting

HTS: high-throughput screening

IGF-1: Insulin-like growth factor-1

iPSCs: pluripotent stem cells

NCX: sodium-calcium exchanger

NF-κB: Via nuclear factor κB

NGM: Nematode Growth Medium

nNOS: neuronal nitric oxide synthase

NOX2: NADPH oxidase 2

MHC: Major histocompatibility complex

MTC: maximum tolerated concentration

PDE5A: phosphodiesterase 5A

PDNL: Prednisolone

PMCA: plasma membrane calcium ATPase

QOL: Quality of life

RyR: ryanodine receptor

SMA: spinal muscular atrophy

THI: 2-acetyl-4(5)-tetrahydroxybutyl imidazole

TLR4: Toll-like receptor 4

TLR7: Toll-like receptor 7

TSS: alternative trasncription sites

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Chapter 1 – Introduction

1. Duchenne Muscular Dystrophy (DMD)

1.1. General aspects

1.1.1. Historical background

The first historical account of muscular dystrophy was described in 1830, when Sir Charles Bell wrote an essay about an illness that causes progressive weakness in boys (1). In the 1850s, evidence of boys who grew progressively weaker, lost the ability to walk, and died at an early age became more frequent in medical journals (2). After one decade, in the 1860s, the French neurologist Guillaume Benjamin Amand Duchenne described for the first time Duchenne Muscular Dystrophy (DMD), when he noticed that 13 boys had the most common and severe form of the disease. At present, the prevalence of DMD is 1 in 3,500 male births worldwide (3).

1.1.2. Pathogenesis

DMD is caused by mutations in the dystrophin gene (*DMD*), which is located on the sexual X chromosome (region p21) (4). Several pathogenic mutations have been referenced on this gene, such as duplications (5%-10%), deletions (60%-65%) or point mutations (5). In DMD, there are frame-shifting mutations that cause an early stoppage of protein translation and result in the absence of the dystrophin protein (loss-of-function). This peculiar feature differs from other muscular dystrophies, like Becker, where in-frame mutation occurs and forms an abnormal, but partially functional dystrophin protein (6). We will dig deeper into the pathogenic mechanisms of *DMD* mutations in the next section.

1.1.3. Inheritance

DMD is inherited in an X-linked recessive pattern and therefore mostly affects males because they have only one copy of the X chromosome. In heterozygous female, because they have two X chromosomes, they can produce enough functional dystrophin protein to either prevent the symptoms of DMD or cause only mild symptoms. These individuals are called DMD "carriers"

who can transmit the mutation to their progeny without having any symptoms of the disease (7). However, in very rare cases, female DMD has been identified in individuals with Turner syndrome or unilateral parental disomy for X chromosome (8).

1.1.4. Diagnosis

After a physical examination that includes the evaluation of any abnormalities in the muscle function and the cardiovascular condition of the patient, three different examinations can be performed to determine the non-functional muscular activity:

1- CK blood test. This diagnosis measures the creatine kinase level in the muscle. If the amount of this enzyme is elevated, muscle is being disintegrated by some abnormal processes, such as a muscular dystrophy or inflammation. From the age of 2 years old, the CK level falls progressively at a rate of 25% per year.

2- Muscle biopsy. The aim of this test is to seek for changes in the amount and look of dystrophin protein and is usually performed by immunohistochemistry of slides of the muscle or by a Western blot test of the muscle protein.

3- Genetic testing. Used for both patients and carriers, this type of testing confirms the diagnosis when a mutation of dystrophin gene is identified. Genetic testing is usually performed on a blood or saliva sample. Genetic testing is the gold standard for diagnosing Duchenne (9).

1.1.5. Clinical characteristics

Patients with DMD show different symptoms that can range from mild skeletal muscle weakness or cardiac involvement to severe weakness or cardiac effects and can begin in childhood or adulthood. The principal symptom of DMD is muscle weakness. It can appear by the age of 2 or 3 years old, first altering the proximal muscles and later affecting the distal limb muscles. Lower external muscles are commonly affected before the upper external muscles. Patients with this symptom might have difficulties in walking, jumping, and running. Progressive weakness and scoliosis lead to impaired pulmonary function, which can eventually provoke acute respiratory failure (9). Other symptoms comprise enlargement of the calves, difficulty with climbing stairs, a waddling gait, frequent falls, and lumbar lordosis. Subsequently, the heart and respiratory muscles

are affected as well, resulting in a dilated cardiomyopathy after the age of 10 years, which is evident in all patients of 18 years old. As the disease progresses, boys become increasingly dependent on others for assistance with their daily activities. Cardiac and respiratory problems continue to worsen, ultimately leading to their death, normally when they reach the age between 20 and 40 years old (10).

1.1.6. Quality of life

As DMD is a progressive and disabling disorder, the life of DMD patients and their families is deeply affected. Several qualities of life (QOL) studies in patients with DMD have been performed, but there are discrepancies (11). Indeed, some of them showed a reduced QOL whereas others have not found any differences compared with healthy individuals. The reason of such discrepancies could lie on different instruments/tools to assess QOL and because health related QOL and QOL are used conversely (12). Despite DMD symptoms, satisfaction with health care, social interaction, and support, adjusted expectations, and acceptance can help mitigate the negative effects of DMD on QOL. Certainly, patients with DMD adapt to their disabilities and develop new ambitions, attitudes, and hopes. On the other hand, families and caregivers tend to underestimate the QOL of the patient with DMD by using their own criteria (13). This fact could be a counter-productive measure when a life-sustaining intervention should be done, especially in acute situations like the COVID-19 pandemic, where the fragile situation in the health system would disadvantage patients with neuromuscular disorders in advance disease stages in the access to an intensive unit (14).

1.2. Dystrophin gene

1.2.1. Dystrophin function

DMD encodes for dystrophin, a protein that constitutes a protein complex responsible of strengthening muscle fibers and protecting them from injury during muscle contraction and relaxation (15). There are three dystrophin isoforms: Dp427m, Dp42c and Dp427p (Figure 1) (16). These isoforms are expressed in muscle, cortical neurons and cerebellar Purkinje cells, respectively (17). In addition to the full-length dystrophin, there are other shorter dystrophin isoforms produced by four alternative transcription start sites (TSS). These shorter dystrophin

isoforms are expressed throughout different tissues. Dp260 is expressed primarily in the retina (18), Dp140 is expressed in the central nervous system, kidney (19) and at high levels in embryonic brain (18), Dp116 is primarily expressed in peripheral nerves and Schwann cells (20), and Dp71 is expressed ubiquitously but at higher levels in neuronal cells than in other cell types (21). Another isoform, Dp40, arises from the same promoter as Dp71 but is polyadenylated in intron 70 (17).

1.2.2. Structure

In Figure 1a, the approximately 2.4 Mb full-length *DMD* genetic locus contains eight promoters that are differently used to transcribe up to 79 exons. Three upstream promoters (Dp427b, Pd427m and Dp427p) allow the transcription of a 11.4 kb full-length cDNA encoding the 427 kDa full-length dystrophin protein. This full-length DMD isoform (Dp427m) is the primary muscle isoform. Four internal promoters (Dp260, Dp140, Dp116 and Dp71) generate N-terminal truncated non-muscle isoforms of dystrophin. Alternative splicing at the 3'-end and alternative polyadenylation (the addition of a poly(A) tail to RNA) yield additional isoforms of dystrophin such as Dp40.

DMD is caused by mutations in the dystrophin gene, known as *DMD*. When this gene is disrupted, the production of the muscle isoform of dystrophin is blocked (promoters Dp427b, Dp427m, Dp427p) (17) (Figure 1a). As previously mentioned, other mutations in the *DMD* gene that preserve the function of crucial domains can cause another disease named Becker muscular dystrophy (BMD, figure 1c). The difference between these two diseases could be explained by the "reading frame rule". First, in DMD, frameshifting mutations or nonsense mutations provoke premature truncation of dystrophin translation, thus the protein is non-functional and unstable (Figure 1b). On the contrary, in BMD, mutations are present in the middle of the gene, so they keep the reading frame and maintain the production of a partially functional dystrophin because crucial domains are present (Figure 1c).

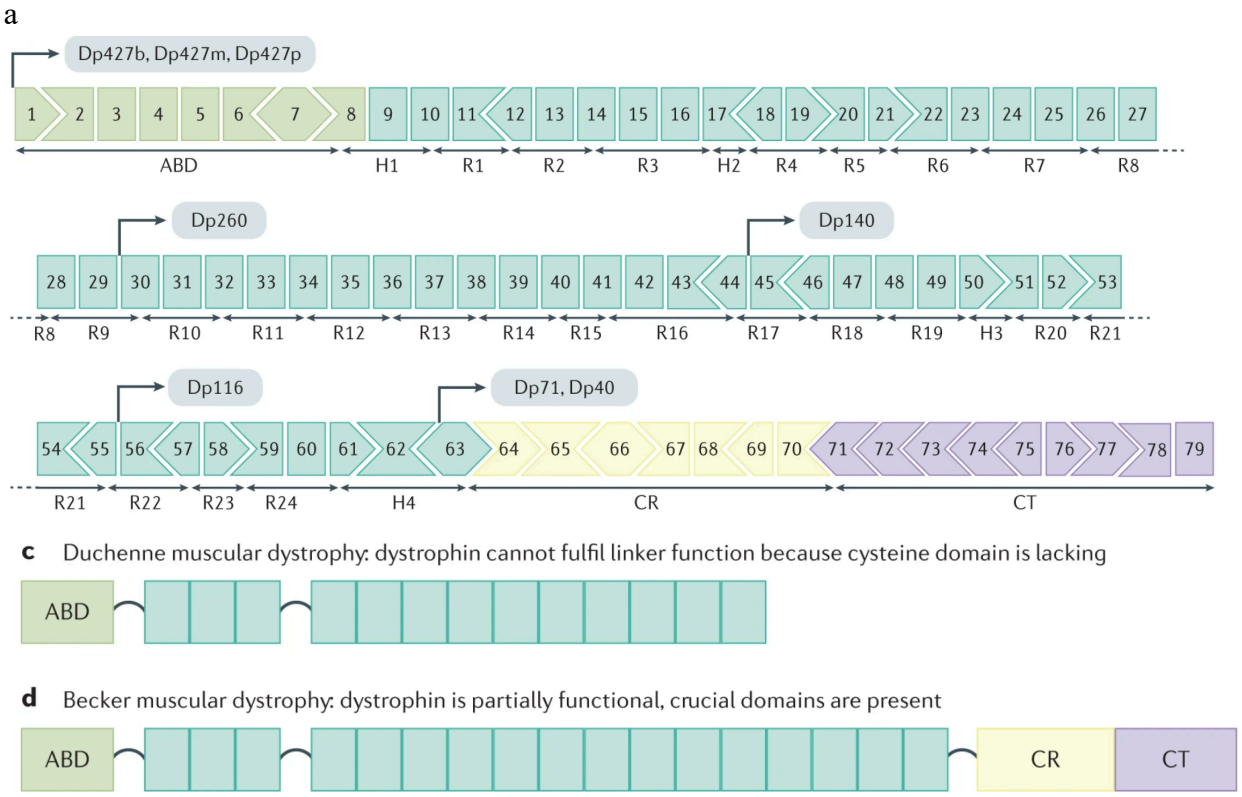


Figure 1. Schematic depiction of DMD and dystrophin protein.

a) DMD gene, with a length of approximatey 2.4Mb, contains eight promoters and 79 exons. There are three upstream promoters (Dp427b, Dp427m and Dp427p) that produce the ~11.4 kb full-length cDNA and the 427 kDa full-length dystrophin protein. Four internal promoters (Dp260, Dp140, Dp116 and Dp71) generate N-terminal truncated non-muscle isoforms of dystrophin. Alternative splicing at the 3'-end and alternative polyadenylation (the addition of a poly(A) tail to RNA) yield additional isoforms of dystrophin such as Dp40. The primary muscle isoform is the full-length protein generated from Dp427m. b) Dystrophin production in patients with DMD is prematurely truncated and the resulting protein is not functional. This leads to the loss of connections between the cytoskeleton and the extracellular matrix. c) On the other hand, in patients with Becker muscular dystrophy, a partially functional dystrophin is produced that contains the crucial domains required to connect to F-actin and the extracellular matrix.

Note. Taken from Duchenne muscular dystrophy by Duan, D., Goemans, N., Takeda, S. et al. (2021). Nat Rev Dis Primers 7, 13 (https://doi.org/10.1038/s41572-021-00248-3).

1.2.3. Mechanisms and pathophysiology

DMD is a progressive muscle degeneration disease. As it progresses, different symptoms appear along a patient's lifetime (Table 1).

Phases of DMD in human patients	Symptoms
Early phase (up to age 7)	Difficulty in sitting, walking, or talking. Children may be more tired than other non-affected children
Transitional phase (ages 6 to 9)	Increasing weakness in muscles. Difficulty in walking, going up stairs, and getting up from a sitting position Children may walk on their toes or have a wobbly or abnormal gait. Heart problems
Loss of ambulation phase (ages 10 to 14)	Teenagers become more and more dependent on a wheelchair. Scoliosis (curving of the spine) may develop, and the heart and breathing ability will be closely monitored
Teenager (age 15+)	Regular heart and lung monitoring is required, medications are often necessary, and breathing support may be needed
Adult (age 30)	Premature death

Table 5. DMD progression in human patients.

1.2.4. Dystrophin-associated protein complex and function

Dystrophin is a 427kDa cytoskeletal protein that is bound at the cytoplasmic face of the sarcolemma, which is the cell membrane of a muscle cell, and is enriched at costameres in muscle fibers (22). This protein has four main functional domains: an actin-binding amino-terminal domain (ABD), a central rod domain, a cysteine-rich domain and a carboxyl-terminus. ABD contains 2 calponin homology domains (CH1 and CH2) (23). The CH-actin binding domain binds directly to F actin, linking dystrophin to the subsarcolemmal actin network (24). All this complex works as a broader cytoskeletal integrator, essential for muscle membrane stability.

The discovery of dystrophin-binding partners is related to a combination of studies in human cell models and animal models along with the study of autopsy and biopsy tissues from patients with muscular dystrophy. For example, in striated muscle, dystrophin interacts with the sarcolemma, cytoskeleton, channel proteins, and signaling or scaffolding proteins either directly or indirectly (Figures 2 and 3). Specifically, dystrophin interacts with the sarcolemma through four binding domains located in spectrin-like repeats R1-3 and R10-12 and in the cysteine rich (CR) and C-terminal (CT) domains (Figure 3) (25). Collectively, dystrophin and its binding partners form the dystrophin-associated protein complex (DAPC) (26), that was discovered in the early 1990s and was called the dystrophin glycoprotein complex (DGC) as several components are glycoproteins. The DGC contains 11 proteins: dystrophin, the dystroglycan subcomplex (α-dystroglycan and β-dystroglycan), the sarcoglycan subcomplex (α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan and δ-sarcoglycan), sarcospan, syntrophin, dystrobrevin and neuronal nitric oxide synthase (nNOS) (27). Since the discovery of the DGC, the knowledge about dystrophin-interacting partners has increased. More binding partners have been identified and the interaction domains of many of these proteins have been mapped. Accordingly, these data have improved our understanding of DMD pathogenesis.

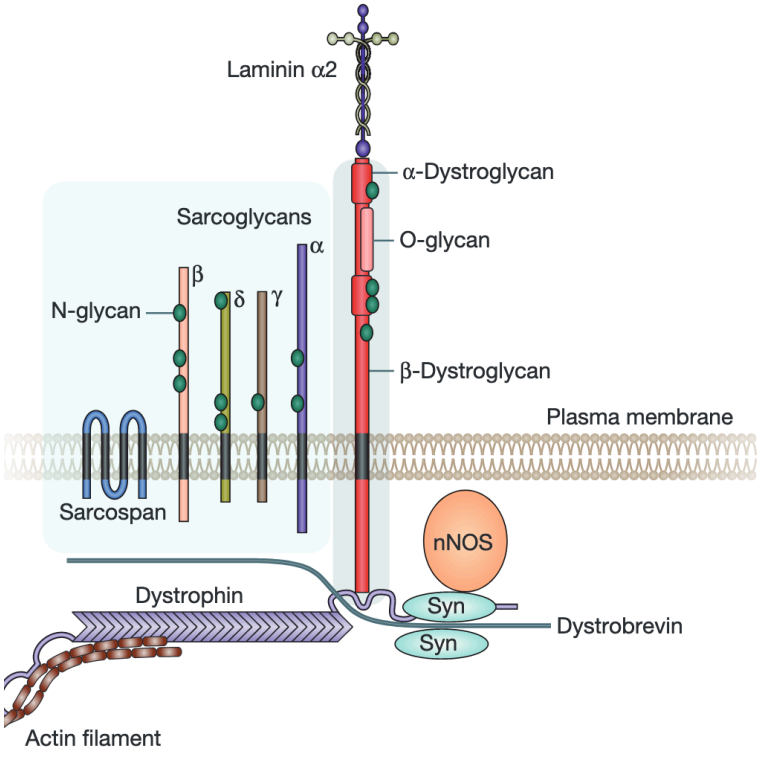


Figure 2. Dystrophin-glycoprotein complex (DGC) in a healthy muscle.

Dystrophin is a large shape protein that connects intracellular cytosqueleton to transmembrane components of the DGC, including dystroglycan and sarcoglycans. Dsytroglycan consists of two subunits, α and β. α-Dystroglycan is an extracellular peripheral membrane protein and a receptor for laminin-2. Besides a structural role, the sarcoglycan complex is also involved in signal transduction and mechanoreception. The syntophins, dystrobrevins, and nNOS are recruited to the C-terminus of dystrophin and participate in signal transduction pathways.

Note. Taken from Sarcoglycanopathies: molecular pathogenesis and therapeutic prospects by Sandonà, D. & Betto, R. (2009). Expert Reviews in Molecular Medicine, 11 (28). (10.1017/S1462399409001203).

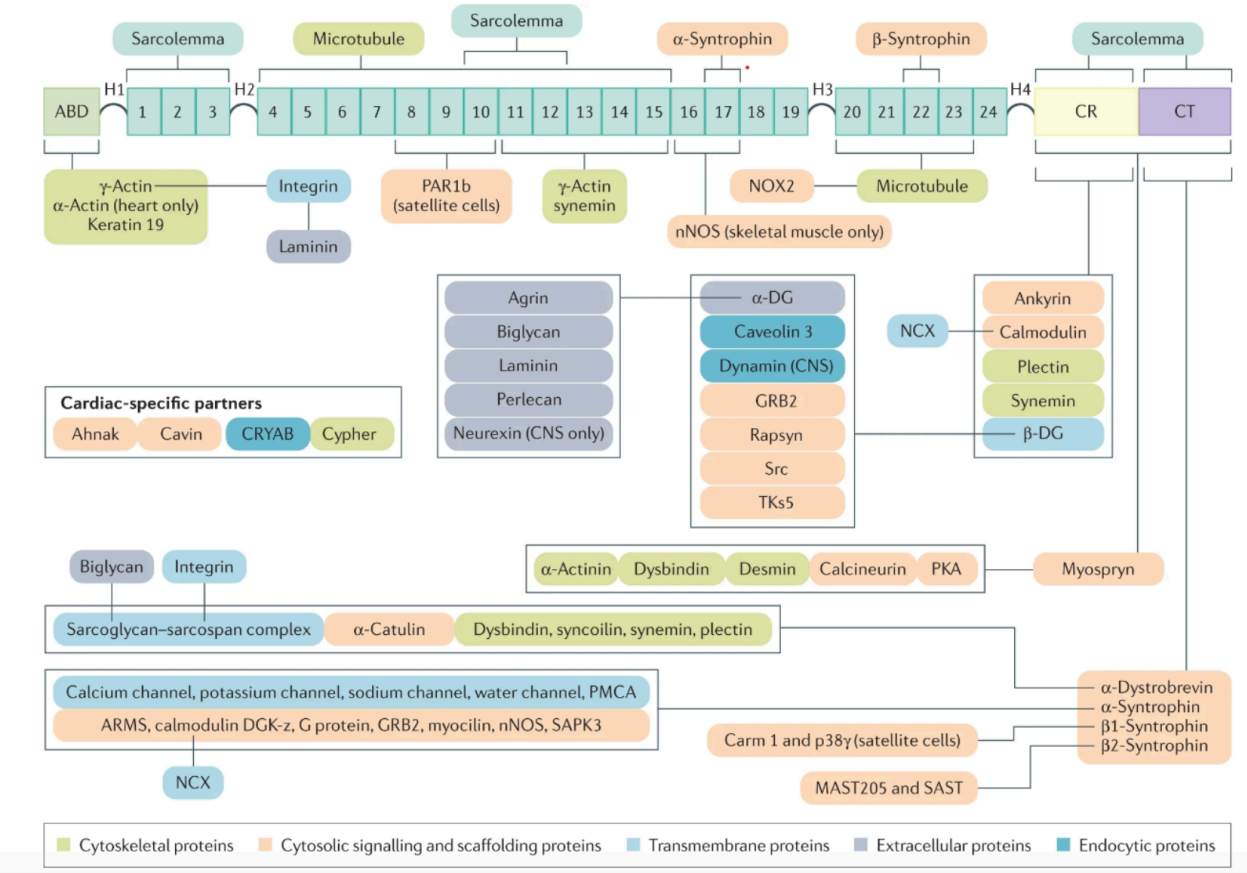


Figure 3. Dystrophin domains.

Dystrophin binding partners can be categorized as cytoskeletal proteins, transmembrane proteins, extracellular proteins, cytosolic signalling and scaffolding proteins, endocytic proteins, and cardiac-specific interacting proteins. ABD, actin-binding domain; CNS, central nervous system; CR, cysteine-rich domain; CT, C-terminal domain; DG, dystroglycan; NCX, sodium-calcium exchanger; nNOS, neuronal nitric oxide synthase; NOX2, NADPH oxidase 2; PMCA, plasma membrane calcium ATPase.

Note. Taken from Duchenne muscular dystrophy by Duan, D., Goemans, N., Takeda, S. et al. (2021). Nat Rev Dis Primers 7, 13 (https://doi.org/10.1038/s41572-021-00248-3).

1.3. Consequences of dystrophin deficiency

When dystrophin is disrupted, it provokes the disassembly of the DAPC and the loss of the interaction between F-actin and the extracellular matrix, leading to different pathogenic consequences on muscle cell integrity and function that will be discussed below. In this section, several studies with DMD mouse will be be explained. To better understand these studies, it should be noted that the classical DMD mouse model is generated by a nonsense mutation in exon 23 of the *dmd* gene, which provokes incomplete proteins translation and futile dystrophin protein, leading to the common features of muscular dystrophy. Further details of this model will be explained in the section named "Animal models of DMD".

1.3.1. Sarcolemma weakening

In normal conditions, the integrity of the sarcolemma is controlled via connections between the cytoskeleton, sarcolemma, and extracellular matrix through the DAPC and the integrin complex (Figure 4). In DMD, DAPC disassembly weakens the sarcolemma, which becomes highly susceptible to contraction damage. Several pieces of evidence support this mechanism. First, sarcolemmal tears (also called "delta" lesions) have been observed using electron microscopy in muscle from patients with DMD (28). Moreover, sarcolemma rupture can be identified by passive leaking-in of circulating proteins or dyes, such as albumin, IgG, and Evans blue, and leaking-out of muscle enzymes, such as creatine kinase (CK) from muscle into the blood, especially after exercise in both humans and mice (29). Moreover, muscle damage correlates with muscle stress (30). For example, the diaphragm, the most stressed muscle, is affected earlier and more severely than less-stressed muscles. Finally, muscle disease is significantly restored in animal models with even a partial recovery of cytoskeleton, sarcolemma and extracellular matrix connection using adeno-associated virus micro-dystrophin gene therapy.

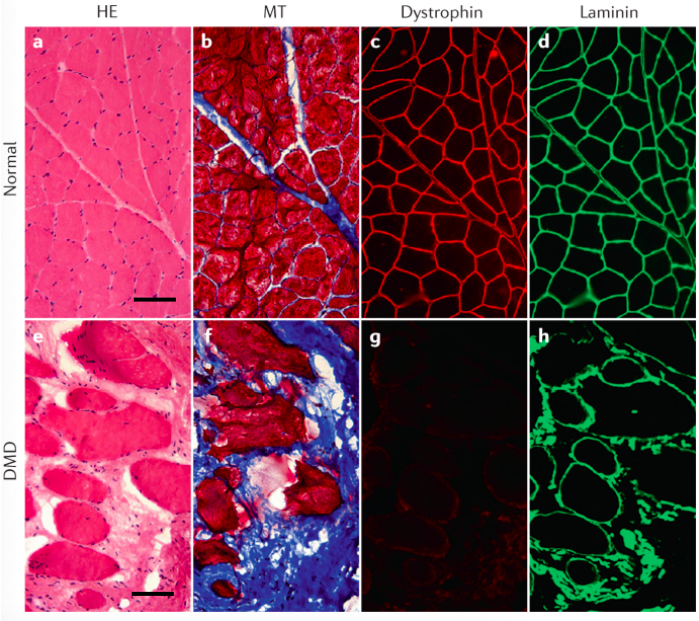


Figure 4. Healthy muscle and DMD muscle histology.

Cross-sectional staining of healthy muscle (panels a–d; scale bar represents 60 μm) and skeletal muscle from a patient with Duchenne muscular dystrophy (panels e–h; scale bar represents 100 μm). Haematoxylin and eosin (HE) staining shows centrally nucleated myofibers, inflammatory cell infiltration, variable myofiber size, and endomysium and perimysium connective tissue deposition (panels a and e). Masson trichrome (MT) staining shows increased fibrosis (blue staining) in a patient with DMD compared with healthy muscle (panels b and f). Immunofluorescence labelling of dystrophin and laminin shows a lack of dystrophin in a patient with DMD compared with healthy muscle (panels c and g) and variation in myofiber size in DMD muscle (panels d and h).

Note. Taken from Duchenne muscular dystrophy by Duan, D., Goemans, N., Takeda, S. et al. (2021). Nat Rev Dis Primers 7, 13 (https://doi.org/10.1038/s41572-021-00248-3).

1.3.2. Inflammatory reactions

In DMD, there is a strong activation of multiple components of the innate immune system after birth and before symptoms appear. This includes altered signaling via Toll-like receptors (TLR4, TLR7) and via nuclear factor κB (NF-κB), and expression of major histocompatibility complex (MHC) class I molecules on muscle cells (31). Some studies showed that associated discharge of cytoplasmic contents into the extracellular space and membrane fluctuation mediate this chronic activation of the innate immune system and associated inflammatory response. A second pathological mechanism is the degeneration and regeneration of myofibers, which takes place concurrently on the chronic proinflammatory level (32). In this process, muscular fibers are infiltrated by neutrophils and phagocytosed by macrophages. Meanwhile, resident myogenic stem

cells are activated and differentiate into myoblasts, and regeneration of the myofiber appears within the pre-existing basal lamina. As the regenerated muscular myofibers remain dystrophin-deficient, this leads to successive rounds of degeneration and regeneration, with a temporally pattern of inflammatory infiltrates (33). Conclusively, with increasing age, the interplay between chronic activation of innate immunity and asynchronous and neighboring rounds of degeneration and regeneration promotes a poor repairing mechanism response that could itself lead to disease progression (34).

1.3.3. Genetic dysregulation

Gene expression profiling can be used to gain insights into the biochemical mechanisms underlying the progressive pathophysiology of a muscular disease (35). In DMD, there is a general metabolic catastrophe in dystrophic muscle, with large-scale downregulation and upregulation of gene expression (36). Particularly, some studies showed that in DMD, the expression of 138 genes is upregulated, whereas the number of the downregulated genes is 137 (37). Among the upregulated genes, 42% of them are genes of cell surface and extracellular proteins (42%). Additional functional groups include genes involved in immune responses (20%) and cell growth, differentiation, and signaling (15%) (38). About 80 downregulated genes, 36% of them were involved in mitochondria function and energy metabolism and approximately 12% of downregulated genes were involved in cell growth, differentiation, and signaling (39).

Recent studies have shown that epigenetic changes occur in DMD-mutant versus control mouse (Figure 5). In DMD-mutant, the epigenetic differentiation network is disrupted due to the lack of dystrophin-α/β-syntrophin-nNOS signaling and the interruption of histone deacetylase HDAC2 S-nitrosylation. Therefore, there is a decrease in the levels of miR-133/1 and miR-29, followed by inhibition of muscle differentiating genes and ROS generation (40). Also, diminished amounts of miR-29 levels correlate with an increase in collagen and fibrotic tissue. The miR-206 level is higher, resulting in an imbalance between proliferative and differentiated states. Additionally, miR-144 and miR-223 are also observed following muscle degeneration and an increase in inflammation. The other miRNA committed in the pathogenesis is miR-206, which targets utrophin mRNA and whose appropriate concentration in skeletal muscle could inhibit the development of DMD (41).

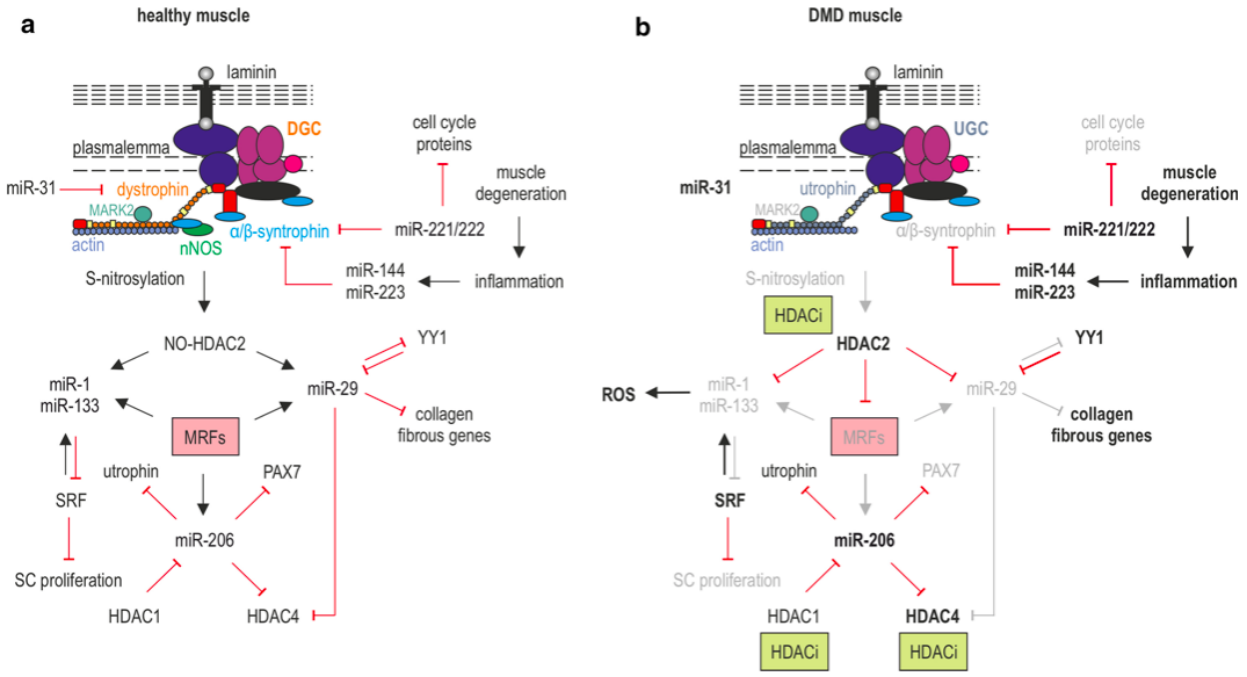


Figure 5. Epigenetic modifications in muscle regeneration and progression of Duchenne muscular dystrophy.

The dystrophin-nNOS signaling regulates the epigenetic profile of myogenesis via S-nitrosylation of HDAC2 (class I HDAC), which affects gene expression through changes in histone acetylation. Both miR-221/222 are involved in the inhibition of cell cycle proteins and miR-222 targets β-syntrophin, while miR-31 temporarily targets dystrophin. Transcription of miR-1 and miR-133 is regulated by the HDAC2 S-nitrosylation state, controlled by nNOS activity. Also, miR-133 targets SRF, which in a self-regulating manner promotes miR-133 expression, and miR-29 is coregulated by the HDAC2 S-nitrosylation state and YY1, while miR-206 is regulated by MRFs and the HDAC1 activity, and supports cell cycle inhibition by targeting PAX7. B) In DMD, the epigenetic differentiation network is disrupted because of the absence of dystrophin-α/β-syntrophin-nNOS signaling and interrupted HDAC2 S-nitrosylation. Consequently, a decrease in the levels of miR-133/1 and miR-29, followed by inhibition of muscle differentiating genes and ROS generation is observed. Also, diminished quantities of miR-29 levels correlate with an augmentation in collagen and fibrotic tissue. The miR-206 level is higher, resulting in an imbalance between proliferative and differentiated states. Moreover, miR-144 and miR-223 are also observed following an elevation in inflammation and muscle degeneration. miRNA and protein names marked in bold and grey indicate their upregulation and downregulation, respectively; black arrows mark activation while red and grey blunt lines inhibition and reduction in inhibition, respectively. HDACi targeting HDACs and the corresponding processes are marked in green boxes.

Note. Taken from Epigenetic modifications in muscle regeneration and progression of Duchenne muscular dystrophy by Rugowska, A., Starosta, A. & Konieczny, P. (2021). Clin Epigenet 13, 13. (https://doi.org/10.1186/s13148-021-01001-z).

1.4. Current therapies

Since no cure for DMD has been yet found, drugs are currently the only compounds capable to alleviate the symptoms of the disease. These drugs are described in the following sections.

1.4.1. Anti-inflammatory

The broad anti-inflammatory treatment is the current corticosteroid therapy, which comprises the use of two different glucocorticoids: prednisone and deflazacort. They are the only medication that has been used for over 20 years to mitigate muscular weakness. Several studies have shown a positive impact in patient lives: lower rate of scoliosis surgery, enhanced lung function, and improved cardiac function (42). However, some adverse effects should be considered. In long-term treatment, the most frequent adverse effect is the reduction in the patient's height. The second most frequent one is weight gain, which is a consequence of reduced mobility. Other side effects include delayed puberty, vertebral fractures, and gastrointestinal problems, among others.

The indicated amount of prednisone is 0.75 mg/kg/day, however when the dose is less than 0.3 mg/kg the effect is reduced, and daily administration seems to be more effective than on alternating days (43). Also, it is recommended to start the treatment once the patient has reached the "plateau phase", which means the patient is between 4 and 6 years old.

Deflazacort is an oxazoline derivate of prednisone and at a dose of 0.9 mg/kg/day, has the same effect as prednisone. Although deflazacort is preferred by patients (44), the risk of development of cataracts is increased in comparison with prednisone. A study showed that 10-30% of cataracts were observed at an average of 3.2 years of treatment with deflazacort (45). Recent findings on the inflammatory mechanisms in DMD are discussed, especially focusing on inflammatory factors released from skeletal muscles, which are regulated by calcium influx, reactive oxygen species (ROS), and nuclear factor-kappa B (NF-κB), which are potential targets for treatment as master regulators (Figure 6). In fact, ryanodine receptor (RyR) channel blockers, antioxidants, and NF-kB inhibitors are under clinical trial (46) and seem to be promising, as they are more effective and less toxic in comparison with the corticosteroid therapy, which can induce

the release of inflammatory factors such as damage-associated molecular patterns (DAMPs) and inflammatory cytokines and the suppression of anti-inflammatory cytokines such as hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1) that arbitrate DMD pathological mechanisms (47).

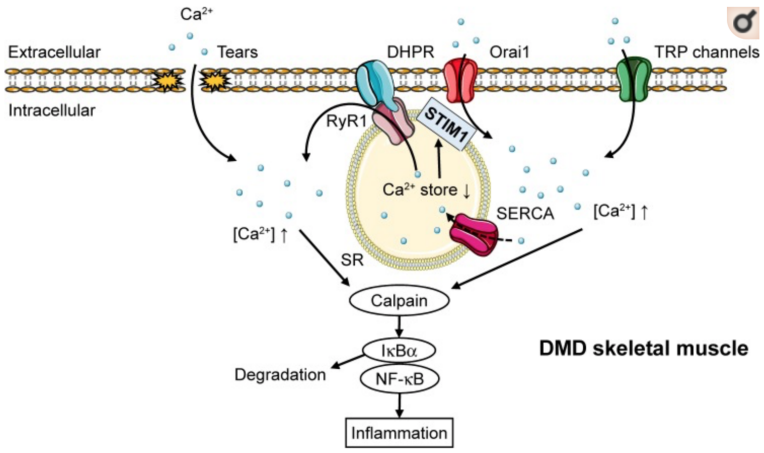


Figure 6. The hypothetical pathway of Ca2+ when DMD muscles respond to inflammation.

There are four pathways for cytosolic Ca^{2+} increase in DMD muscles. First, membrane tears promoted in the dystrophin-deficient muscles allow direct Ca^{2+} entry by disrupted plasma membrane. Second, overactivated "leaky" RyR1 releases Ca^{2+} from SR to cytoplasm. In healthy people, membrane depolarization in EC coupling provokes the voltage-sensitive DHPR activation, subsequent opening of RyR1 and transient Ca^{2+} release into cytoplasm, followed by Ca^{2+} reuptake into SR lumen by SERCA. On the contrary, in DMD patients, overactivated RyR1 releases Ca^{2+} into cytoplasm constitutively. Third, the depletion of ER/SR Ca^{2+} store promotes the translocation of STIM1 in ER/SR membrane to regions close to the plasma membrane, where STIM1 activates Orai1 overexpressed in DMD, allowing Ca^{2+} influx through plasma membrane into cytosol. Fourth, overexpressed TRP calcium channels on plasma membrane in DMD induce cytosolic Ca^{2+} increase from extracellular. The upregulated cytoplasmic Ca^{2+} leads to calpain overactivation, inducing NF-κB activation mediated by IkBα degradation, and eventually stimulating inflammatory cytokines production and release.

Note. Taken from Anti-inflammatory drugs for Duchenne muscular dystrophy: focus on skeletal muscle-releasing factors by Miyatake, S., Shimizu-Motohashi, Y., Takeda, S., & Aoki, Y. (2016). Drug design, development and therapy, 10, 2745–2758 (https://doi.org/10.2147/DDDT.S110163).

1.4.2. Gene therapy

A lot of research is being done in order to find a potential gene therapy whereby a wild-type version of the dystrophin gene is introduced/injected. However, several technical challenges

are dampening the progress of this therapeutic strategy. First, the dystrophin gene is a large gene (2.4 Mbp) and cannot fit into classical adeno-associated virus (AVVs) that are commonly used for gene therapy approaches. Also, another main challenge is to get enough gene therapy product into muscle tissues to be effective.

In 2020, some companies were using gene therapy for the treatment of DMD, and their studies could be in late-stage clinical trials by next year (48). Scientists have engineered smaller modified genes that produce a shortened version of functional dystrophin. Delivered at high doses, these AVVs are supposed to escort the genetic instructions through the blood and into various muscles. This kind of therapy could help patients to produce these proteins for potentially many years.

1.4.3. Exon skipping

One of the most promising therapeutic strategies to overcome the deletion of dystrophin in DMD is exon-skipping therapy using antisense oligonucleotides (ASOs). These molecules can switch splicing patterns by targeting specific sequences at the level of the pre-mRNA, that are involved in exon recognition (49). Targeting splice sites or putative exon splicing enhancers with ASOs can provoke the elimination of exons containing pathogenic mutations from the mature DMD transcript, and therefore generate an internally deleted but partly functional dystrophin (50).

ASOs have been largely tested in disease models and are presently being examined in several clinical trials (51). However, as each ASO targets one particular mutation, that means there will be one drug per ASO; thus, ASOs for different exons must go through long and costly clinical trial stages. Lately, two different single exon duplications (involving exon 44 and exon 45) and a multiple exons duplication (exons 52-62) have been modulated by ASOs in cultured patient cells (52). Notably, the response to ASOs treatment turned out to be exon-specific and linked to the level of spontaneous exon skipping. Thus, the transcriptional behaviour of the different duplications could guide the use of ASOs, leading to the design of different therapeutic approaches.

Currently exon skipping therapy is addressing the skipping of exon 51 because this approach is applicable to specific mutations shared by about 13% of patients, the largest group of

DMD patients. Eteplirsen (brand name *Exondys 51*) is the first FDA-approved DMD treatment for patients whose mutation in the dystrophin gene can be treated by skipping exon 51 (53). Although this approach seems to be applicable to a large volume of patients (54), it should be noted that this will not offer a categorical cure but an advance towards a BMD-like phenotype depending on the activity of the recovered dystrophin.

1.4.4. Vitamin D supplement

Patients with DMD are characterized by vitamin D deficiency, commonly observed probably due to decreased sunlight exposition (55). Bone mineral density diminishes because of reduced mobility, thus there is a higher risk of osteoporosis. Therefore, a calcium and equilibrated vitamin D diet with physical exercise are recommended to alleviate some of DMD symptoms, especially in patients with vitamin D deficit (serum 25 hydroxyvitamin D < 50 mg/ ml) (56).

In conclusion, although there is a wide range of therapies for DMD, there is still no curative treatment available. Active research on this topic is currently underway. For now, the only molecules that seem to be effective in decreasing the progression of this disease are corticosteroids (prednisone and deflazacort). However, the identification of new small molecule therapies would be interesting since they could be a drug-specific treatment for each patient and the first-class medication while moving toward gene therapy.

2. Animal models of DMD

2.1. Classical preclinical animal models

For many years, scientists have been performing their studies using different DMD animal models. Here we show a table that summarizes the benefits and challenges of using some of these animals.

ANIMAL MODEL	ADVANTAGES	DISADVANTAGES

Mice	Easy to house and care for with a relatively short lifespan. High genetic similarity to humans DGC	Minimal clinical symptoms (no loss of ambulation and muscle weakness is not displayed until ~15 months) and lifespan is not majorly reduced
Rat	Still relatively easy to house and care for. Convenient physical size as they are larger than mice. High genetic similarity to humans including a DGC	Not a well-established model and characterisation is still ongoing.
Pig	Genetic, physiological, and anatomical very similar with humans. Cardiac system almost identical to humans.	Expensive because of gestation period (114 days), federal regulations restrictions for the use of genetically modified pigs, and variations in the genome-editing method
Rabbit	Suitable for productivity and cost. Larger physical characteristics than rodents with humans.	More expensive than rodents (higher body size and 31 day-gestation period)
Monkey	Useful for pharmacological analysis, safety examination, and the testing of treatments that commonly augment muscle expansion	Expensive to maintain and absence of a convenient monkey model of DMD
Dog	Higher genetic similarity to humans compared to other mammalian models. Case reports showing that DMD occurs naturally in these animals as well.	Expensive to maintain, not easily genetically manipulable and many ethical concerns.
C. elegans	Easy and cheap to maintain, short lifespan, high throughput experiments. Similar muscle	Have a very simple body plan and nonconventional circulatory system. Are unable to regenerate muscle as

	structure and has orthologues for most human DGC proteins.	they lack satellite cells and do not have a conventional immune system.
Drosophila melanogaster	Suitable for large-scale, high-throughput drug screening. Easy to maintain and care.	Missing high amount of mammalian organs.
Danio rerio	Easy to house and care for, high throughput experiments possible. High skeletal muscle content and expresses orthologues of most human DGC proteins	Missing several mammalian organs, are ectothermic and are influenced heavily by their environment.

Table 2. Benefits and restrictions of different DMD animal models.

2.1.1. Mice

The most commonly used mouse model for DMD is the strain C57BL/10ScSn-*Dmd*^{mdx}/J (BL10-*mdx*) mouse. This strain carries a nonsense mutation in exon 23 of the *dmd* gene, which provokes an incomplete protein translation and futile dystrophin, leading to the common features of muscular dystrophy (57). Nevertheless, even though BL10-*mdx* mice have a defective dystrophin, the disease symptoms are much more benign than that of patients with DMD. The reason why it occurs is, among other causes, the big differences in body size, muscle loading, length of growth state between species and possible discrepancies in capacity for muscle regeneration across species (58). Since there is a marked difference in disease pathology, extrapolating therapeutic benefits obtained in BL10-*mdx* mice to the human disease should be done with carefulness (59).

Lately, a new DMD mouse model has been generated by crossing BL10-*mdx* mice on a DBA/2J [D2-wild type (WT)] genetic background (60). This new strain D2.B10-*Dmd*^{mdx}/J (D2-*mdx*) mice exhibit a more serious dystrophic symptoms than BL10-*mdx* mice, comprising damaged muscle function and regeneration, diminished muscle weight, and increased levels of fibrotic tissue

in skeletal muscles (61). Fundamentally, these mice may provide a promising alternative to the BL10-*mdx* model. Despite the potential use of the D2-*mdx* strain for research, more study of this model has to be done, although the number of preclinical studies using this model is exponentially increasing.

The advantages of the mdx mouse are linked to their steady phenotype and comparatively modest cost, allowing numerous variables to be analyzed. However, the mice's small size may limit examination of scalable variables such as cell migration or drug diffusion. In addition, preclinical trials in mdx mice have mostly found significantly immunologic side effects of gene and cell therapies (62).

2.1.2. Rats

A rat model could serve as potential research alternative due to their size (rats are 10 times bigger than mice) and could better show the lesions and functional disorders observed in DMD patients. DMD mutated rats (dmd^{mdx}) were generated by using TALENs targeting exon 23. Muscles of these animals show undetectable amounts of dystrophin by western blot and less than 5% of the protein by immunohistochemistry. Forelimb and hindlimb muscular strength and spontaneous activity are significantly diminished in these rats. At 3 months, limb and diaphragm muscles show necrosis and regeneration. At 7 months, these muscles are characterized by severe fibrosis and some adipose tissue replacement. Moreover, as opposed to mice, rats present dilated cardiomyopathy caused by necrotic and fibrotic muscular changes (63). Some other constraints can appear when generating a *dmd*-mutant rat. For example, individuals within strains are not isogenic, and there are differences in the levels of genetic variation. There are mosaic mutations that require careful consideration (64).
Although there are some disadvantages of using rats, the weak muscle strength and muscular lesions in these mutated rats firmly mimic those observed in DMD patients.

2.1.3. Pigs

As rodent models are not fully capable of mimicking DMD patient phenotypes, there are several studies that use larger animal models of DMD such as pigs. Since pigs share genetic,

physiological, and anatomical characteristics with humans compared to other animals, they seem to be an interesting animal model candidate (65). Specifically, the cardiac system in the porcine world is almost identical to humans, which represents advantages in possibly designing the cardiac features of DMD. In 2013, Klymiuk et al. generated *dmd* exon 52-deleted pigs that principally died within the first week of life (66).

The most efficient technology used to generate DMD mutant pigs is CRISPR/Cas9 (67), which was first developed by Yu et al. in 2016 (68). Yu and his team microinjected Cas9 mRNA and a gRNA targeting *dmd* exon 27 into the cytoplasm of 1-cell stage embryos from Diannan miniature pigs, afterwards transferring the embryos into surrogate mothers. Out of 98 injected embryos, two piglets were successfully born. With one of them having the mutation, they both died after their birth. The mutant pig died at day 52 after birth and carried four different indel mutations in the *dmd* gene that differed in distribution and level across tissues. So, the productivity of frame-disrupting mutations in skeletal muscle was 60%. The amount of dystrophin in the DMD pig was lower than the age-matched wild-type in the heart, intestine, and biceps femoris, but not significant. Common dystrophic aspects such as necrosis, reduced myofiber size, and myofiber disorganization were also detected in skeletal muscles. The acutely reduced lifespan of the mutant pig generated by Yu et al. is comparable to the pigs that were studied by Klymiuk et al. As this notoriously diminished survival inhibits the use and adequate production of porcine DMD models for pre-clinical study, research into why this occurs or variations in the genome-editing method may help surmount the problem. The lengthy gestation period of pigs at 114 days, their requirement for bigger living spaces and costly maintenance, as well as federal regulations restricting the use of genetically modified pigs (69) are all challenges that must be overcome for using this DMD model in research.

2.1.4. Rabbit

In contrast to pigs, rabbits are more convenient in terms of productivity and cost while sharing greater similarity at the physiological, genetic, and anatomical levels to humans than mice (70). Rabbits have a gestational period of 31 days, the capability to breed throughout the year, and have diminished space requirements than dogs or pigs. Their average dimensions also bring the advantages that come with augmented body size without much of the financial handicap (71). Sui

et al. (2018) generated a DMD rabbit model by co-injecting Cas9 mRNA and two gRNAs targeting *dmd* exon 51 into rabbit zygotes (72). A total of 33 live rabbits were obtained out of 128 microinjections, 26 of which had at least one mutated *dmd* allele. Apart from having indels of several sizes, the mutant rabbits had markedly lower *dmd* transcript levels, entire dystrophin function loss as well as noticeably diminished α-sarcoglycan and glycosylated α-dystroglycan at skeletal muscle membranes (73).

Notably diminished mobility in the 1-hour walking and step-climbing experiments were detected in the DMD rabbits, along with forelimb paralysis. Survival was reduced, with 42.6% of the rabbits dying by 20 weeks. In comparison with wild-type, 5-month-old mutant rabbit skeletal muscle had dystrophic phenotypes in addition to a notably larger percentage of centrally nucleated fibers (CNFs), augmented fibrosis, fat restoration, and significantly diminished fiber areas to less than half the standard wild-type amounts (74). More particularly, in contrast to *mdx* mice that do not have evident cardiac dysfunction until 1 year of age (75), DMD rabbits present notably diminished ejection fraction and fractional shortening at 4 months old in contrast to wild type. Histological experiments of cardiac muscle showed dystrophic evidence, despite the absence of notable variations in gross heart anatomy between DMD mutant and healthy control rabbits. These characteristics make DMD rabbits a preferable model for analysing the cardiac phenotype of the disease and for examining treatments that focus on the dystrophic heart. In conclusion, this model is yet to be used for therapeutic testing.

2.1.5. Monkey

Monkeys have mostly been needed for pharmacological analysis, safety examination, and the testing of treatments that commonly augment muscle expansion (76). The absence of a convenient monkey model of DMD has limited more detailed assessments of dystrophin-targeting cures in these mammals. This context changed in 2015 with the generation of DMD mutant rhesus monkeys (*Macaca mulatta*) by Chen et al. (77). Chen and his team used CRISPR/Cas9 to insert mutations in exons 4 and 46 of the monkey *dmd* gene. A total of 2 stillborn and 9 live monkeys out of 179 microinjected embryos resulted from the editing process. Mosaic mutations were noted in some live monkeys. As both wild-type and mutant stillborn monkeys were detected, the authors did not link this phenotype to CRISPR/Cas9 action. Histological material of the stillborn monkeys

(1 female, 1 male) was examined, showing that this presented 3 different frameshifting mutations in exon 4: a deletion of 2 bp, and insertions of 2 bp and 20 bp. Modified mutation rates of each type were observed throughout tissues and between monkeys, with 87% (female) or 67% (male) of *dmd* alleles in skeletal muscle mutated in total as analyzed by RT-PCR. The decrease in dystrophin protein in muscle positively correlated with mutation load. Histological analysis of skeletal muscle samples from the stillborn monkeys revealed a general reduction in fiber size, showing hypertrophic myofiber clusters, 12.5/17.5% CNFs, and augmented interstitial space area. It is noted that these dystrophic phenotypes started appearing at a prior time before birth. Phenotypic studies were not executed for the live monkeys, which presented a mixture of frameshifting mutations in exons 4 and/or 46. Despite these previous studies, no work with monkeys in the therapeutic field has been published yet.

2.1.6. Dog

The use of canine DMD models started with the golden retriever. Before the *dmd* gene and dystrophin protein were discovered in the 1980s, reported animal models were chosen based on analogies in the pattern of inheritance and shared phenotypic characteristics. In the 1970s and 1980s, Golden retriever dogs presented an apparent X-linked degenerative myopathy, later named golden retriever muscular dystrophy (GRMD) due to the presence of common symptoms with those of human DMD. These features, including elevated serum enzymes, sarcolemmal deficiency, and complex repetitive discharges (CRDs) on electromyography (EMG), were in accordance with the membrane theory of DMD disease pathogenesis (78). Rusty, a dog with GRMD identified at the University of Georgia in 1981, was the founder for initial colonies at Cornell University. Further studies confirmed genetic homology with DMD, and several additional colonies were generated in the USA and around the world (79). In comparison with mice, dogs are more severely affected, often stabilizing the phenotype after 6 months of age.

These pros and cons are substantially inverted for dystrophic dogs. The expenses for dog maintenance and required facilities restraints the number of dogs that can be used. As with preclinical studies, data from these animal models have not consistently translated to DMD patients.

In conclusion, the GRMD model has been useful in outlining complications of pharmacologic treatment and immunologic reactions to cell and gene therapies (80).

2.2. Alternative preclinical animal models

Ongoing drug discovery efforts for DMD are principally based on preclinical mouse models. This is feasible when testing with one or two drug candidates. Yet, it becomes very difficult when analyzing an extensive range of amounts of a drug or to find a hit from hundreds of compounds. The time-consuming and activity-demanding nature of the mice research also excludes the chance of intensely examining all known compounds that reside in the same category of a lead drug. The use of non-mammalian DMD models is a good choice for high-throughput screening of extensive libraries of chemical compounds.

Three most frequently developed non mammalian models are nematode (*C. elegans*), Drosophila (*D. melanogaster*) and zebrafish (*D. rerio*). These models possess many attractive characteristics that make them remarkably desirable for high throughput drug screening. These comprise their large progeny size, short life cycle, easy maintenance, genetic tractability, and early-onset visible phenotypes. Also, the dystrophin gene is vastly conserved between mammals and non-mammals (81).

2.2.1. *Caenorhabditis elegans*

The first *C. elegans* DMD model *dys-1(cx18)*, *hlh-1(cc561ts)* was generated in 2000 by Segalat (82). With a null mutation in the dystrophin gene *dys-1(cx18)* and a thermosensitive mutation in the MyoD gene *hlh-1(cc561ts),* this double mutant worm presents a time-dependent mobility and muscle deterioration. Mobility change can be identified by eyeballing. Muscle impairment can be analyzed with phalloidin staining. Using this approach, the Segalat lab blindly tested approximately 1,000 approved drugs (83). They found more than 20 candidate drugs that were able to block muscle deterioration and/or ameliorate motility. Moreover, these drugs not only comprised the ones that were known already (such as prednisone, cyclosporin A and calcium antagonist Nifedipine) (84), but also consisted of many new compounds such as antidepressants (serotonin and tricyclic compounds) and carbonic anhydrase inhibitors (methazolamide and

dichlorphenamide) (85). Further validation works in mdx mice verified muscle preservation effect of antidepressants and carbonic anhydrase inhibitors (86).

Lately, Hewitt et al. described a new worm DMD model only deficient for dystrophin *dys-1(cx33)* (86). The *dys-1(cx33)* mutant carries a new dystrophin null mutation and shows a more severe phenotype than the *dys-1(cx18)* mutant. Developing an automatic force measurement system called NemaFlex, the authors confirmed strength diminution in the *dys-1(cx33),* but not *dys-1(cx18)* mutant. The author in addition corroborated this new model as a drug screening model using compounds that are known to improve muscle force in the DMD model (such as prednisone and melatonin). In conclusion, the authors proposed that future drug screening should be done on the *dys-1(cx33)* model.

2.2.2. *Drosophila melanogaster*

A broad compilation of Drosophila DMD models has been developed. According to the FlyBase website, around 17 knockdown models have been developed by using RNA interference (87). In addition, at least 7 genetic loss-of-function mutants are accessible including Dys^{8-2}, Dys^{E17}, Dys^{det-1}, Dys^{EP3397}, Dys^{Df}, Dys^{kx43}, and $Dys^{Exel6184}$ (88). These models show gradual loss and muscle deterioration. Moreover, dystrophin plays an important role in wing vein development in Drosophila (89), so morphological modifications in the wing vein can be a marker for phenotypic screening (90).

As mentioned before, dilated cardiomyopathy is a considerable health characteristic in DMD patients (91). Two compound mutants Dyskx43/DysExel6184 and Dyskx43/Dys^{8-2} exhibit cardiac dysfunction simulating dilated cardiomyopathy (92). Interestingly, heart disease in the fly DMD model can be restored by Dp116, a naturally truncated dystrophin isoform carrying only spectrin-like repeats 22, 23, 24, hinge 4, the cysteine-rich domain and the C-terminal domain. According to the fact that Dp116 aggravates skeletal muscle disorder in dystrophin-null mdx4cv mice (92), considerations should be taken when using Dyskx43/Dys^{8-2} and Dyskx43/DysExel6184 as a DMD cardiomyopathy model.

Although the fly DMD model has been recommended for large-scale, high-throughput drug screening (93), no unbiased screening has ever been conducted using a large chemical compound

library yet. However, developing a candidate drug approach, Pantoja et al. showed that drugs involved in the signaling of sphingosine 1-phosphate, including 2-acetyl-4(5)-tetrahydroxybutyl imidazole (THI), THI-oxime (a derivative of THI), and FTY720 (gilenya), can abolish dystrophic phenotypes in several fly DMD models (94). A consecutive study in mdx mice confirmed these findings (95).

2.2.3. Zebrafish

Zebrafish is the most commonly non mammalian animal used as a DMD model for high-throughput drug testing (96). Comparable to the fly model, both knockdown and genetic null DMD models are accessible in zebrafish. In the knockdown model, dystrophin function is diminished by inhibiting mRNA translation using a morpholino oligomer (97). In total, five dystrophin null zebrafish DMD models have been generated in the genetic screen following ethylnitrosourea (ENU) mutagenesis (98). These are *sapje* (dmd^{ta222a}), *sapje-like* (sap^{cl100}), dmd^{pc1}, dmd^{pc2}, and dmd^{tm90c} (99). Four of these carry nonsense mutations in exons 4 (*sapje*), 21 (dmd^{pc1}), 32 (dmd^{pc2}) and 53 (dmd^{tm90c}), respectively (100). The *sapje-like* model contains a mutation in the donor splice junction of exon 62 (101).

DMD zebrafish share many features of human patients. They die prematurely prior to reproductive age. Their muscles not only present tissue lesions (degeneration/regeneration, necrosis, inflammation and fibrosis), but also are remarkably sensitive to physical effort (102). Also, both absolute and specific muscle force are diminished (103). Notably, the amount of force loss is similar to what was described in GRMD dogs (104).

As a result of premature death, the zebrafish model is raised as heterozygotes. Thus, dystrophin-null mutant zebrafish (homozygous genotype) are obtained by crossing heterozygous animals. Zebrafish do not present defined sexual chromosomes (105) and dmd genetic inheritance can be considered as autosomal (with 25% of the offspring from heterozygotes being homozygous). Muscle dystrophy can be identified using a birefringence assay at 3 to 4 days post-fertilization (pdf) in altered zebrafishes. The birefringence assay is a sensitive, economical non-intrusive assay to rapidly examine muscle integrity in translucent zebrafish larvae (106). Non-affected larval zebrafish show a bright, remarkably ordered array of birefringence under polarized light. On the other hand, affected larvae exhibit diminished birefringence in areas corresponding

to injured muscle and these regions appear as black non-homogeneous spots within muscle fibers. In addition to the birefringence approach, the dystrophic phenotype can also be examined by quantifying survival, muscle strength, and the touch-evoked escape response (107). High throughput drug screening in the dystrophic zebrafish model has highly improved from the birefringence assay. Currently, at least five compound libraries have been tested in DMD zebrafish: Prestwick collection 1 (1280 compound), Prestwick collection 2 (1120 compounds), NINDS 2 compound library (1040 compounds), ICCB Known Bioactives 2012 (480 compounds), and FDA Approved Drugs (640 compounds) (108). These screens have provided at least 25 hit molecules (108-110).

3. Zebrafish as a model

3.1. History

Zebrafish is originally from the river basins in and surrounding East India. It was reported as an animal model first by Streisinger et al. in the 1970s as a potential tool for genetic analysis to vertebrate development (111). In the past decades, zebrafish have developed into a profitable tool to dissect embryogenesis. Experimental advantages of zebrafish for this use comprise rapid external development (eggs development occurs externally into free swimming larvae within 5 dpf), amenability to mutagenesis, small genome, relatively simple husbandry skills, and high fecundity (a single breeding session typically yields 100-300 fertilized eggs) (112). By using these advantages, scientists have discovered key factors in developmental events, from early germ layer patterning to how tissues emerged from these layers gain form and function (113). Lately, zebrafish have been developed to investigate additional aspects of biology, comprising behavior, stem cells, and disease (114).

3.2. Advantages for drug-screening purposes

Zebrafish drug screening represents a small but growing fraction of phenotype-based screens. One of the reasons is because they display unique advantages that come from screening in an intact animal.

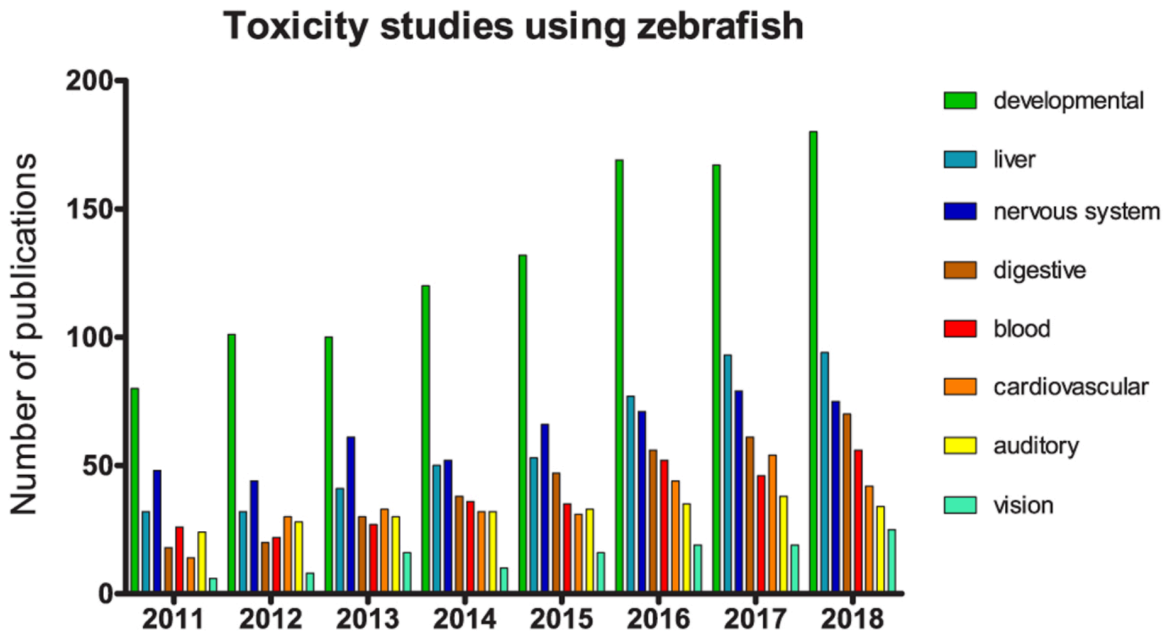


Figure 7. Number of publications using zebrafish as animal model.

Note. Taken from Use of Zebrafish in Drug Discovery Toxicology by Cassar S., Zon L. I., et al. (2019). Chemical Research in Toxicology, 33, 3, 95-118 (https://doi.org/10.1021/acs.chemrestox.9b00335).

3.2.1. Broad range of accessible biology

Most phenotypic tests are performed in cultured cells, which reduces the analyses to cell-autonomous phenotypes or those end points that can be identified in comparably simple culture systems. Zebrafish tests are mostly performed in living zebrafish embryos or larvae, which display a varied repertoire of biological processes and carry fully integrated vertebrate organ systems. Fundamentally, a much more extensive range of phenotypes can be screened in zebrafish than in cultured cells. Sedation, vascular tone, tumour metastasis, pain, and gut motility are examples of disease-significant phenotypes that are observable in zebrafish still simply unfeasible to modelling in cultured cells. The advantages of the intact animal as a target for screening are especially evident for neuroscience drug discovery, in which the intricacy of cell–cell connections and endocrine signalling challenge even modelling with patient-derived induced pluripotent stem cells (iPSCs) (115).

3.2.2. Early insight into toxicity

Zebrafish screens often report understandings about the absorption, distribution, metabolism, excretion, and toxicity of screening compounds, whereas cell-based assays provide limited information about these pharmacological features. Zebrafish larvae carry functional livers, kidneys, and blood–brain barriers (116). To generate *in vivo* phenotypes in zebrafish screening, molecules must display the ability to be absorbed, reach the target tissue, and evade rapid metabolism and excretion (117). This fact may justify the observation that some compounds that were discovered in zebrafish screenings have been quickly translated to *in vivo* mammalian models with minimal optimization of pharmacological characteristics (118).

3.3. A good model for human drug discovery

During the last few years, we have questioned the similarities between zebrafish and humans in terms of targets, physiology, drug metabolism and pharmacology, specifically during the first few days of life (when it is feasible to keep them in multi-well plates for screening). However, other relevant questions persist regarding the use of zebrafish in drug screening.

3.3.1. Molecular targets

Now that a high-quality zebrafish genome is available, it seems that 71% of human proteins (and 82% of disease-causing human proteins) present an orthologue in zebrafish (119). Orthologous zebrafish proteins are sensibly comparable to their human equivalents, especially within functional domains. For instance, the protein targets of the ten most-prescribed compounds have zebrafish orthologs with sequence identity going from 54% (glucocorticoid receptor) to 91% (thyroid receptor). Despite the sequence discrepancy between zebrafish and human proteins, one could expect a smaller rate of conservation of pharmacological effect. But, in fact, the rates of conservation are bigger. One explanation could be that the target similarity is larger at active sites in the enzymes, channels and receptors that are commonly the targets of drugs because these sites have retained the capacity, through evolution, to bind to the same molecules. For instance, the zebrafish glucocorticoid receptor, which is only around 50% identical to the human receptor, is

74% the same in the carboxy-terminal half of the protein, which carries the ligand-binding domain (120).

3.3.2. Physiology

Among other animal models that are susceptible to screening, zebrafish seem to be the most appropriate due to their highly conserved integrative physiology. Zebrafish is particularly interesting to look at its observable organ systems - livers, hearts, kidneys, and pancreases, among others. Although there are some other differences regarding the aquatic-living conditions, most zebrafish organs possess the same functions as their human counterparts and display well-conserved physiology. For example, the zebrafish pancreas encloses islets containing α, β, δ, and ε cells that regulate glucose homeostasis by secreting glucagon, insulin, somatostatin and ghrelin, like in humans (121). Drugs that have a role in the glucose homeostasis in humans have been shown to have the same effects in fish (122).

Cardiovascular physiology is also remarkably conserved between humans and zebrafish at anatomical, cellular, and membrane-biology levels. Surprisingly, the cardiac electrophysiology of humans is more comparable to that of zebrafish than it is to that of mice or rats (123). Regarding to muscle, in zebrafish fast and slow muscle fibers position to different parts of the developing fish. Zebrafish have been used to distinguish which progenitor cells are destined to differentiate into different muscle fiber types (fast or slow). Fish slow muscle is generally found just underneath the skin, whereas the fast muscle is located more internally (124). On the contrary, in mammals, slow and fast muscle fibers are interlaced. In both fish and mammals, fast muscle fibers have an anaerobically activity and are utilized for short powerful bursts of energy, whereas slow muscle fibers work aerobically and are used for continued activity (125).

Some effort has been made to describe the similarities between different stages of zebrafish development and those of humans. In these cases, the key molecular transitions in each of the organs for disease characterisation or drug metabolism appear to occur in sequence, but often much more quickly in zebrafish than in humans. For example, the sequential electrophysiological development of the zebrafish heart, which has been identified at the resolution of individual ionic

currents, occurs within 96 hours post-fertilization, whereas some of the analogue events are not completed until adolescence in humans (126).

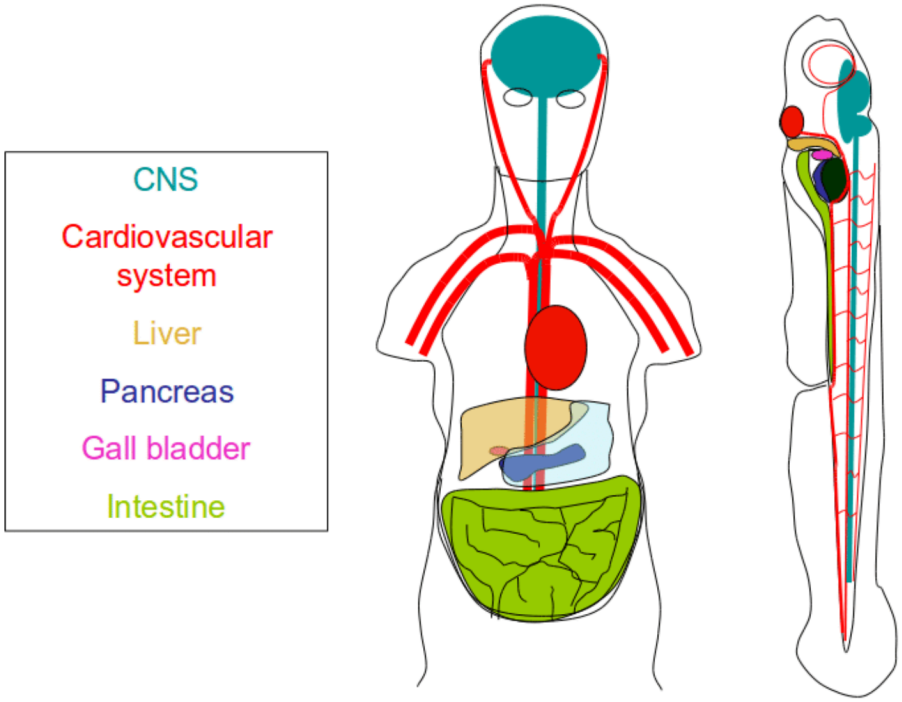


Figure 8. Similarities in zebrafish and human physiology.

The zebrafish is a multifaceted animal model that is advantageous for drug discovery as well as for assessing biochemical safety and toxicity.

Note. Taken from Multi-Step Usage of in Vivo Models During Rational Drug Design and Discovery by Williams H.C, Hong C International Journal of Molecular Sciences. 12(4):2262-74,(2011). (10.3390/ijms12042262).

At a structural level, zebrafish skeletal muscle is very similar to human muscle, and zebrafish carry the same muscle disease genes as humans. The mechanisms of skeletal muscle regeneration and repair can provoke a disorder, and both zebrafish and mammals share associated proteins such as annexins for sarcolemmal repair. Another example of analogous proteins is Pax-7, a protein expressed in skeletal muscle stem cells, or satellite cells, which are necessary for mammalian skeletal muscle regeneration. In the same way, Zebrafish express Pax-7 protein that migrates into zones of muscle degeneration (126).

3.3.3. Pharmacology

The question about how often a compound discovered in zebrafish will retain its efficacy in humans has not yet been answered because only a few molecules discovered in zebrafish have been tested in humans. Nonetheless, it has been feasible to screen an acceptable number of human drugs for conserved effects in zebrafish. For instance, 23 drugs known to display repolarization cardiotoxicity were tested for their effects on fish, and 22 out of the 23 compounds provoked repolarization-related toxicity (127). Correspondingly, drugs with specific actions on cardiac contractility and vasomotion in humans frequently recapitulate these effects in the zebrafish (128). Moreover, there are some publications that show two different actions for one compound. An example of that is pimozide, a drug used to initially treat schizophrenia, but currently discovered as a drug for amyotrophic lateral sclerosis (ALS) by using worms and fish animal models (129).

3.3.4. Drug distribution, metabolism, and excretion

As small compounds were methodically screened in the zebrafish, it seems that not only the effects of individual human drugs were replicated in fish, but so too were the majority of drug–drug interactions. These data illustrate that the distribution, metabolism, and excretion of drugs might also be available in zebrafish modelling. There are now robust proofs not only of conserved partitioning of drugs into different compartments based on physicochemical aspects, but also of the presence of the regulation of drug distribution across active physiological ambits such as the blood–brain barrier and by conserved tissue-specific transporters (129, 130).

Chapter 2 – The project

1. Background

1.1. Rationale: phenotypic vs target-based screening

Over the past 100 years, drug discovery has dramatically changed the practice of medicine and has had a repercussion on many aspects of our lives (131). With the apparition of modern

molecular biology, new approaches and the knowledge of the human genome, drug discovery has broadly transitioned into a hypothesis-driven target-based method, a progress which is correlated by relevant environmental changes in the pharmaceutical industry. Laboratories have become progressively automated and computerized, and world-wide research places are now increasingly associated into large centers to acquire technological and biological synergies. Currently, academia, the regulatory institutions, and the pharmaceutical industry all have a role in drug discovery, and, to transcribe the basic science into new medical treatments, the pharma must have a huge number of distinguished scientists working in many therapeutic fields and technologies. The importance for the pharmaceutical companies to design drugs is matched by the rising numbers of first-in-class drugs approved in the past years and mirrors the impact of modern drug discovery methods, genomics, and technologies (132).

One of the most complicated scientific areas that connect to many contrasting scientific fields is modern drug discovery. This area of medicine started by the end of the nineteenth century, in the experimental biological and medical research of Louis Pasteur, Claude Bernard, Robert Koch, Paul Ehrlich, and Joseph Lister, along with the great advances in organic chemistry, and has since then completely revolutionize the practice of medicine. About 1500 exclusive compounds are now known which act through more than 350 different mechanisms (133). Therefore, a big number of diseases can now be treated, or at least be regulated at the symptomatic level, including parasitic, bacterial, and viral infections, asthma, rheumatoid arthritis, osteoporosis, thrombosis and other cardiovascular diseases, psychiatric diseases, diabetes, and various cancers. Furthermore, drugs have facilitated many surgical operations of medicine and even made cell and solid organ transplantation feasible (134).

The first century of modern drug discovery was generally primarily driven by target-based methods, which are approaches based on a specific known chemical or molecule class which served as a starting point for further research (135). These compounds were either discovered through natural ligands and substances or from ethnobotanical knowledge. Nonetheless, serendipity was also a relevant success aspect in many cases. Aspirin, ergotamine, penicillin, steroid hormones are examples of drugs that were discovered during that period (136).

At present, there are new techniques like high-throughput screening (HTS), fragment-based screening or crystallography in combination with molecular modeling, that have generated a huge diversity of compounds lead structures very different from the classical chemicals used as starting points for drug discovery in the past. Furthermore, these new compounds can be utilized as a source to find new drug candidates through phenotypic screening (137).

Since 2011, research has been focused on phenotypic drug approaches, which are not based on the identity of a specific drug target or a hypothesis about its pathological role. In the case of phenotypic drug discovery, the starting points rely on biological assays that use phenotypic biomarkers as functional readouts. Thus, the concept of chain of translatability is one of the core features of this kind of drug screening, that refers to the molecular-level union between the mechanisms that dictates the assay phenotype, the preclinical disease model, and human patients (138). This target-agnostic method is the main difference in contrast to target drug screening, which is based on a hypothesis-driven method (139).

The transformation of phenotypic target screening into an established research method discipline was introduced by Eder and his team in analysis of first-in-class new molecular entities between years 1999 and 2013 (139). In this study, phenotypic testing was defined as the analysis of a large number of molecules (most of them randomly chosen) in a system-based method using a mechanistic agnostic protocol.

This type of approach has identified new medicines with novel mechanisms for disease like spinal muscular atrophy (SMA), cystic fibrosis (CF), and hepatitis C (140). For instance, the pathology of SMA resides on the defective exon splicing of SMN2 RNA. A cellular assay screen called “black box” was used by two different teams, leading them to discover new small molecules which could rectify SMN2 RNA splicing and increase levels of SMN2 protein (141). Another interesting fact is that Novartis and Roche assays on SMA were performed by simple cell-based reporter gene assays. What facilitated creating a disease-relevant, but simple HTS was the mechanistic understanding of the disease, showing that phenotypic-based screenings do not have to be complicated when models are mechanistically well defined (142).

The discovery of new targets and mechanisms of action is another relevant advantage of phenotypic drug screening. In addition to discovering unexpected novel targets, this approach can ameliorate existing therapies by establishing novel physiology for a known target, scrutinizing targets that belong to well known drug target classes or discovering novel mechanisms of action (143).

Another interesting point is that rare diseases have become an attractive target for the application of phenotypic drug discovery. Apart from the strong chain of translatability, there are other important aspects to consider: support from charities and patient organisations, access to funding implemented by governments, high disease molecular knowledge provided by highly engaged scientist and physician teams, and the competence of generating animal models, pluripotent stem cells (iPS)-derived or gene-edited cell models that can be easily examined in screening centres (144).

On the other hand, one of the most critical points of phenotype-based screening for first-in-class drugs is target validation (145). Reaching a robust target validation is a serious challenge because there is a low linkage of a molecular target to the disease biology, meaning that approximately 50% of clinical failures are caused by an absence of efficacy (146).

These target-agnostic and empirical aspects of both phenotypic and target-based screening are summarized in these table:

	Target-based screening	**Phenotype-based screening**
Cost	Low	High
Speed	Rapid	Moderate
Structure-activity relationship	Easy	More difficult
Translatability	Generally low	Generally high

Molecular target	Known	May not be identified

Table 6. Comparison between target-based screening and phenotype-based screening.

1.2. Previous work in the DMD drug discovery program at Modelis

The identification of the hit molecules that were tested on the DMD fish model was achieved by a previous work on a DMD worm model. In order to manipulate *C. elegans*, strains were raised on Nematode Growth Medium (NGM) agar plates at 20°C on *Escherichia coli* OP50 lawns.

With the aim of defining a significant motor deficiency phenotype, first we compared the movement of the DMD strain to N2 worms in a M9 liquid screen using WMicrotracker screen system. Age-synchronized young adult DMD worms were distributed in 96-wells plate (an average of 50 animals / well to a final volume of 100 µl) in either M9 buffer or test compounds and incubated for up to 10 hours at 20°C during which the movement of the worms was tracked. Measurements were performed in 3 batches of experiments.

DMD strains were assessed for motor-deficiency phenotypes using an infrared tracking device (WMicrotracker, Designplus SRL, Argentina). This machine tracks the activity of *C. elegans* automatically in 96-well-microtiter plates. Two infrared light rays cross each microtiter well from top to bottom and a detector determine how often the light rays are interrupted by worms moving in the well. The signal was used to calculate a movement score, which is the amount of animal movement in a fixed period. The average movement score was compared to a no-compound control and the average movement score of the whole plate. Candidates that increased movement abilities significantly in the second screen were counted as positive. Together, a minimum of 250 animals were scored per genotype and condition (vehicule and drug groups). The motility phenotype that has been used for the screening consisted in touching worms with an aluminium wire pick and then evaluate their behaviour. If the stimulation triggered a full-body response, the

motility was considered normal. If only the head was moving, worm was paralysed. And if the worm was not moving it was considered as dead.

Chemicals used in this study are derived from libraries including Prestwick Chemicals library (1280 compounds 100µL at 10mM concentration in DMSO), Sigma-Aldrich Lopac 1280 library (1280 compounds at 10mM in DMSO), Product LO3300-1KT, Custom U.S. collection including 880 compounds 125µL at 10mM concentration in DMSO (MicroSource Discovery systems Inc), ENZO LIFE SCIENCES, INC. BML-2865 Natural Products Library 100µL/well (10mM in DMSO). Among this collection of almost 3500 molecules, 26 presented a significant improvement of DMD-mutant worm motility. These compounds were represented in a high variable list of molecules (Figure 12) and were later tested on our DMD fish model.

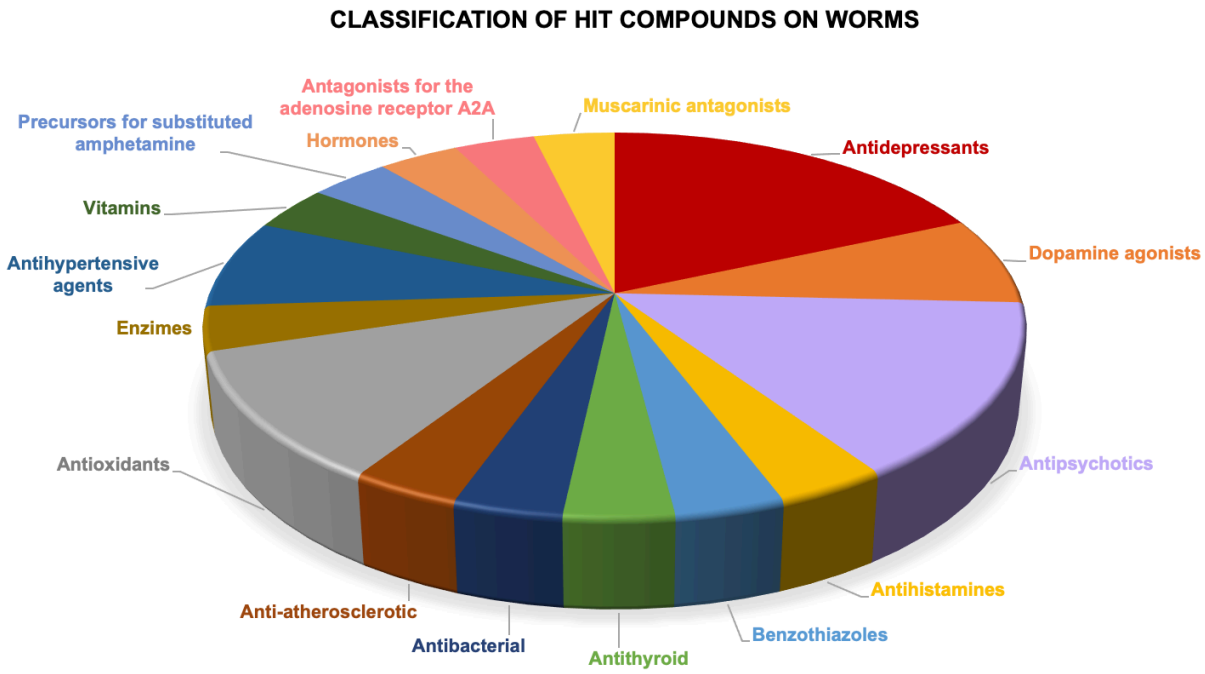


Figure 9. Classification of the hits identified on worms prior testing on fish.

We observe a high variety of molecules that are distributed quite equally. However, there are three categories of compounds that are represented more than the rest, which are antidepressants, antipsychotics and antioxidants, suggesting an advantageous therapeutical effect for the treatment of the muscular disease.

1.3. Objectives

One of the projects which is being studied at Modelis is DMD. The project started with a high-throughput screening strategy on worms DMD mutant that led to the identification of 26 molecules restoring disease-related phenotype (ameliorated motility). Following Modelis drug discovery program, the next step of this study is to validate these candidate compounds in a DMD fish model.

The objectives of the current research project are:

1- To generate a *dmd*-mutant zebrafish model and to characterize its phenotype.

2- To confirm the effects of the 26 candidate compounds identified above in zebrafish.

3- To establish a stable *dmd*-mutant zebrafish line has been generated via CRISPR technology.

4- To confirm that the hit compound exhibits clinically relevant phenotypes in the context of DMD (as it happens in humans) with survival tests, swimming tests and assessment of muscle integrity.

5- To screen the effect of molecules *in vivo*.

6- To perform toxicity assay for each compound that determine the maximum tolerated concentration (MTC) that zebrafish larvae can tolerate.

7- To check the effect of each candidate molecule on different *dmd*-mutant phenotypic readouts in order to know if they ameliorate them.

Of note that many of these molecules are FDA approved, which could help expedite further preclinical and clinical testing.

1.4. Materials and methods

1.4.1. Generation of a CRISPR-KO DMD fish model

In order to generate DMD mutant fish, the CRISPR/Cas-9 technology has been applied. Once the mutation was stable, the mutant fish line was generated for further analysis.

1.4.2. Phenotypic characterisation

Fish were raised in tanks of 5 L of water at 25ºC at the fish facility of CRCHUM.

For the survival test, the DanioDesign® plate technology was applied to 300 5 dpf embryos and after being genotyped with HRM (Roche LightCycler® 96), they were isolated according to their genotype in three 2 L tanks.

To analyze motor defects, we took advantage of an automated recording chamber (DanioVison, Noldus) to track the swimming pattern of dmd-/- *versus* their siblings.

For the muscle integrity test, 35 DMD fish were randomly selected at 5 dpf of age from a 1-liter breeding tank and were placed in a Petri dish of fish water supplemented with an anesthetic (Tricaine 0.15%.). Using this simple and non-invasive assay, we could identify some "translucent" zebrafish larvae with intact muscle fibers and others dark patches in skeletal muscle, corresponding to disruption of the organization of myosin fibers. After that, we performed an immuno-fluorescence experiment using the phalloidin (Alexa Fluor 488 Phalloidin) to show F-actin in muscle cells from untreated (n=4) and treated DMD fish larvae (n=4).

1.4.3. Drug screening approach

The 26 drug hits identified from our initial testing on worms were screened on *dmd*-mutant fish. First, a toxicity assay was performed to determine the maximum tolerated dose we could utilize on the zebrafish model. In this previous study, we used a polystyrene Petri dish 6 well cell culture plate, sterile. Each compound was administrated in every well with 7 different concentrations: 1µM, 5µM, 10µM, 20µM, 30µM, 50µM and 100µM. The non-geometrical progression of these concentrations was determined according to two reasons. First, the progression of 1µM, 5µM, 10µM, 20µM was designated because in the literature, the molecules with potential effects are at these concentrations. Then, we determined 30µM, 50µM and 100µM to ensure the maximum tolerated dose for our model. As a medium, we used 6 mL of drug treatment water per well. In addition, we deposited 10 dechorionated 1dpf *wild type* fish in each

well and finally, we inoculated different amounts of drug to reach the expected concentration in each well. Drug was administered daily for 4 days prior to renewing drug treatment water.

A concentration was considered as toxic if at least 10% of the larvae show lack of posture, cardiac edema or lack of touch response. In addition, their general dorsal-up posture and general morphology were evaluated. According to these aspects, on the fifth day of treatment, we established the maximum tolerated concentration for which fish were not intoxicated to perform subsequent experiments.

Once each compound concentration was determined, drug screening was performed on our *dmd*-mutant zebrafish model. The experiment consisted in a 4-day drug treatment on 100 1dpf dechorionated DMD fish raised in 100 ml of raising medium. Drug was administered/renewed, and we evaluated the muscle integrity under birefringence conditions at day 5 for both treated and untreated groups. We expected in each group to obtain 25% of fish with disruptive fiber muscles. If a compound reduces this percentage by 2.5 fold (thus we obtain 10% of fish with muscular defects), then this molecule represents a good candidate and a subsequent swimming assay would be performed. Thus, we focused on muscle integrity as a primary readout since this is the main hallmark of the disease.

In each experiment we quantified the number of larvae with muscle integrity defects (using birefringence) upon treatment with a hit candidate molecule or vehicle as control. Each experiment was repeated three times, on independent batches of larvae, using fresh stock for candidate compounds.

In order to analyze accurately the birefringence results obtained from each group, we established a threshold under which we would consider a compound as having a significant effect on the muscle phenotype. Indeed, we estimate that treatments leading to a reduction of a 2.5 fold reduction in the number of larvae with muscle defects should be considered as a confirmed hit.

1.4.4. Statistical analyses

Both swimming test and drug screening were analyzed statistically one-way ANOVA on Noldus EthoVision XT 13® and GraphPad Prism®.

1.5. Results

1.5.1. Generation of a CRISPR-KO DMD fish model

In this study we generated our own DMD mutant fish and not the *sapje* zebrafish due to the impossibility of importing this type of fish to Canada and in our institute, the CRCHUM.

1.5.1.1. sgRNA design and validation in vivo

The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-associated (Cas) machinery is the main gene editing technology. This revolutionary system requires an appropriate CAS endonuclease protein and a sequence-specific guide RNA (gRNA). However, low cleavage efficiency and off-target effects can hamper the development and application of CRISPR/Cas systems (148). As it is shown in Figure 10, in fish, the dystrophin gene is located on chromosome 1 (10824kb-11075kb) (149).

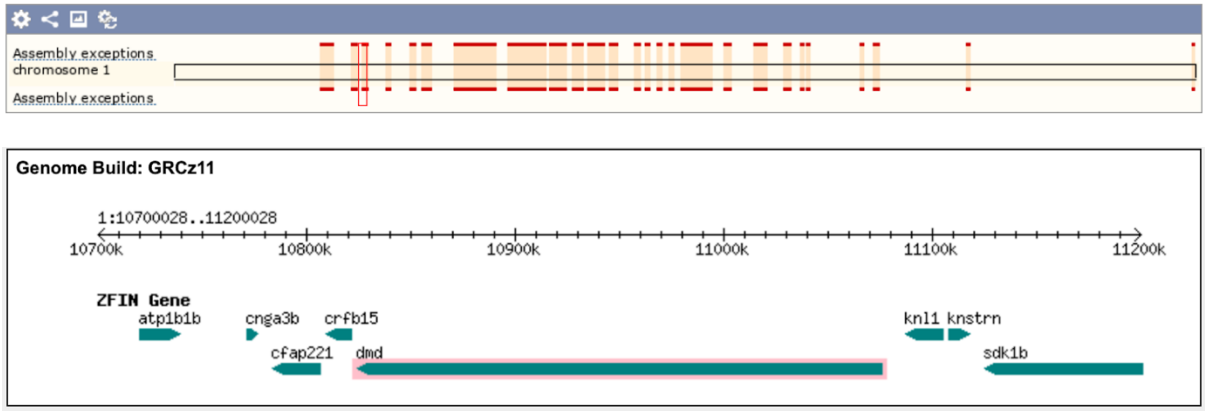


Figure 10. Physical map of dystrophin gene in zebrafish.

The image above shows the entire chromosome 1 with the region of the dystrophin gene framed in red. Below, a more specific image about the dystrophin gene is shown in a large and green slightly pink colored line.

Note. Taken from Ensembl and ZFIN. (http://useast.ensembl.org/Danio_rerio/Location/View?db=core;g=ENSDARG00000008487;r=1:10824351-11075405; https://zfin.org/action/mapping/detail/ZDB-GENE-010426-1)

The next important step is to find a suitable gRNA that will be used for CRISPR/Cas9 mutagenesis. A gRNA sequence was designed using the online tool CRISPRscan to target the following early coding sequence of the *dmd* gene (Table 4), which is located at this position in the zebrafish genome: chr1:11024589-11024611. More importantly, this gRNA targets exon 5 of the *dmd* transcript that encodes for the N-terminal calponin-homology (CH) functional domain of the protein. As a result, the generation of indels at this locus is highly susceptible to be deleterious.

Target site sequence (with Peptidylglycine Alpha-Amidating Monooxygenase)	**GAG**GAGCTGACATTGTAGATGGG
Oligo sequence	taatacgactcactataGGGGAGCTGACATTGTAGATgttttagagctagaa

Table 7. CRISPRscan predictions on genes (CDS) for Cas9 (NGG) with T7 promoter (Gg18NGG-62).

Note. Taken from UCSC. (http://genome.ucsc.edu/).

1.5.1.2. Stable line generation

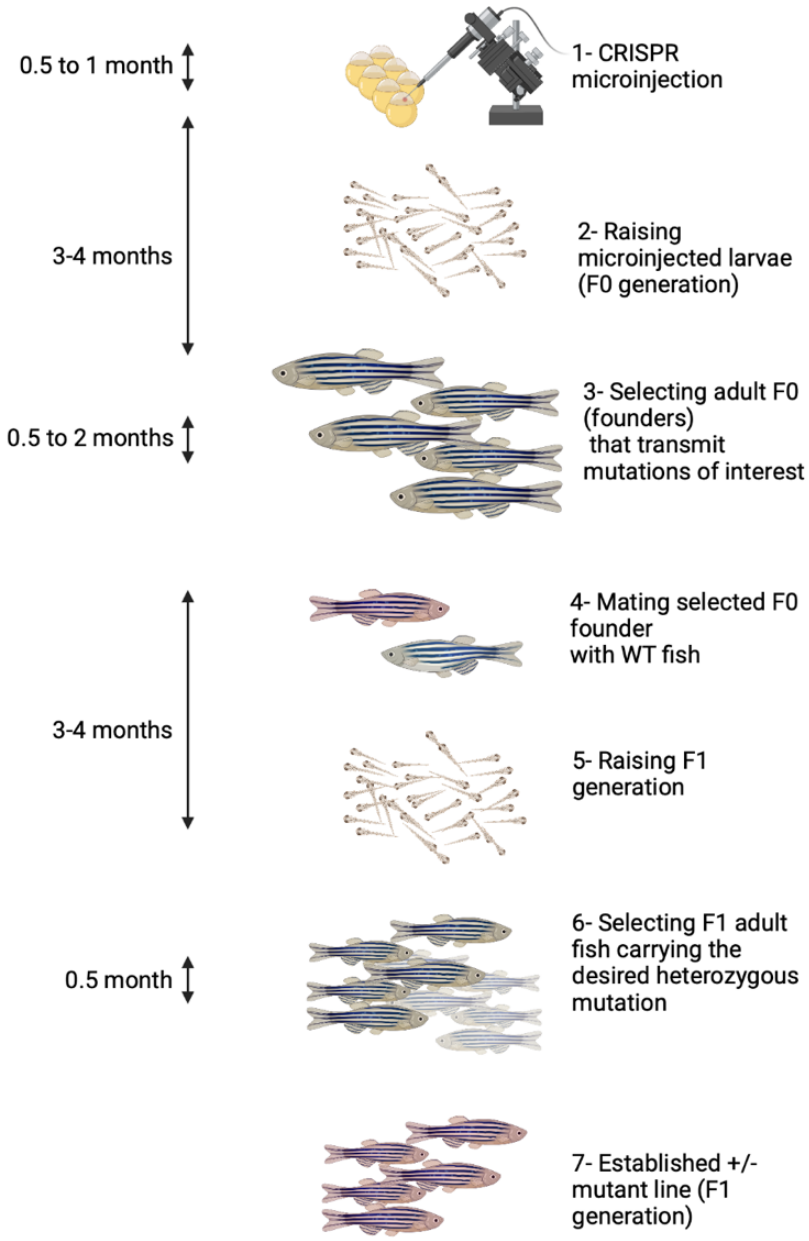


Figure 11. Schematic process to obtain a stable fish line.

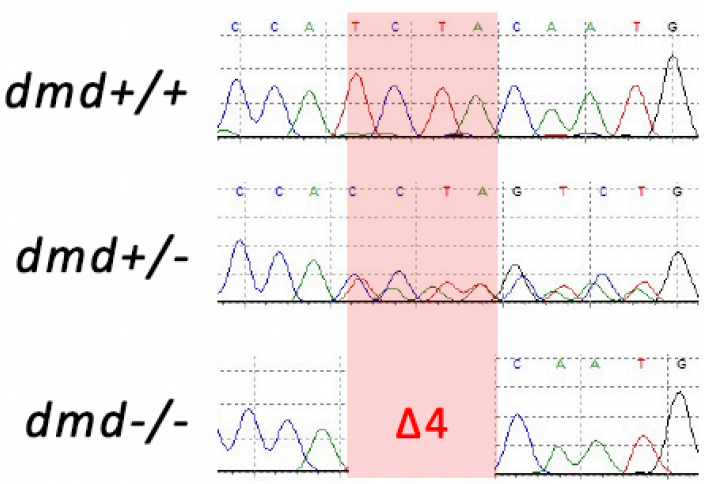
In this process, all begins with the CRISPR microinjection on fish eggs, obtaining after 3-4 months CRISPR-KO DMD line (F0) (steps 1 and 2). After examining the somatic mutation, F0 is crossed with WT fish to 1) identify the founder (F0) and 2) obtain the next generation, F1 (steps 3, 4 and 5). After selecting

HT DMD mutant fish by fin-clipping genotyping (step 6), the mutant line is established and ready to be tested (step 7).

In order to perform reliable experiments and reduce the experimental variability, we aimed at establishing a stable *dmd*-mutant fish line. The first step is the microinjection of one-cell stage zebrafish embryos. To do that, we microinjected about 100 freshly-layed zebrafish embryos with a mixture of gRNA (targeting *dmd* locus) and mRNA encoding CAS9 endonuclease. These injected embryos will carry several different random indels (insertion/deletion) and can therefore be considered as F0 mosaic mutants. Thus, the next step is to identify an adult F0 that carries and transmits a frameshifting mutation at the locus of interest (e.g exon 5). To do so, we crossed F0 adult CRISPR-injected fish with WT and screen their progeny for the presence of indel mutations. This screening was done by High Resolution Melting assays (HRM) (Roche LightCycler® 96) and further confirmed by Sanger sequencing. We tested 5 adult F0 fish and found that each of them was transmitting mutations in *dmd*. We determined that the best founder was transmitting a frameshifting mutation, consisting in 4-nucleotide deletion (Figure 12a), at a high rate to the progeny (>50%). After crossing this founder fish with WT, we raised the F1 generation and selected heterozygous fish among this population by fin-clipping (HRM and Sanger sequencing). These heterozygous fish were further incrossed for our analysis.

When incrossed, we confirmed that stable *dmd*+/- fish generate three different genotypes among their offspring according to Mendel's laws of recessive inheritance. Therefore, we obtained 25% *dmd*+/+, 25% *dmd*-/- and 50% *dmd*+/- (Figure 12b).

a



b

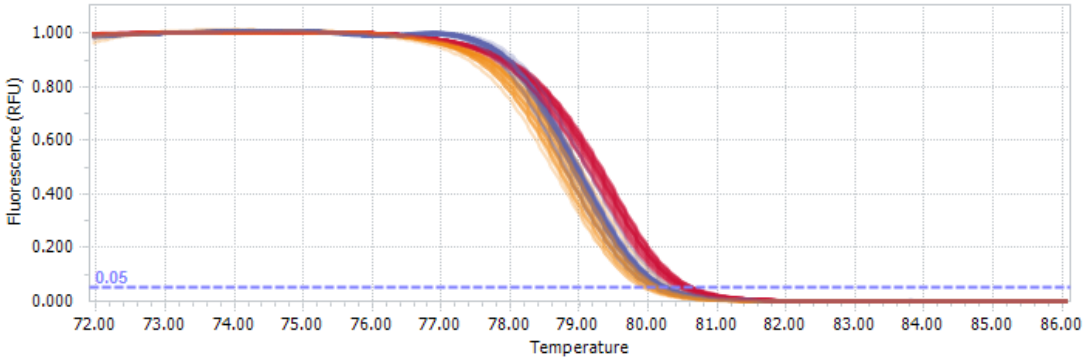


Figure 12. *dmd* mutation sequence and chromatogram.

a) After evaluating 5 adult F0 zebrafish, we found all of them were transmitting *dmd* gene mutated. The best founder was transmitting a frameshifting mutation (4-nucleotide deletion) at a high rate to the progeny (<50%). This mutation causes a premature stop codon at position 121, thus avoiding the production of dystrophin protein. b) Chromatogram of DMD genotypes: *dmd*+/+ (orange), *dmd*-/- (red), and *dmd*+/- (blue), following this proportion representation: 25%, 25%, and 50%, respectively. Curves present different melting temperatures due to the size of *dmd* gene (sometimes absent when genotype was *dmd*-/-).

1.5.2. Phenotypic characterization

1.5.2.1. Survival experiment

After raising F2 *dmd*-mutant fish obtained from F1 *dmd*+/- incross for one month, *dmd*-/- fish were not identified, suggesting they were not surviving. Therefore, to obtain more information about *dmd*-/- it was necessary to perform a survival test. In this study, the objective was to monitor

the survival of *dmd*-/- larvae compared to their siblings under normal conditions. To do that, we incrossed *dmd*+/- fish and separated the three different genotypes (*dmd*+/+, *dmd*+/- and *dmd*-/-) from the offspring by tail-clipping 3 dpf embryos using a microblade. Then, each genotype was raised in a 1-liter tank at 25ºC, and their survival was monitored over 30 days.

As shown in Figure 16, all three genotypes start dying with at a slow rate, but at day 13 homozygous fish start dying abruptly, reaching the 50% of survival at day 14, whereas heterozygous and *wild type* fish reached the plateau phase. At day 21, all *dmd*-/- fish were dead while 90% of siblings were still alive.

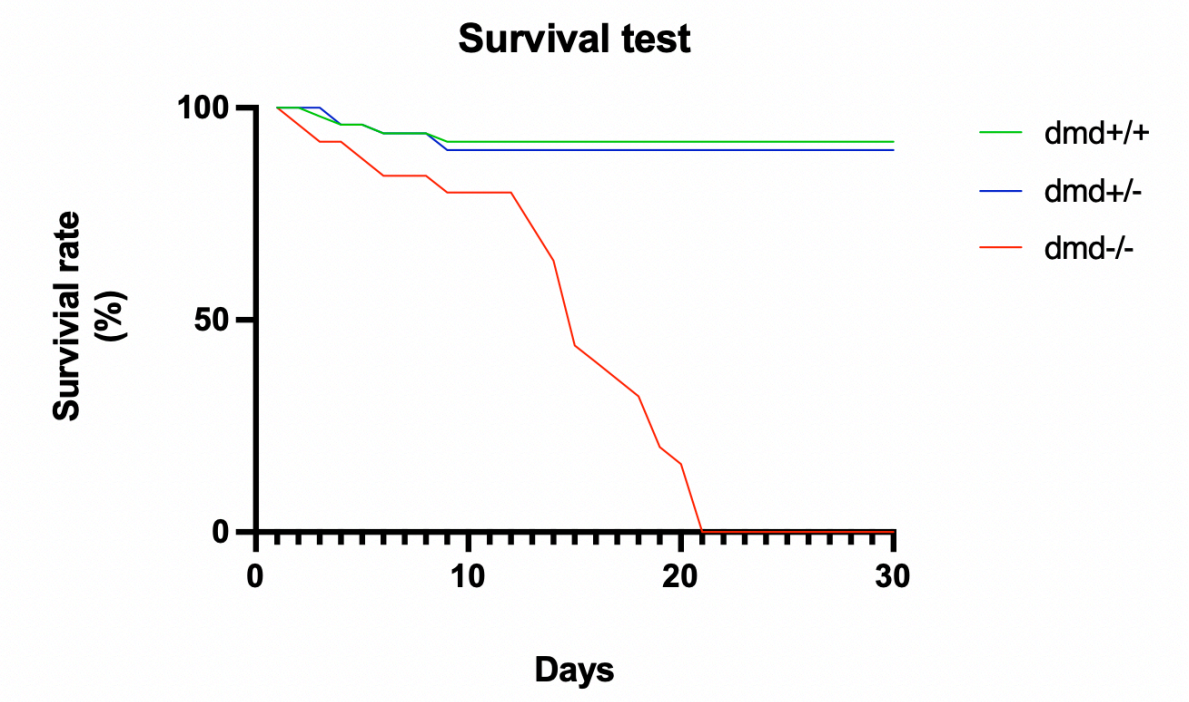


Figure 13. Survival curve in *dmd*-mutant fish.

The three different DMD genotype lines, *dmd*+/+ (n=48), *dmd*+/- (n=50), and *dmd*-/- (n=45), were separated in one-liter tank respectively, and raised at 25ºC during 30 days. They were monitored every day to establish the number of deaths/day/genotypes.

1.5.2.2. Motor defects

Because *dmd*-/- fish do not survive as we showed previously, we could only raise heterozygous fish until adulthood and had to continue working with larvae obtained from heterozygous incrosses. As a result, all behavioral experiments were done blindly of the genotype and the larvae were only genotyped post-acquisition using HRM analysis as previously described by Samarut et al. 2016 (150).

Because DMD pathology is associated with muscle weakening and motor dysfunction, we wanted to check if *dmd*-/- larvae displayed any swimming defects. To do so, we took advantage of an automated recording chamber (DanioVison, Noldus) to track the swimming pattern of *dmd*-/- *versus* their siblings. We decided to check the swimming at 5 dpf because this is a stage at which zebrafish larvae are freely swimming and already developed a robust musculature. We recorded their swimming following a 1h:1h dark:light cycle and quantify the total distance swam.

As shown in Figure 14, during the dark period, the motion pattern of *dmd*-/- genotype is significantly reduced compared to the two other genotypes (*dmd*+/+ and *dmd*+/-). In contrast, after one hour in complete darkness, the light provoked a linear progression of the motion of the three genotypes during the first 20 minutes. However, the general motility of *dmd*-/- larvae was still significantly reduced compared to their siblings.

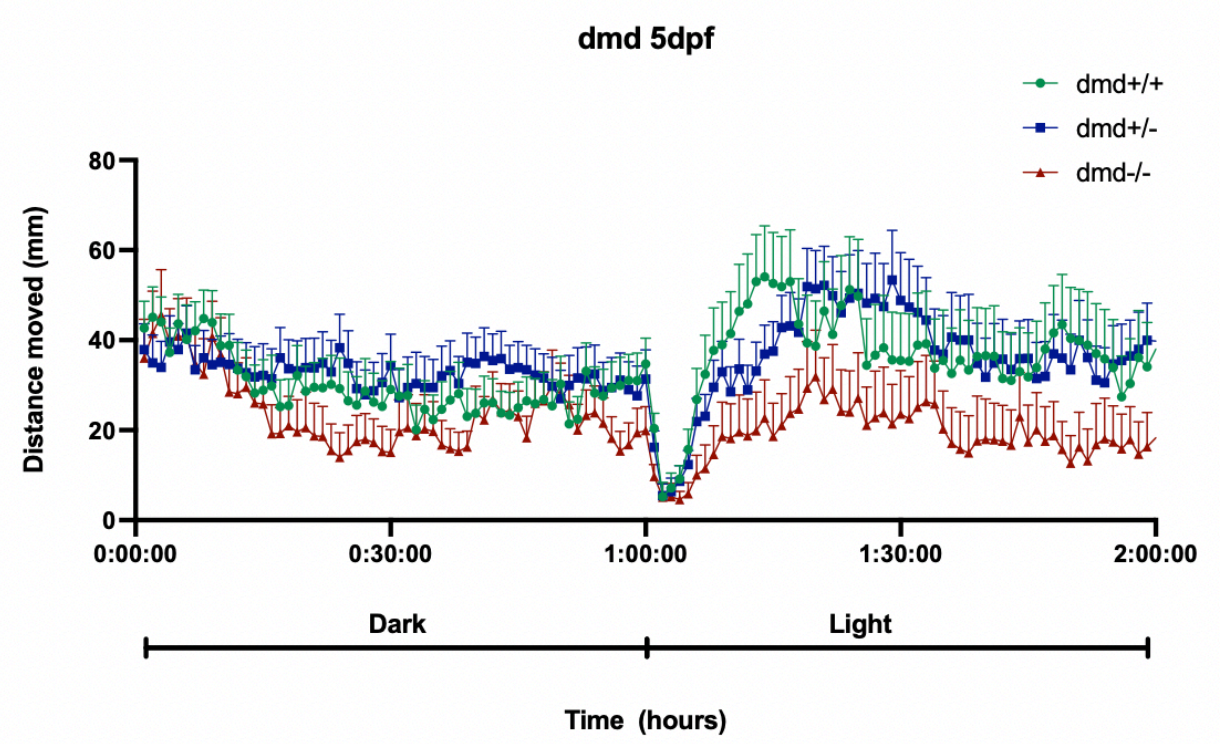


Figure 14. Swimming test on DMD 5pdf fish.

In this experiment, a dark:light photoperiod of 1:1 hour was performed in a 96-well plate by Noldus EthoVision XT 13®, which measured the distance moved (mm) per each fish (n=87). In the dark, the distance moved by the three genotypes remains low (between 18 and 50 mm), whereas in the light period the distance moved is higher and more different between genotypes groups. Green and blue curves, *dmd*+/+ (n=25) *dmd*+/- (n=37), respectively, show a greater distance moved (between 38 and 62 mm) in light conditions, whereas red curve, *dmd*-/- (n=25), remains almost on the same range than in the dark period (between 18 and 38 mm).

A notable characteristic observed on *dmd*-/- is their poor response in both light and dark conditions, comparing to siblings. They show almost the same swimming pattern during the entire experiment, stopping their movement only for a few minutes when light turns on. More specifically, they depict a general hypoactivity that is even more visible during dark phases. When the total distance swam was binned over the entire dark or light cycle (1-hour binning, Figure 18), we showed a statistical difference between *dmd*-/- and their siblings both during light and dark cycles.

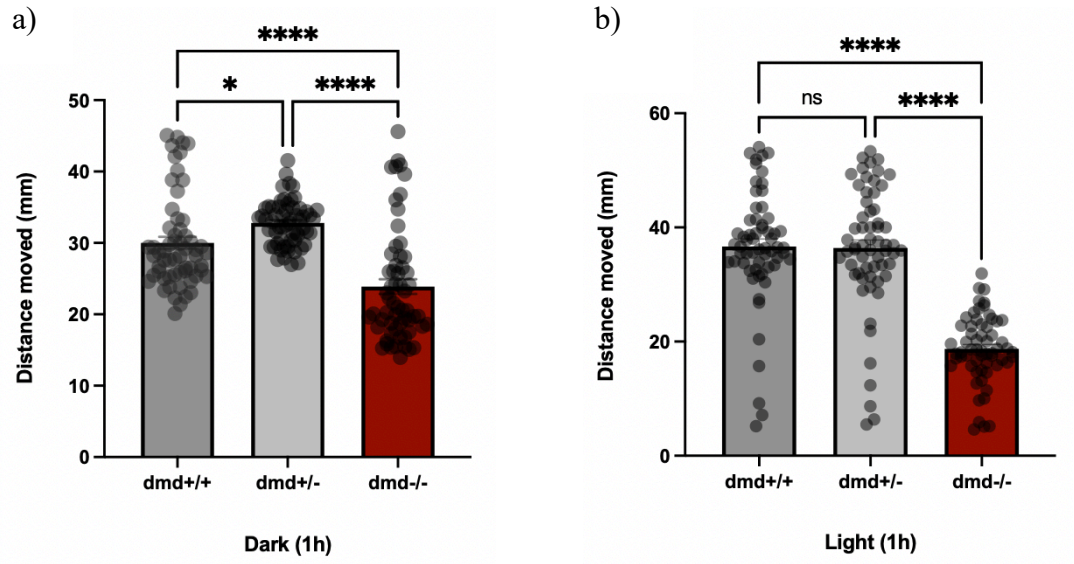


Figure 15. Swimming test on DMD 5pdf fish.

a) In the dark period, there is a high significant difference between all three groups (****), except *dmd*+/+ (dark grey bar) versus *dmd*+/- (light grey bar) (*). The group with less amount of distance moved accumulated (mm) is *dmd*-/- (red bar). b) In the light period, there is a high significant difference between all three groups (****), except *dmd*+/+ (dark grey bar) versus *dmd*+/- (light grey bar) (ns). In this case, *dmd*-/- (red bar) shows the lowest distance moved accumulated (mm).

1.5.2.3. Muscle integrity

The third and last study performed aims at assembling the muscle fiber integrity of our *dmd*-mutant fish under birefringence conditions. As dystrophin protein is responsible of the muscle assembling, it is very opportune to use this technique due to its simplicity and rapid readout. Here, DMD fish were randomly selected at 5 dpf of age from a 1-liter breeding tank and were placed in a Petri dish of fish water supplemented with an anesthetic (Tricaine 0.15%.). Birefringence is a physical trait in which light is rotated as it crosses organized matter. In this case, light passes through the pseudo-crystalline and well-arranged array of muscle sarcomeres (151). Using this simple and non-invasive assay, we could identify some "translucent" zebrafish larvae with intact muscle fibers and others dark patches in skeletal muscle, corresponding to disruption of the organization of myosin fibers (Figure 16). These defects are particularly relevant in regard to DMD (Figure 16). The genotype of each larva was subsequently assessed by HRM. We found that 97.1% of fish with non-disrupted muscle fibers were either *dmd*+/+ or *dmd*+/-, while 100% (n=192) of

those with disrupted muscle fibers corresponded to *dmd-/-* fish. This strong correlation shows that *dmd-/-* zebrafish present obvious muscle fiber defects when observed under birefringence that can be explained by the disorganization of sarcomeres.

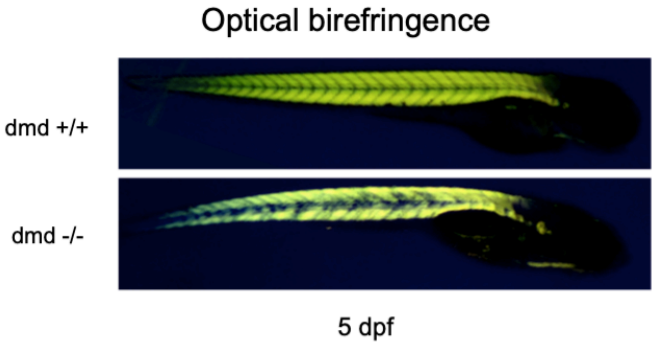


Figure 16. Optical birefringence captures of DMD fish at 5dpf age.

Fish were anesthetized with tricaine 4% before obtaining pictures (n=35). Above, *dmd*+/+ fish with perfectly well-structured muscle fibers. Below, *dmd*-/- fish with injured muscle fibers.

Despite the advantages the advantages of the birefringence assay for skeletal weakness detection, this approach presents some limitations. The most important restriction in this assay was the scale at which we were evaluating their muscle. The stereo zoom microscope could not provide an accurate image of the myosin fibers. So, we were limited by the zoom of the microscope and therefore, it was not possible to distinguish if the disrupted muscle appeared only at 5 dpf or before.

Moreover, as dystrophin is involved in the arrangement of myosin fibers, we decided to perform a phalloidin staining on zebrafish larvae in order to analyze the integrity and arrangement of myosin fibers specifically.

Phalloidin is a peptide from the family of toxins that belongs to the deadly *Amanita phalloides* mushroom and is frequently used in imaging experiments to selectively label F-actin in permeabilized tissues. In our experiment, we performed an immuno-fluorescence experiment using the phalloidin (Alexa Fluor 488 Phalloidin) to show F-actin in muscle cells from untreated and treated DMD fish larvae (152).

As it is shown in Figure 17, we found disrupted muscle fibers at the early age of 2dpf, which represents a relevant advantage versus the detection of disrupted muscle at 5dpf with birefringence assay. With the phalloidin approach, we could determine more precisely the muscular effect of drugs in both treated and untreated DMD fish.

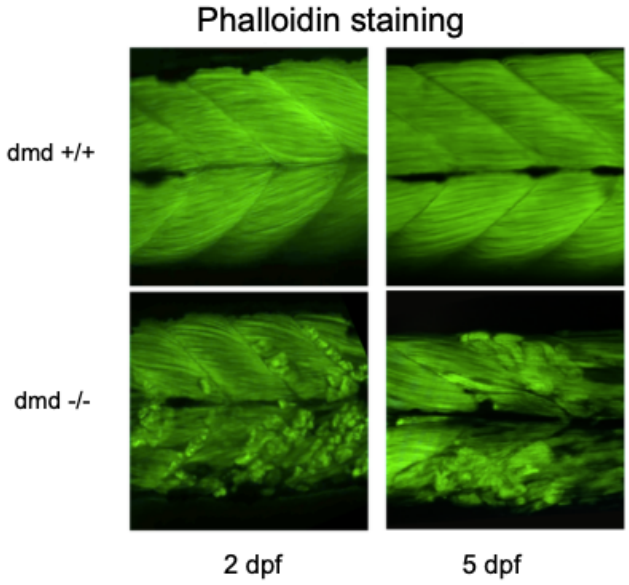


Figure 17. Phalloidin staining on *dmd*+/+ and *dmd*-/- fish.

In *dmd*+/+ fish (n=4), muscle fibers were perfectly distributed along the muscle, whereas in *dmd*-/- fish (n=4), fibers were disrupted, resulting in dark patches and disorganized muscle tissue. Differences were observed between muscle fibers of *dmd*-/- fish at 2dpf in comparison with the same group at 5dpf, revealing the progressive muscular degeneration of the disease.

Although phalloidin staining provided more detailed information about muscle fibers at earlier development phases, this methodology presents some disadvantages to have in consideration. First, the timeline of the immuno-fluorescence experiment was higher (2 days) than with birefringence method (less than an hour). Second, the low cost-effectiveness of phalloidin method was notable compared to birefringence assay. Lastly, phalloidin staining presented manipulative limitations because samples could not be used for more experiments once they had been permeabilized and fixed. For these reasons, the eligible approach to perform further analysis was birefringence method.

1.5.3. Drug screening approach

In order to screen our list of hit compounds on DMD zebrafish model, we designed an approach that comprised 4 steps:

1- First, we performed a toxicity test to determine the maximum tolerated drug concentration for our fish model.

2- Birefringence assay. During this phase of the drug screening approach, we counted under birefringence conditions fish with muscle defects and fish with normal muscular appearance in both treated and untreated groups.

3- 10% threshold. In the untreated groups, we obtained 25% of fish with disrupted muscle versus 75% of fish with normal muscle fibers. In addition, in the treated groups we expected our compounds to ameliorate the 25% of fish with disrupted muscle fibers by 2.5 fold, which represents a threshold of 10%.

4- Decision-making. If the percentage of fish with muscle defects were 10% or lower, we could proceed for the next phase of the experiment: swimming test. If the percentage was higher than 10%, then the drug was discarded (Figure 21).

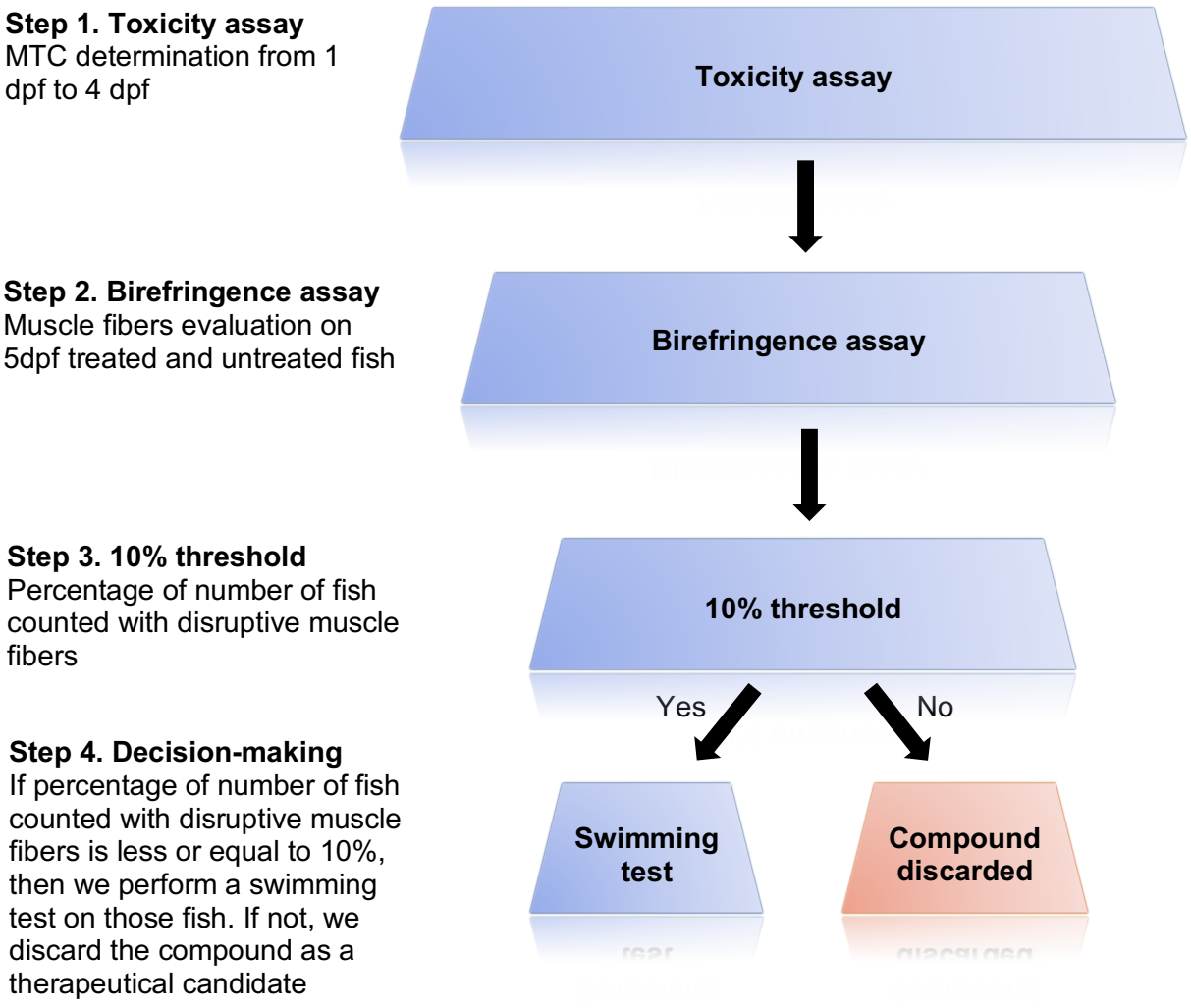


Figure18. Experimental pipeline of the drug screening on DMD fish.

First step consisted in determining which MTC concentration would be administrated to our animal model. To do that, we utilized a polystyrene Petri dish 6 well cell culture plate, sterile, from 1 dpf to 4 dpf. When fish were 5 dpf, we assessed their muscle integrity under the stereo microscope provided by birefringent lens. After counting number of fish with disrupted muscle fibers, we established to discard those compounds for which fish presented more than 10% of disruptive muscle fibers and to continue analysis on fish with the 10% or less of their muscle fibers injured.

1.5.3.1. Toxicity assay

As a first step before testing the 26 promising molecules that improved motility of *dystrophin*-mutant worms (*dys-1*), it was necessary to determine the maximum tolerated

concentration (MTC) for each compound on zebrafish larvae described previously in the section 1.4.3.

Compound	MTC (µM)	Compound	MTC (µM)	Compound	MTC (µM)
MOD1	10	MOD2	20	MOD3	20nM
MOD4	25	MOD5	3	MOD6	8
MOD7	5	MOD8	1	MOD9	0.125
MOD10	15	MOD11	30	MOD12	0.5
MOD13	3	MOD14	50	MOD15	5
MOD16	15	MOD17	100	MOD18	0.5
MOD19	2	MOD20	20	MOD21	100
MOD22	100	MOD23	50	MOD24	50
MOD25	5	MOD26	100		

Table 8. Maximum tolerated concentration for each candidate drug.

1.5.3.2. Phenotypic assessment of muscle integrity

This method consists in a visual evaluation of untreated and treated *dmd*-mutant fish with the subsequent genotype characterization of each fish in order to determine the effect of each compound on muscle integrity, one main hallmark of DMD pathology.

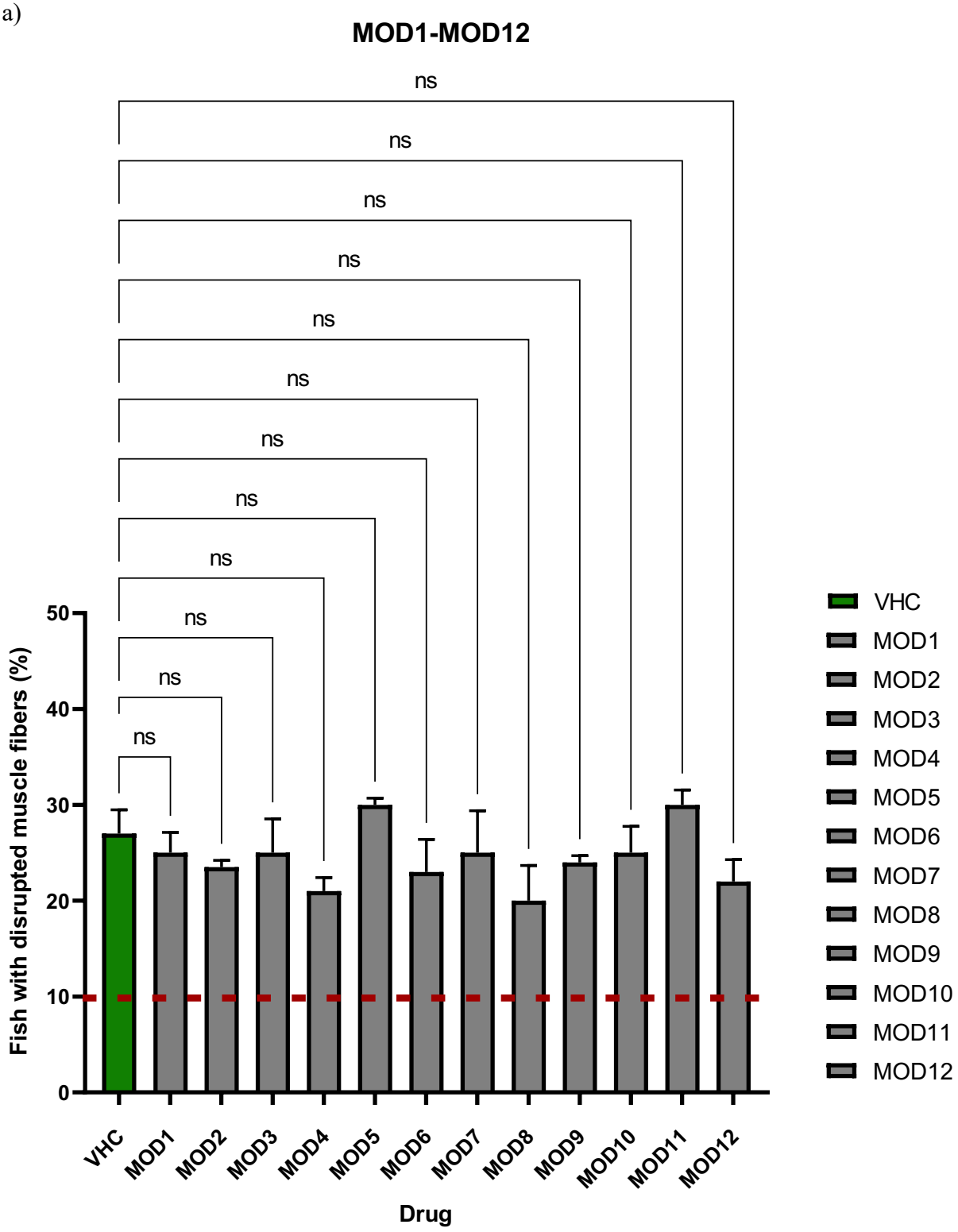
1.5.3.3. Drug Screening Results

Although the number of larvae depicting muscle defects was averaging 25% in control groups (as expected), we were not able to identify any amelioration of this phenotype with any of the compounds tested.

As a result, we decided not to test the effect of these candidate compounds on further swimming or survival assay.

It is worth being noted that I completed these assays by February 2020, just before the COVID-19 pandemic. As a result, based on these negative results, we decided to stop this project since it seems not adequate in identifying promising hit small molecules.

a)


MOD1-MOD12
ns
ns
ns
ns
ns
ns
ns
ns
ns
ns
ns
Fish with disrupted muscle fibers (%)
0
10
20
30
40
50
VHC
MOD1
MOD2
MOD3
MOD4
MOD5
MOD6
MOD7
MOD8
MOD9
MOD10
MOD11
MOD12
Drug
VHC
MOD1
MOD2
MOD3
MOD4
MOD5
MOD6
MOD7
MOD8
MOD9
MOD10
MOD11
MOD12

b)

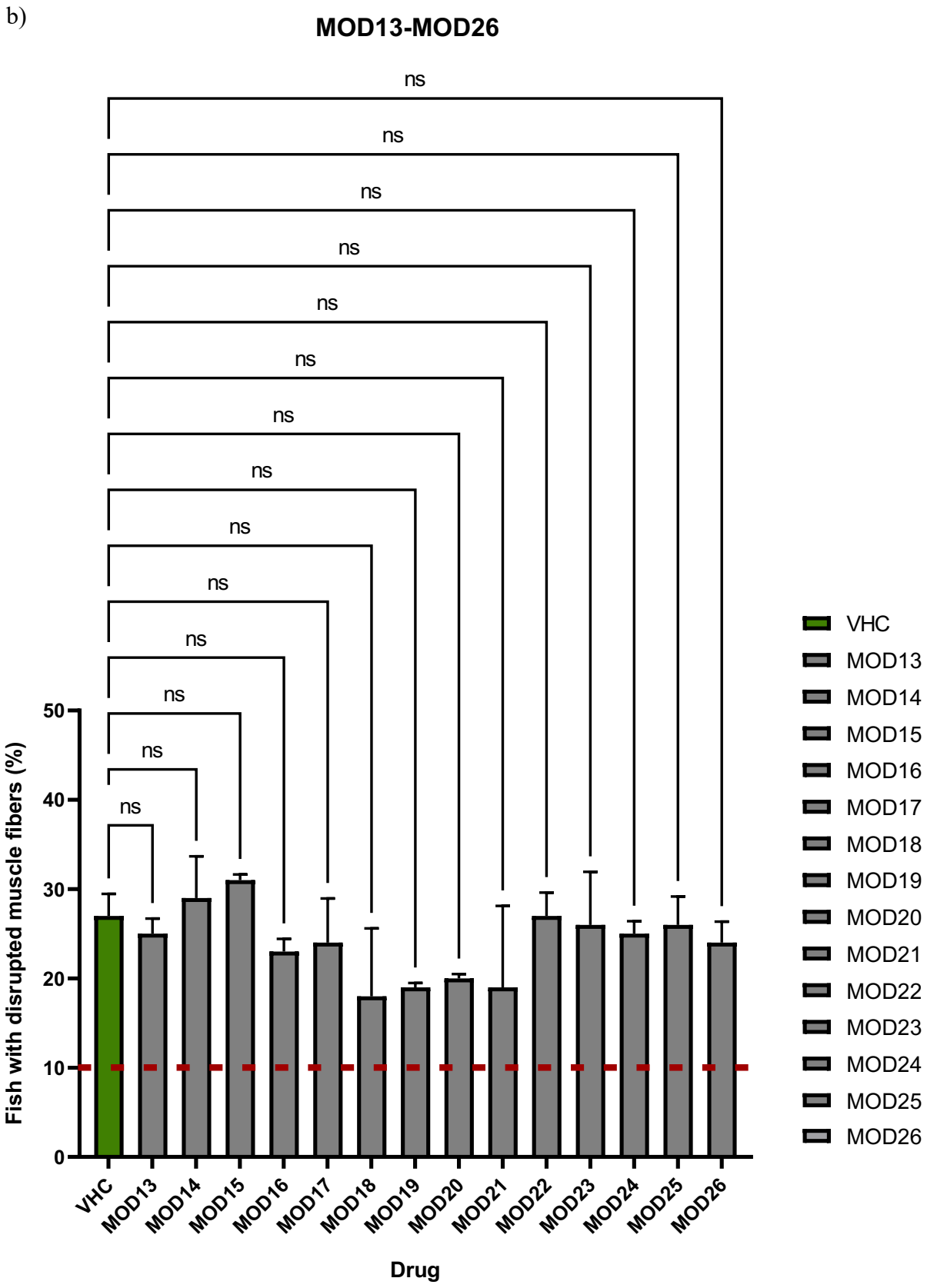

MOD13-MOD26
ns
Fish with disrupted muscle fibers (%)
0
10
20
30
40
50
VHC
MOD13
MOD14
MOD15
MOD16
MOD17
MOD18
MOD19
MOD20
MOD21
MOD22
MOD23
MOD24
MOD25
MOD26
Drug

Figure19. Drug screening results for the 26 hit compounds found previously on worms.

a) MOD1 to MOD12 drug screening. b) MOD13 to MOD26 drug screening. Bars show the percentage of fish with disrupted muscle fibers (n=100-120 per group). Each batch of experiments was performed with 2 vehicle-treated groups and each compound was tested at least 3 times in different batches. The red dashed line shows the threshold (10%) under which the compound would be considered suitable to perform further experiments. Although some compounds were close to the threshold, statistically there was no difference between each group and the vehicle-treated group, showing the compounds didn't have a significant impact on the pathology's characteristics of the disease.

Chapter 3 – Discussion

Our experiments on swimming behaviour, life expectancy and muscular condition provided a better understanding of the disease in DMD fish, such as a diminished capacity of swim, a shorter life expectancy and the apparition of disrupted muscular fibers at early ages. Unfortunately, none of the molecules utilized on drug screening experiments presented any amelioration at a muscular level. Thus, further experiments should be done in order to find the cure of DMD.

1. Possible explanations for the negative results

Although the previous work on worm DMD models we identified 26 potential hit molecules able to ameliorate their motor phenotype, unfortunately, none of these small molecules had a positive impact on muscle fiber health in our DMD zebrafish model. This fact could be explained by the points described in the following sections.

1.1. Clinically relevant phenotypes of the zebrafish model

Zebrafish with mutations in the dystrophin gene are good models for the study of DMD (153). Their muscle integrity can be easily assessed on living larvae as early as few days post-fertilization, they are easy to raise, care and manipulate. Thus, their cost-effectiveness is very attractive for research. Besides that, their possess a high skeletal muscle content, and they express

orthologues of most of human dystrophin glycoprotein complex. Therefore, zebrafish are suitable and relevant for high-throughput chemical screens to identify drug candidates adequate for the correction of the muscle phenotype (154).

In this study, the two most clinically significant phenotypes of the zebrafish model are their motility behaviour and their muscle condition. The data of our swimming tests shows a significant correlation between dystrophin null-fish (*dmd*-/-) and the diminution swimming activity, whereas siblings (*dmd*+/- and *dmd*+/+) display a normal swimming behaviour. In addition, their muscular characteristics can be accurately quantified under birefringence conditions. Indeed, when performed blindly (and genotyped post experimentally), we found that fish with disrupted muscle fibers correspond to dystrophin null-fish (*dmd*-/-), whereas most fish with healthy fibers muscle correlate to *dmd*+/- and *dmd*+/+ genotypes.

In the field of DMD fish, examining swimming behaviour and using phalloidin to determine muscle condition are two common approaches utilized by researchers. In fact, all studies concerning DMD fish present phalloidin experiments, and show a global sarcomeric disorganization of muscle fibers (155), which is a phenotype we found in our current study. On the contrary, our experiment regarding to swimming behaviour is slightly different comparing to other laboratories. Whereas we have used statistical data from almost 100 *larvae,* most of the research groups show a video of a single DMD larvae (156), demonstrating effectively a diminution of their motility. This fact could be explained because there is a high level of variability when a 100-fish swimming experiment is performed, thus researchers opt to show only a video of one affected fish. Furthermore, it is worthy of mention that in none of the current published studies, DMD fish were generated by using CRISPR/Cas9 technology, but by morpholino injections. This fact could also explain the variability of the results on our swimming test and the response of our model to drug screening. Despite of these discrepancies, all the phenotypes we found in our model are reminiscent of human DMD features, such as short life expectancy, motility disfunction, and sarcomeric disorganization.

The small molecules utilized here were obtained from four commercially available libraries: Sigma, Prestwick, Microsource and Biomol. Although the primary screening on worm

DMD models identified 26 potential hit molecules capable of improving their motor phenotype, none of these small molecules had a positive effect on muscle fiber health in our DMD zebrafish model.

One could wonder if zebrafish DMD models are clinically relevant. In this study, we generated our own DMD mutant fish and not the *sapje* zebrafish due to the difficulty of importing this type of fish to Canada. *Sapje* zebrafish is a mutant obtained by random mutagenesis. Dystrophic mutants were identified by Basett et al. (2004), who demonstrated the *sapje* mutant contained a nonsense mutation in exon 4 of the zebrafish dystrophin gene. They reported that the progressive muscular degeneration in zebrafish was provoked by the division of somatic fibers from their attachment sites at the embryonic myotendinous junctions (157). Many fibers detached at one end and contract to a fraction of their original length, showing compression or even collapse of the sarcomeric banding. Despite presenting the phenotype of muscle degeneration, at the cellular level the disease of *sapje* mutant zebrafish differs from the human pathological muscular degeneration, where membrane damage occurs along the length of the fiber. In zebrafish, the *dystrophin*-associated protein complex (DAPC) is contained embryonically to the ends of muscle fibers prior it becomes appreciable at the sarcolemma, suggesting that loss of the DAPC may compromise muscle attachments and, possibly provoking the detachment is observed on *sapje* mutant zebrafish (157).

Comparing to our DMD zebrafish model, confocal imaging of skeletal muscle expressing green fluorescent protein (GFP) showed lesions where the ends of DMD mutant muscle fibers divide from their attachment sites. We believe that in our DMD zebrafish mutant, the mutation is more deleterious than in *sapje* mutant line because other works demonstrated to have found a subset of *sapje* fish that can live much longer than one month (158).

Some directions to consider for future analysis are (1) to perform parallelly experiments with worms and zebrafish and (2) to investigate some other drug side effects in zebrafish not related with the muscle or the heart that can be affected.

We conclude that our model is relevant and that it is unlikely to explain the negative results of our screen because of a problem at the level of our mutant model.

1.2. Complementary tests on worms

It was unexpected that none of the hit compounds identified in worms showed a positive effect on our fish model. Indeed, other projects have been conducted at Modelis, from worms to fish, and this situation where none of the hits identified in worm show, at least a trend of amelioration, never happened. It should be mentioned that there are some differences in the experimental procedure for the drug screening on *dmd*-mutant worms in comparison with fish. In the study with worms, the information about their response to hundreds of compounds was obtained from an acute treatment, in opposition of the chronic treatment we performed with fish. In addition, the motility on worms was monitored for few hours in liquid culture, whereas we looked at their motility every day of the treatment.

Hit compounds did not improve the muscle health in fish as compared to the worm model. This could be due to species-specific responses to drugs, or it could be that the worm approach focusing on acute motility might not be a robust read-out as it appears that all these candidate chemicals are false positive. Thus, it shows that we should not simply rely on a “basic” motility phenotype in worms, but rather complement these assays with some more specific phenotypes to discard any potential false positive hits (i.e randomly affecting the motility of worms, not specifically improving muscle health).

In our experiments, treated fish did not show muscle amelioration after being treated, and in some cases, drugs even deteriorated notably their health. As we believe it is not a problem at the level of the fish model itself, we believe that the molecules should have been selected based on multiple and complementary assay in worms. In this study, they have been selected on their potential to ameliorate the general motility of DMD worms. Apparently, this is not specific enough to determine whether a molecule has a real therapeutic potential or not. Thus, it is necessary to acquire complementary and maybe more specific phenotypes in worms before testing in fish. For

example, we could check muscle integrity on worm that could help selecting molecules that have an effect not only on the general motility, but at a muscle level (Figure 20).

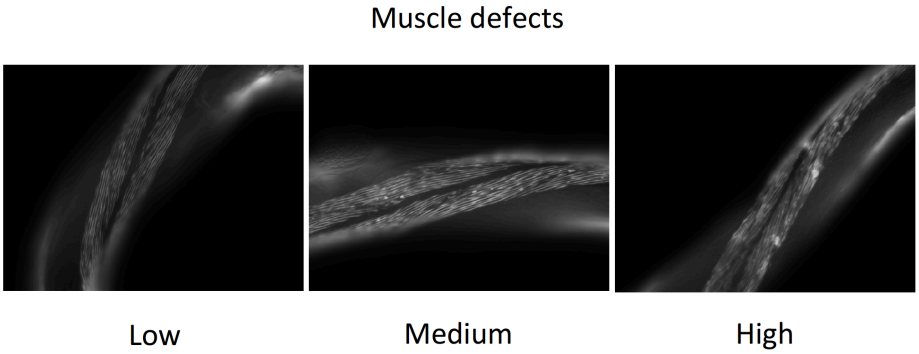


Figure 20. A complementary assay in worm: using the DM8005 strain containing a GFP-tagged myo-3 protein.

The effect of drugs can be quantified using scores of muscle integrity as “low, “medium” or high”.

1.3. Radiation mutagenesis mutant: day-33: potential off-targets

Another reason that could explain the difficulty on identifying a hit compound for fish is the initial screening on the DYS-1 (eg33) worm strain, that was obtained by chemical random mutagenesis. As this technique is not targeted, it exists the risk that an off-target mutation segregates with the selected *dmd* mutation we are studying. That means maybe the motility phenotype is not due (or at least not at 100%) to the mutation in *dmd* gene, but maybe it is the result of unknown off-target mutations.

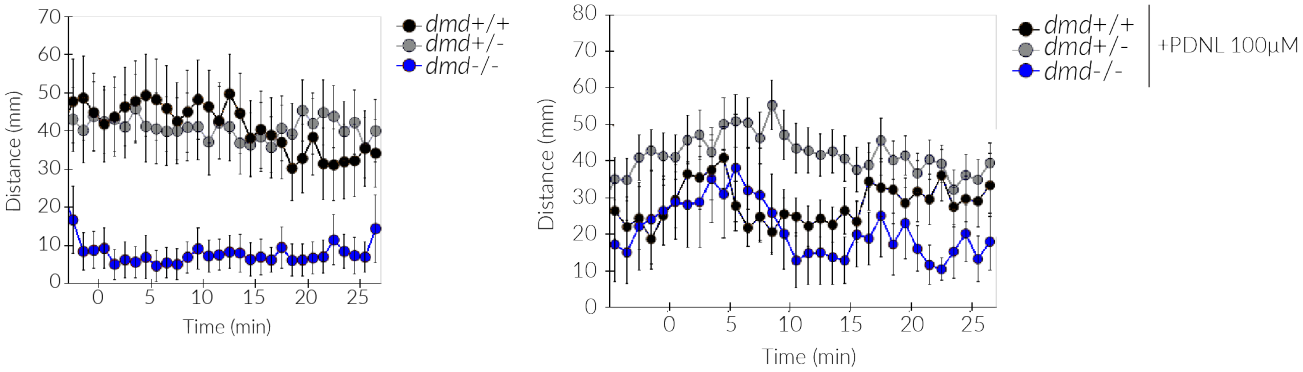
In order to avoid these off-target mutations, we could double check the effect of our hits on other DMD worm mutants. A high variety of genes when mutated in worms present phenotypes such as *dys-1* mutants (head bending, hyperactivity and a tendency to hyper contract), showing the possibility they may have the same biological function as *dys-1*. These genes are translated in proteins that belong to the DAPC or interact with it, focusing the relevance of maintaining the integrity of DAPC. These include *dyb-1* (dystrobrevin), *dyc-1* (capon), *stn-1/2* (syntrophins), *sgn-1* (sarcoglycan), *snf-6* (an acetylcholine transporter), *slo-1* (a potassium channel) and *islo-1* (interactor of *slo-1*) (158).

Finally, we could have generated a novel mutant worm line using a targeted mutagenesis method, like CRISPR/Cas9 technology. The generation of this line is currently in process, and we will proceed to cross-validate the list of hit compounds on this new CRISPR mutant.

1.4. Variability in phenotypic assessment

Both swimming tests and muscular observations under birefringence conditions present some variability that can make drug screening assay difficult.

In the swimming test, different values were obtained in the same type of experiment (same fish age, same experiment conditions, and same treatment). This issue makes the interpretation of the data difficult and therefore it urges the team to increase the number of replicates, obtaining in most cases no consistent data, resulting in a deceleration of the study. Even with FDA approved drugs for Duchenne syndrome in humans, such as Prednisolone (PDNL), swimming tests show different values with the same experiment conditions. As we can see in Figure 24, PDNL induces a significant improvement on swimming, but it does not show amelioration at the muscle level. Thus, the lack of a clear positive control can be misleading.



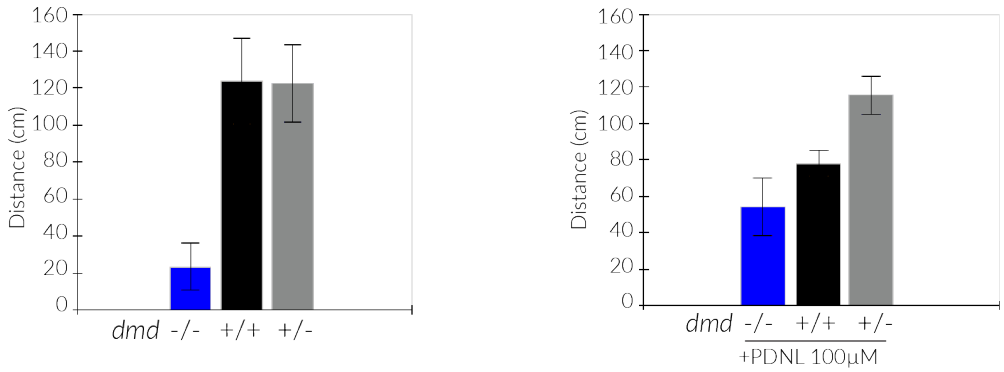


Figure 21. Effect of PDNL on *dmd* mutant fish.

Although *dmd* mutant fish do not show any motor deficit at early stages (until 3 days), they depict a severe hypomotility phenotype from day 4 (data from day 5 is shown below). Interestingly, this hypomotility can be partially rescued after exposing the larvae with prednisolone (PDNL) at 100 µM ($p < 0.05$).

Comparing worms with zebrafish animal model, we have noted some differences between them. The first difference is the rapid development of worms comparing to zebrafish, so the possible therapeutic results of drugs could be rapidly screened. Furthermore, worms present a more robust response to the drugs because they need a few environmental conditions to be raised, so there are less factors to take in consideration in their development that could affect to their response to drugs.

2. Challenges to do phenotypic screening in DMD fish

2.1. Variability due to bias in embryo selection

In the birefringence screen for treated fish, some compounds have the same unaltered non restoring effect in all the replicates, whereas some other compounds show a noticeable variability among their replicates. Thus, in some experiments, these compounds provoke a significant diminution of the percentage of fish with altered fiber muscle (less than 10%), but in other replicates this percentage does not show any ameliorations, therefore invalidating the result (overall not significant). To exemplify this fact, it can be interesting to mention the variability of the effect of MOD18. In this case, 5 experiments were performed, and while the two first showed a diminution of fish with altered fiber muscle being around the 10% threshold (Table 5), the next

experiments, were not showing any improvement. This could be explained by bias in the selection of eggs at day 0, when we collect fish and raise them in beakers. In that case, we collected randomly 100 fish per beaker and maybe there was some bias in the way of separating the fish. That means we could eventually have different proportions of homozygous in each beaker, when each of them should present 25% of homozygous.

MOD18 0.5 µM	Deffects (%)	Normal (%)	Average	SEM	P value
Experiment 1	12.6	77.4	17.9	7.6	0.2
Experiment 2	11.8	88.2			
Experiment 3	25	75			
Experiment 4	26	74			
Experiment 5	27	73			

Table 9. Results obtained with drug MOD18 on five experiments.

Drug was administrated according to our drug screening approach and results were obtained once fish were assessed under birefringence conditions at 5 dpf. Note that the average for all experiments is 17.9%, a number above the threshold (10%), invalidating the potential therapeutical effect of this drug on our fish model (P value < 0.05).

In the laboratory, we tried to anesthetise fish after birefringence assay in order to genotype them or to perform swimming tests, but even with little amounts of anesthesia, fish didn't recover well and at the end that is not good for muscle health, so at the end it is not convenient. Ideally, we would like to genotype them before doing the assay, so we are only assessing muscle integrity in the homozygous population. In the laboratory, we have been working on establishing a new non-invasive genotyping protocol on 4 dpf larvae (unpublished). Although this protocol is currently ready, at the time these experiments we performed it was still being tested on fish.

2.2. Environmental factors affecting disease's progression

One possible reason about these facts is that DMD is a progressive muscular disease, so different environment-adaptive reactions can be reported at later phases of fish development. Therefore, it might be interesting to perform the experiments at juvenile fish stages. However, if

we adopt this new approach, then drug screening could be less effective because at early development stages, fish do not present any the symptoms of the disease, but they appear while disease progresses (Figure 25). In addition, in neurodegenerative disease like DMD, it is common to expect slight difference in environmental conditions between batches (number of larvae per dish, properly cleaning of the beaker, etc.) that can influence the general health of the larvae and therefore the progression of the disease.

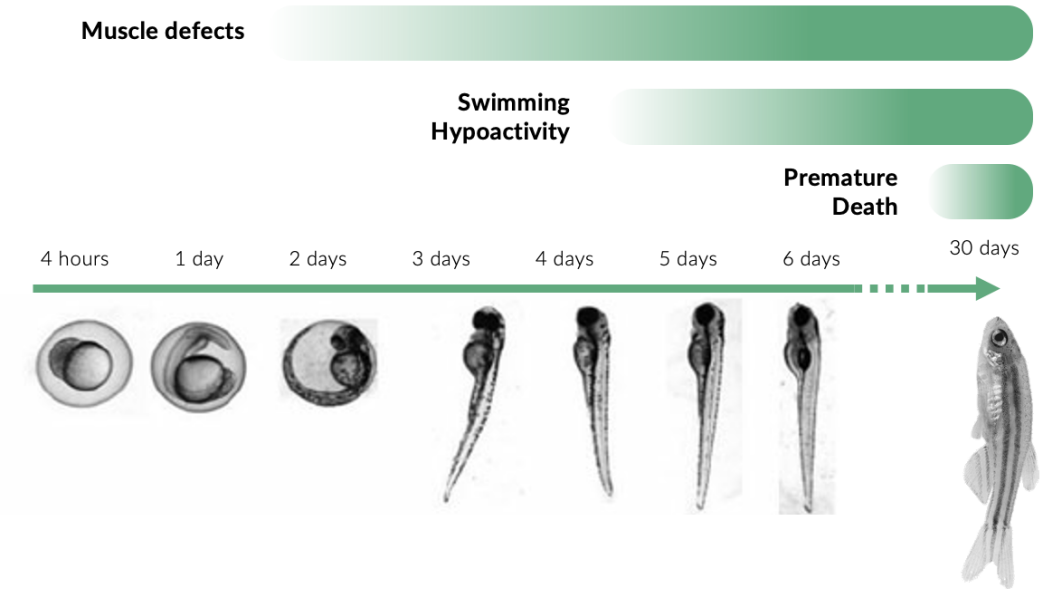


Figure 22. Neurodegenerative disease progression in zebrafish.

The fact the phenotypes appear progressively might induce more variability and complexity standardised drug screening. Some of the symptoms of the disease are early muscle fiber disorganization (from 2 dpf) and progressive hypomotility (swimming function impaired) at 3-4 dpf, bringing fish to a premature death at 15-30 dpf).

2.3. Future directions in the experimental procedure

In order to elaborate a more accurate experiment and therefore to dampen this perpetual variability, some changes could be considered.

First, the diminution of the number of fish in each experiment. Every study performed, swimming test and birefringence observation, included around 100 fish per group of treatment. This number of fish increases the possibility of having more variability data because they had to

be raised in a higher and less controlled environment (500 mL beakers), so their interactions and response to this new environment could affect the results. In addition, most experiments that have succeeded with fish drug screening were performed in smaller and more stable and organised plates. Subsequently, the containers where they are raised should have less dimensions and more robust storage. An example of this type of container is the 6-well plate, in which a notable previous and rewarding studies have been completed.

Regarding the toxicity assay, it is important to mention that a substantial change could be done. In this study, the toxicity assay was done on *wild type* zebrafish, but not on DMD mutant zebrafish. So, the suitable drug concentration could slightly be different due to the distinct subject of the study. As *wild type* fish carry full dystrophin gene, they resist more for higher drug concentrations, so the potential impact of a given concentration of drug in *wild type* fish cannot be expected identical on DMD mutant fish. In fact, in a few compounds, we observed they didn't resist until the last day of the treatment, showing a rapid health deterioration due to the high amount of drug inoculated, previously tested on *wild type* fish. In this case, it would be appropriate to do a toxicity test on DMD mutant fish to identify the most suitable drug concentration, apart from the toxicity test on *wild type* fish. Unfortunately, we could not do the toxicity test on mutant fish due to a lack of sufficient drugs to perform future experiments.

Given the negative results of the drug screening, we believe that by ameliorating the phenotypic readouts used for worm drug screening and by treating specifically homozygous *dmd* mutant fish, the experimental variability we encountered could be surpassed. Additional studies need to be extended to find a therapeutic molecule that restores pathological effects of Duchenne Muscular Disease, the most common among rare disease affecting children.

Bibliography

1. Kini, U., & Cilliers, D. (2012). Authors muscle in on history: The History of a Genetic Disease Duchenne Muscular Dystrophy or Meryon's disease. *European Journal of Human Genetics*, *20*(11), 1201. https://doi.org/10.1038/ejhg.2012.111
2. Kakulas B. A. (2008). A brief history of muscular dystrophy research: a personal perspective. *Neurology India*, *56*(3), 231–235. https://doi.org/10.4103/0028-3886.43440
3. Falzarano, M. S., Scotton, C., Passarelli, C., & Ferlini, A. (2015). Duchenne Muscular Dystrophy: From Diagnosis to Therapy. *Molecules (Basel, Switzerland)*, *20*(10), 18168–18184. https://doi.org/10.3390/molecules201018168
4. Barakat-Haddad, C., Shin, S., Candundo, H., Lieshout, P. V., & Martino, R. (2017). A systematic review of risk factors associated with muscular dystrophies. *Neurotoxicology*, *61*, 55–62. https://doi.org/10.1016/j.neuro.2016.03.007
5. Allen, D. G., Whitehead, N. P., & Froehner, S. C. (2016). Absence of Dystrophin Disrupts Skeletal Muscle Signaling: Roles of Ca2+, Reactive Oxygen Species, and Nitric Oxide in the Development of Muscular Dystrophy. *Physiological reviews*, *96*(1), 253–305. https://doi.org/10.1152/physrev.00007.2015
6. Guiraud, S., Aartsma-Rus, A., Vieira, N. M., Davies, K. E., van Ommen, G. J., & Kunkel, L. M. (2015). The Pathogenesis and Therapy of Muscular Dystrophies. *Annual review of genomics and human genetics*, *16*, 281–308. https://doi.org/10.1146/annurev-genom-090314-025003
7. Govoni, A., Magri, F., Brajkovic, S., Zanetta, C., Faravelli, I., Corti, S., Bresolin, N., & Comi, G. P. (2013). Ongoing therapeutic trials and outcome measures for Duchenne muscular dystrophy. *Cellular and molecular life sciences: CMLS*, *70*(23), 4585–4602. https://doi.org/10.1007/s00018-013-1396-z
8. Fujii, K., Minami, N., Hayashi, Y., Nishino, I., Nonaka, I., Tanabe, Y., Takanashi, J., & Kohno, Y. (2009). Homozygous female Becker muscular dystrophy. *American journal of medical genetics. Part A*, *149A*(5), 1052–1055. https://doi.org/10.1002/ajmg.a.32808
9. Giliberto, F., Radic, C. P., Luce, L., Ferreiro, V., de Brasi, C., & Szijan, I. (2014). Symptomatic female carriers of Duchenne muscular dystrophy (DMD): genetic and clinical

characterization. *Journal of the neurological sciences*, *336*(1-2), 36–41. https://doi.org/10.1016/j.jns.2013.09.036

10. Aartsma-Rus, A., Ginjaar, I. B., & Bushby, K. (2016). The importance of genetic diagnosis for Duchenne muscular dystrophy. *Journal of medical genetics*, *53*(3), 145–151. https://doi.org/10.1136/jmedgenet-2015-103387
11. Uttley, L., Carlton, J., Woods, H. B., & Brazier, J. (2018). A review of quality of life themes in Duchenne muscular dystrophy for patients and carers. *Health and Quality of Life Outcomes*, *16*(1). https://doi.org/10.1186/s12955-018-1062-0
12. Zamani, G., Heidari, M., Azizi Malamiri, R., Ashrafi, M. R., Mohammadi, M., Shervin Badv, R., Hosseini, S. A., Salehi, S., Shahrokhi, A., Qorbani, M., & Fathi, M. R. (2016). The quality of life in boys with Duchenne muscular dystrophy. *Neuromuscular disorders : NMD*, *26*(7), 423–427. https://doi.org/10.1016/j.nmd.2016.05.004
13. Lim, Y., Velozo, C., & Bendixen, R. M. (2014). The level of agreement between child self-reports and parent proxy-reports of health-related quality of life in boys with Duchenne muscular dystrophy. *Quality of life research : an international journal of quality of life aspects of treatment, care and rehabilitation*, *23*(7), 1945–1952. https://doi.org/10.1007/s11136-014-0642-7
14. Aartsma-Rus, A., Van Deutekom, J. C., Fokkema, I. F., Van Ommen, G. J., & Den Dunnen, J. T. (2006). Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule. *Muscle & nerve*, *34*(2), 135–144. https://doi.org/10.1002/mus.20586
15. Gao, Q. Q., & McNally, E. M. (2015). The Dystrophin Complex: Structure, Function, and Implications for Therapy. *Comprehensive Physiology*, *5*(3), 1223–1239. https://doi.org/10.1002/cphy.c140048
16. Chelly, J., Hamard, G., Koulakoff, A., Kaplan, J. C., Kahn, A., & Berwald-Netter, Y. (1990). Dystrophin gene transcribed from different promoters in neuronal and glial cells. *Nature*, *344*(6261), 64–65. https://doi.org/10.1038/344064a0
17. Duan, D., Goemans, N., Takeda, S., Mercuri, E., & Aartsma-Rus, A. (2021). Duchenne muscular dystrophy. *Nature reviews. Disease primers*, *7*(1), 13. https://doi.org/10.1038/s41572-021-00248-3

18. Górecki, D. C., Monaco, A. P., Derry, J. M., Walker, A. P., Barnard, E. A., & Barnard, P. J. (1992). Expression of four alternative dystrophin transcripts in brain regions regulated by different promoters. *Human molecular genetics*, *1*(7), 505–510. https://doi.org/10.1093/hmg/1.7.505

19. Doorenweerd, N., Mahfouz, A., van Putten, M., Kaliyaperumal, R., T' Hoen, P., Hendriksen, J., Aartsma-Rus, A. M., Verschuuren, J., Niks, E. H., Reinders, M., Kan, H. E., & Lelieveldt, B. (2017). Timing and localization of human dystrophin isoform expression provide insights into the cognitive phenotype of Duchenne muscular dystrophy. *Scientific reports*, *7*(1), 12575. https://doi.org/10.1038/s41598-017-12981-5

20. D'Souza, V. N., Nguyen, T. M., Morris, G. E., Karges, W., Pillers, D. A., & Ray, P. N. (1995). A novel dystrophin isoform is required for normal retinal electrophysiology. *Human molecular genetics*, *4*(5), 837–842. https://doi.org/10.1093/hmg/4.5.837

21. Byers, T. J., Lidov, H. G., & Kunkel, L. M. (1993). An alternative dystrophin transcript specific to peripheral nerve. *Nature genetics*, *4*(1), 77–81. https://doi.org/10.1038/ng0593-77

22. Hugnot, J. P., Gilgenkrantz, H., Vincent, N., Chafey, P., Morris, G. E., Monaco, A. P., Berwald-Netter, Y., Koulakoff, A., Kaplan, J. C., & Kahn, A. (1992). Distal transcript of the dystrophin gene initiated from an alternative first exon and encoding a 75-kDa protein widely distributed in nonmuscle tissues. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(16), 7506–7510. https://doi.org/10.1073/pnas.89.16.7506

23. Gao, Q. Q., & McNally, E. M. (2015). The Dystrophin Complex: Structure, Function, and Implications for Therapy. *Comprehensive Physiology*, *5*(3), 1223–1239. https://doi.org/10.1002/cphy.c140048

24. Prins, K. W., Humston, J. L., Mehta, A., Tate, V., Ralston, E., & Ervasti, J. M. (2009). Dystrophin is a microtubule-associated protein. *The Journal of cell biology*, *186*(3), 363–369. https://doi.org/10.1083/jcb.200905048

25. Zhao, J., Kodippili, K., Yue, Y., Hakim, C. H., Wasala, L., Pan, X., Zhang, K., Yang, N. N., Duan, D., & Lai, Y. (2016). Dystrophin contains multiple independent membrane-binding domains. *Human molecular genetics*, *25*(17), 3647–3653. https://doi.org/10.1093/hmg/ddw210

26. Mokri, B., & Engel, A. G. (1975). Duchenne dystrophy: electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber. *Neurology*, *25*(12), 1111–1120. https://doi.org/10.1212/wnl.25.12.1111
27. Naito, M., Mori, M., Inagawa, M., Miyata, K., Hashimoto, N., Tanaka, S., & Asahara, H. (2016). Dnmt3a Regulates Proliferation of Muscle Satellite Cells via p57Kip2. *PLoS genetics*, *12*(7), e1006167. https://doi.org/10.1371/journal.pgen.1006167
28. Nowak, K. J., & Davies, K. E. (2004). Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO reports*, *5*(9), 872–876. https://doi.org/10.1038/sj.embor.7400221
29. Aartsma-Rus, A., & van Putten, M. (2014). Assessing functional performance in the mdx mouse model. *Journal of visualized experiments : JoVE*, (85), 51303. https://doi.org/10.3791/51303
30. Pétrilli, V., Dostert, C., Muruve, D. A., & Tschopp, J. (2007). The inflammasome: a danger sensing complex triggering innate immunity. *Current opinion in immunology*, *19*(6), 615–622. https://doi.org/10.1016/j.coi.2007.09.002
31. Hyldahl, R. D., Nelson, B., Xin, L., Welling, T., Groscost, L., Hubal, M. J., Chipkin, S., Clarkson, P. M., & Parcell, A. C. (2015). Extracellular matrix remodeling and its contribution to protective adaptation following lengthening contractions in human muscle. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *29*(7), 2894–2904. https://doi.org/10.1096/fj.14-266668
32. Dadgar, S., Wang, Z., Johnston, H., Kesari, A., Nagaraju, K., Chen, Y. W., Hill, D. A., Partridge, T. A., Giri, M., Freishtat, R. J., Nazarian, J., Xuan, J., Wang, Y., & Hoffman, E. P. (2014). Asynchronous remodeling is a driver of failed regeneration in Duchenne muscular dystrophy. *The Journal of cell biology*, *207*(1), 139–158. https://doi.org/10.1083/jcb.201402079
33. De Paepe, B., Creus, K. K., Martin, J. J., & De Bleecker, J. L. (2012). Upregulation of chemokines and their receptors in Duchenne muscular dystrophy: potential for attenuation of myofiber necrosis. *Muscle & nerve*, *46*(6), 917–925. https://doi.org/10.1002/mus.23481
34. Wang, L., Zhou, L., Jiang, P., Lu, L., Chen, X., Lan, H., Guttridge, D. C., Sun, H., & Wang, H. (2012). Loss of miR-29 in myoblasts contributes to dystrophic muscle pathogenesis.

Molecular therapy : the journal of the American Society of Gene Therapy, *20*(6), 1222–1233. https://doi.org/10.1038/mt.2012.35

35. Kennedy, T. L., Moir, L., Hemming, S., Edwards, B., Squire, S., Davies, K., & Guiraud, S. (2017). Utrophin influences mitochondrial pathology and oxidative stress in dystrophic muscle. *Skeletal muscle*, *7*(1), 22. https://doi.org/10.1186/s13395-017-0139-5
36. Dhuri, K., Bechtold, C., Quijano, E., Pham, H., Gupta, A., Vikram, A., & Bahal, R. (2020). Antisense Oligonucleotides: An Emerging Area in Drug Discovery and Development. *Journal of clinical medicine*, *9*(6), 2004. https://doi.org/10.3390/jcm9062004
37. Gualandi, F., Neri, M., Bovolenta, M., Martoni, E., Rimessi, P., Fini, S., Spitali, P., Fabris, M., Pane, M., Angelini, C., Mora, M., Morandi, L., Mongini, T., Bertini, E., Ricci, E., Vattemi, G., Mercuri, E., Merlini, L., & Ferlini, A. (2009). Transcriptional behavior of DMD gene duplications in DMD/BMD males. *Human mutation*, *30*(2), E310–E319. https://doi.org/10.1002/humu.20881
38. Rugowska, A., Starosta, A. & Konieczny, P. (2021). Epigenetic modifications in muscle regeneration and progression of Duchenne muscular dystrophy. *Clin Epigenet 13*(13) https://doi.org/10.1186/s13148-021-01001-z
39. Colussi, C., Mozzetta, C., Gurtner, A., Illi, B., Rosati, J., Straino, S., Ragone, G., Pescatori, M., Zaccagnini, G., Antonini, A., Minetti, G., Martelli, F., Piaggio, G., Gallinari, P., Steinkuhler, C., Clementi, E., Dell'Aversana, C., Altucci, L., Mai, A., Capogrossi, M. C., … Gaetano, C. (2008). HDAC2 blockade by nitric oxide and histone deacetylase inhibitors reveals a common target in Duchenne muscular dystrophy treatment. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(49), 19183–19187. https://doi.org/10.1073/pnas.0805514105
40. Lai, Y., Zhao, J., Yue, Y., & Duan, D. (2013). α2 and α3 helices of dystrophin R16 and R17 frame a microdomain in the α1 helix of dystrophin R17 for neuronal NOS binding. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(2), 525–530. https://doi.org/10.1073/pnas.1211431109
41. Guerron, A. D., Rawat, R., Sali, A., Spurney, C. F., Pistilli, E., Cha, H. J., Pandey, G. S., Gernapudi, R., Francia, D., Farajian, V., Escolar, D. M., Bossi, L., Becker, M., Zerr, P., de la Porte, S., Gordish-Dressman, H., Partridge, T., Hoffman, E. P., & Nagaraju, K. (2010). Functional and molecular effects of arginine butyrate and prednisone on muscle and heart in

the mdx mouse model of Duchenne Muscular Dystrophy. *PloS one*, *5*(6), e11220. https://doi.org/10.1371/journal.pone.0011220

42. Thomas, G. D., Ye, J., De Nardi, C., Monopoli, A., Ongini, E., & Victor, R. G. (2012). Treatment with a nitric oxide-donating NSAID alleviates functional muscle ischemia in the mouse model of Duchenne muscular dystrophy. *PloS one*, *7*(11), e49350. https://doi.org/10.1371/journal.pone.0049350
43. Kobayashi, Y. M., Rader, E. P., Crawford, R. W., Iyengar, N. K., Thedens, D. R., Faulkner, J. A., Parikh, S. V., Weiss, R. M., Chamberlain, J. S., Moore, S. A., & Campbell, K. P. (2008). Sarcolemma-localized nNOS is required to maintain activity after mild exercise. *Nature*, *456*(7221), 511–515. https://doi.org/10.1038/nature07414
44. Kole, R., Krainer, A. R., & Altman, S. (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. *Nature reviews. Drug discovery*, *11*(2), 125–140. https://doi.org/10.1038/nrd3625
45. Aartsma-Rus, A., Fokkema, I., Verschuuren, J., Ginjaar, I., van Deutekom, J., van Ommen, G. J., & den Dunnen, J. T. (2009). Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Human mutation*, *30*(3), 293–299. https://doi.org/10.1002/humu.20918
46. Moxley, R. T., 3rd, Pandya, S., Ciafaloni, E., Fox, D. J., & Campbell, K. (2010). Change in natural history of Duchenne muscular dystrophy with long-term corticosteroid treatment: implications for management. *Journal of child neurology*, *25*(9), 1116–1129. https://doi.org/10.1177/0883073810371004
47. Beytía, M., Vry, J., & Kirschner, J. (2012). Drug treatment of Duchenne muscular dystrophy: available evidence and perspectives. *Acta myologica : myopathies and cardiomyopathies : official journal of the Mediterranean Society of Myology*, *31*(1), 4–8.
48. Markham, L. W., Kinnett, K., Wong, B. L., Woodrow Benson, D., & Cripe, L. H. (2008). Corticosteroid treatment retards development of ventricular dysfunction in Duchenne muscular dystrophy. *Neuromuscular disorders : NMD*, *18*(5), 365–370. https://doi.org/10.1016/j.nmd.2008.03.002
49. Bushby, K., Finkel, R., Birnkrant, D. J., Case, L. E., Clemens, P. R., Cripe, L., Kaul, A., Kinnett, K., McDonald, C., Pandya, S., Poysky, J., Shapiro, F., Tomezsko, J., Constantin, C., & DMD Care Considerations Working Group (2010). Diagnosis and management of

Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. *The Lancet. Neurology*, *9*(1), 77–93. https://doi.org/10.1016/S1474-4422(09)70271-6

50. Barthélémy, F., Wang, R. T., Hsu, C., Douine, E. D., Marcantonio, E. E., Nelson, S. F., & Miceli, M. C. (2019). Targeting RyR Activity Boosts Antisense Exon 44 and 45 Skipping in Human DMD Skeletal or Cardiac Muscle Culture Models. *Molecular therapy. Nucleic acids*, *18*, 580–589. https://doi.org/10.1016/j.omtn.2019.09.020
51. Miyatake, S., Shimizu-Motohashi, Y., Takeda, S., & Aoki, Y. (2016). Anti-inflammatory drugs for Duchenne muscular dystrophy: focus on skeletal muscle-releasing factors. *Drug design, development and therapy*, *10*, 2745–2758. https://doi.org/10.2147/DDDT.S110163
52. Arechavala-Gomeza, V., Kinali, M., Feng, L., Guglieri, M., Edge, G., Main, M., Hunt, D., Lehovsky, J., Straub, V., Bushby, K., Sewry, C. A., Morgan, J. E., & Muntoni, F. (2010). Revertant fibres and dystrophin traces in Duchenne muscular dystrophy: implication for clinical trials. *Neuromuscular disorders : NMD*, *20*(5), 295–301. https://doi.org/10.1016/j.nmd.2010.03.007
53. Mendell, J. R., Rodino-Klapac, L. R., & Malik, V. (2010). Molecular therapeutic strategies targeting Duchenne muscular dystrophy. *Journal of child neurology*, *25*(9), 1145–1148. https://doi.org/10.1177/0883073810371005
54. Elangkovan, N., & Dickson, G. (2021). Gene Therapy for Duchenne Muscular Dystrophy. *Journal of neuromuscular diseases*, *8*(s2), S303–S316. https://doi.org/10.3233/JND-210678
55. Echevarría, L., Aupy, P., & Goyenvalle, A. (2018). Exon-skipping advances for Duchenne muscular dystrophy. *Human molecular genetics*, *27*(R2), R163–R172. https://doi.org/10.1093/hmg/ddy171
56. Debruin, D. A., Andreacchio, N., Hanson, E. D., Timpani, C. A., Rybalka, E., & Hayes, A. (2019). The Effect of Vitamin D Supplementation on Skeletal Muscle in the *mdx* Mouse Model of Duchenne Muscular Dystrophy. *Sports (Basel, Switzerland)*, *7*(5), 96. https://doi.org/10.3390
57. Sicinski, P., Geng, Y., Ryder-Cook, A. S., Barnard, E. A., Darlison, M. G., & Barnard, P. J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science (New York, N.Y.)*, *244*(4912), 1578–1580. https://doi.org/10.1126/science.2662404

58. Grounds, M. D., Radley, H. G., Lynch, G. S., Nagaraju, K., & De Luca, A. (2008). Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy. *Neurobiology of disease*, *31*(1), 1–19. https://doi.org/10.1016/j.nbd.2008.03.008

59. De Luca A. (2012). Pre-clinical drug tests in the mdx mouse as a model of dystrophinopathies: an overview. *Acta myologica : myopathies and cardiomyopathies : official journal of the Mediterranean Society of Myology*, *31*(1), 40–47.

60. Fukada, S., Morikawa, D., Yamamoto, Y., Yoshida, T., Sumie, N., Yamaguchi, M., Ito, T., Miyagoe-Suzuki, Y., Takeda, S., Tsujikawa, K., & Yamamoto, H. (2010). Genetic background affects properties of satellite cells and mdx phenotypes. *The American journal of pathology*, *176*(5), 2414–2424. https://doi.org/10.2353/ajpath.2010.090887

61. Coley, W. D., Bogdanik, L., Vila, M. C., Yu, Q., Van Der Meulen, J. H., Rayavarapu, S., Novak, J. S., Nearing, M., Quinn, J. L., Saunders, A., Dolan, C., Andrews, W., Lammert, C., Austin, A., Partridge, T. A., Cox, G. A., Lutz, C., & Nagaraju, K. (2016). Effect of genetic background on the dystrophic phenotype in mdx mice. *Human molecular genetics*, *25*(1), 130–145. https://doi.org/10.1093/hmg/ddv460

62. van Putten, M., Putker, K., Overzier, M., Adamzek, W. A., Pasteuning-Vuhman, S., Plomp, J. J., & Aartsma-Rus, A. (2019). Natural disease history of the D2-*mdx* mouse model for Duchenne muscular dystrophy. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *33*(7), 8110–8124. https://doi.org/10.1096/fj.201802488R

63. Lim, K., Nguyen, Q., Dzierlega, K., Huang, Y., & Yokota, T. (2020). CRISPR-Generated Animal Models of Duchenne Muscular Dystrophy. *Genes*, *11*(3), 342. https://doi.org/10.3390/genes11030342

64. Selsby, J. T., Ross, J. W., Nonneman, D., & Hollinger, K. (2015). Porcine models of muscular dystrophy. *ILAR journal*, *56*(1), 116–126. https://doi.org/10.1093/ilar/ilv015

65. Whitworth, K. M., Lee, K., Benne, J. A., Beaton, B. P., Spate, L. D., Murphy, S. L., Samuel, M. S., Mao, J., O'Gorman, C., Walters, E. M., Murphy, C. N., Driver, J., Mileham, A., McLaren, D., Wells, K. D., & Prather, R. S. (2014). Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. *Biology of reproduction*, *91*(3), 78. https://doi.org/10.1095/biolreprod.114.121723

66. Yu, H. H., Zhao, H., Qing, Y. B., Pan, W. R., Jia, B. Y., Zhao, H. Y., Huang, X. X., & Wei, H. J. (2016). Porcine Zygote Injection with Cas9/sgRNA Results in DMD-Modified Pig with Muscle Dystrophy. *International journal of molecular sciences*, *17*(10), 1668. https://doi.org/10.3390/ijms17101668

67. Klymiuk, N., Blutke, A., Graf, A., Krause, S., Burkhardt, K., Wuensch, A., Krebs, S., Kessler, B., Zakhartchenko, V., Kurome, M., Kemter, E., Nagashima, H., Schoser, B., Herbach, N., Blum, H., Wanke, R., Aartsma-Rus, A., Thirion, C., Lochmüller, H., Walter, M. C., … Wolf, E. (2013). Dystrophin-deficient pigs provide new insights into the hierarchy of physiological derangements of dystrophic muscle. *Human molecular genetics*, *22*(21), 4368–4382. https://doi.org/10.1093/hmg/ddt287

68. Wells D. J. (2018). Tracking progress: an update on animal models for Duchenne muscular dystrophy. *Disease models & mechanisms*, *11*(6), dmm035774. https://doi.org/10.1242/dmm.035774

69. Wang, Y., Fan, N., Song, J., Zhong, J., Guo, X., Tian, W., Zhang, Q., Cui, F., Li, L., Newsome, P. N., Frampton, J., Esteban, M. A., & Lai, L. (2014). Generation of knockout rabbits using transcription activator-like effector nucleases. *Cell regeneration (London, England)*, *3*(1), 3. https://doi.org/10.1186/2045-9769-3-3

70. Esteves, P. J., Abrantes, J., Baldauf, H. M., BenMohamed, L., Chen, Y., Christensen, N., González-Gallego, J., Giacani, L., Hu, J., Kaplan, G., Keppler, O. T., Knight, K. L., Kong, X. P., Lanning, D. K., Le Pendu, J., de Matos, A. L., Liu, J., Liu, S., Lopes, A. M., Lu, S., … Mage, R. (2018). The wide utility of rabbits as models of human diseases. *Experimental & molecular medicine*, *50*(5), 1–10. https://doi.org/10.1038/s12276-018-0094-1

71. Guiraud, S., & Davies, K. E. (2017). Pharmacological advances for treatment in Duchenne muscular dystrophy. *Current opinion in pharmacology*, *34*, 36–48. https://doi.org/10.1016/j.coph.2017.04.002

72. Chen, Y., Zheng, Y., Kang, Y., Yang, W., Niu, Y., Guo, X., Tu, Z., Si, C., Wang, H., Xing, R., Pu, X., Yang, S. H., Li, S., Ji, W., & Li, X. J. (2015). Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9. *Human molecular genetics*, *24*(13), 3764–3774. https://doi.org/10.1093/hmg/ddv120

73. Wang, S., Ren, S., Bai, R., Xiao, P., Zhou, Q., Zhou, Y., Zhou, Z., Niu, Y., Ji, W., & Chen, Y. (2018). No off-target mutations in functional genome regions of a CRISPR/Cas9-

generated monkey model of muscular dystrophy. *The Journal of biological chemistry*, *293*(30), 11654–11658. https://doi.org/10.1074/jbc.AC118.004404

74. Fellows, L. K., Foster, B. J., & Chalk, C. H. (2003). Clinical significance of complex repetitive discharges: A case-control study. *Muscle & nerve*, *28*(4), 504–507. https://doi.org/10.1002/mus.10425
75. Kornegay J. N. (2017). The golden retriever model of Duchenne muscular dystrophy. *Skeletal muscle*, *7*(1), 9. https://doi.org/10.1186/s13395-017-0124-z
76. Wagner, K. R., McPherron, A. C., Winik, N., & Lee, S. J. (2002). Loss of myostatin attenuates severity of muscular dystrophy in mdx mice. *Annals of neurology*, *52*(6), 832–836. https://doi.org/10.1002/ana.10385
77. Sparrow, J., Hughes, S. M., & Segalat, L. (2008). Other model organisms for sarcomeric muscle diseases. *Advances in experimental medicine and biology*, *642*, 192–206. https://doi.org/10.1007/978-0-387-84847-1_14
78. Gieseler, K., Grisoni, K., & Ségalat, L. (2000). Genetic suppression of phenotypes arising from mutations in dystrophin-related genes in Caenorhabditis elegans. *Current biology : CB*, *10*(18), 1092–1097. https://doi.org/10.1016/s0960-9822(00)00691-6
79. Giacomotto, J., Brouilly, N., Walter, L., Mariol, M. C., Berger, J., Ségalat, L., Becker, T. S., Currie, P. D., & Gieseler, K. (2013). Chemical genetics unveils a key role of mitochondrial dynamics, cytochrome c release and IP3R activity in muscular dystrophy. *Human molecular genetics*, *22*(22), 4562–4578. https://doi.org/10.1093/hmg/ddt302
80. Carre-Pierrat, M., Lafoux, A., Tanniou, G., Chambonnier, L., Divet, A., Fougerousse, F., Huchet-Cadiou, C., & Ségalat, L. (2011). Pre-clinical study of 21 approved drugs in the mdx mouse. *Neuromuscular disorders : NMD*, *21*(5), 313–327. https://doi.org/10.1016/j.nmd.2011.01.005
81. Carre-Pierrat, M., Mariol, M. C., Chambonnier, L., Laugraud, A., Heskia, F., Giacomotto, J., & Ségalat, L. (2006). Blocking of striated muscle degeneration by serotonin in C. elegans. *Journal of muscle research and cell motility*, *27*(3-4), 253–258. https://doi.org/10.1007/s10974-006-9070-9
82. Carre-Pierrat, M., Mariol, M. C., Chambonnier, L., Laugraud, A., Heskia, F., Giacomotto, J., & Ségalat, L. (2006). Blocking of striated muscle degeneration by serotonin in C. elegans.

Journal of muscle research and cell motility, *27*(3-4), 253–258. https://doi.org/10.1007/s10974-006-9070-9

83. Hewitt, J. E., Pollard, A. K., Lesanpezeshki, L., Deane, C. S., Gaffney, C. J., Etheridge, T., Szewczyk, N. J., & Vanapalli, S. A. (2018). Muscle strength deficiency and mitochondrial dysfunction in a muscular dystrophy model of *Caenorhabditis elegans* and its functional response to drugs. *Disease models & mechanisms*, *11*(12), dmm036137. https://doi.org/10.1242/dmm.036137
84. Shcherbata, H. R., Yatsenko, A. S., Patterson, L., Sood, V. D., Nudel, U., Yaffe, D., Baker, D., & Ruohola-Baker, H. (2007). Dissecting muscle and neuronal disorders in a Drosophila model of muscular dystrophy. *The EMBO journal*, *26*(2), 481–493. https://doi.org/10.1038/sj.emboj.7601503
85. Taghli-Lamallem, O., Akasaka, T., Hogg, G., Nudel, U., Yaffe, D., Chamberlain, J. S., Ocorr, K., & Bodmer, R. (2008). Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype. *Aging cell*, *7*(2), 237–249. https://doi.org/10.1111/j.1474-9726.2008.00367.x
86. Pantoja, M., Fischer, K. A., Ieronimakis, N., Reyes, M., & Ruohola-Baker, H. (2013). Genetic elevation of sphingosine 1-phosphate suppresses dystrophic muscle phenotypes in Drosophila. *Development (Cambridge, England)*, *140*(1), 136–146. https://doi.org/10.1242/dev.087791
87. Christoforou, C. P., Greer, C. E., Challoner, B. R., Charizanos, D., & Ray, R. P. (2008). The detached locus encodes Drosophila Dystrophin, which acts with other components of the Dystrophin Associated Protein Complex to influence intercellular signalling in developing wing veins. *Developmental biology*, *313*(2), 519–532. https://doi.org/10.1016/j.ydbio.2007.09.044
88. Kucherenko, M. M., Marrone, A. K., Rishko, V. M., Magliarelli, H., & Shcherbata, H. R. (2011). Stress and muscular dystrophy: a genetic screen for dystroglycan and dystrophin interactors in Drosophila identifies cellular stress response components. *Developmental biology*, *352*(2), 228–242. https://doi.org/10.1016/j.ydbio.2011.01.013
89. McNally, E. M., Kaltman, J. R., Benson, D. W., Canter, C. E., Cripe, L. H., Duan, D., Finder, J. D., Groh, W. J., Hoffman, E. P., Judge, D. P., Kertesz, N., Kinnett, K., Kirsch, R., Metzger, J. M., Pearson, G. D., Rafael-Fortney, J. A., Raman, S. V., Spurney, C. F., Targum, S. L.,

Wagner, K. R., … Parent Project Muscular Dystrophy (2015). Contemporary cardiac issues in Duchenne muscular dystrophy. Working Group of the National Heart, Lung, and Blood Institute in collaboration with Parent Project Muscular Dystrophy. *Circulation*, *131*(18), 1590–1598. https://doi.org/10.1161/CIRCULATIONAHA.114.015151

90. Judge, L. M., Haraguchiln, M., & Chamberlain, J. S. (2006). Dissecting the signaling and mechanical functions of the dystrophin-glycoprotein complex. *Journal of cell science*, *119*(Pt 8), 1537–1546. https://doi.org/10.1242/jcs.02857

91. Pantoja, M., & Ruohola-Baker, H. (2013). Drosophila as a starting point for developing therapeutics for the rare disease Duchenne Muscular Dystrophy. *Rare diseases (Austin, Tex.)*, *1*, e24995. https://doi.org/10.4161/rdis.24995

92. Ieronimakis, N., Pantoja, M., Hays, A. L., Dosey, T. L., Qi, J., Fischer, K. A., Hoofnagle, A. N., Sadilek, M., Chamberlain, J. S., Ruohola-Baker, H., & Reyes, M. (2013). Increased sphingosine-1-phosphate improves muscle regeneration in acutely injured mdx mice. *Skeletal muscle*, *3*(1), 20. https://doi.org/10.1186/2044-5040-3-20

93. Kawahara, G., & Kunkel, L. M. (2013). Zebrafish based small molecule screens for novel DMD drugs. *Drug discovery today. Technologies*, *10*(1), e91–e96. https://doi.org/10.1016/j.ddtec.2012.03.001

94. Guyon, J. R., Mosley, A. N., Zhou, Y., O'Brien, K. F., Sheng, X., Chiang, K., Davidson, A. J., Volinski, J. M., Zon, L. I., & Kunkel, L. M. (2003). The dystrophin associated protein complex in zebrafish. *Human molecular genetics*, *12*(6), 601–615.

95. Guyon, J. R., Goswami, J., Jun, S. J., Thorne, M., Howell, M., Pusack, T., Kawahara, G., Steffen, L. S., Galdzicki, M., & Kunkel, L. M. (2009). Genetic isolation and characterization of a splicing mutant of zebrafish dystrophin. *Human molecular genetics*, *18*(1), 202–211. https://doi.org/10.1093/hmg/ddn337

96. Berger, J., Berger, S., Jacoby, A. S., Wilton, S. D., & Currie, P. D. (2011). Evaluation of exon-skipping strategies for Duchenne muscular dystrophy utilizing dystrophin-deficient zebrafish. *Journal of cellular and molecular medicine*, *15*(12), 2643–2651. https://doi.org/10.1111/j.1582-4934.2011.01260.x

97. Bassett, D. I., Bryson-Richardson, R. J., Daggett, D. F., Gautier, P., Keenan, D. G., & Currie, P. D. (2003). Dystrophin is required for the formation of stable muscle attachments in the

zebrafish embryo. *Development (Cambridge, England)*, *130*(23), 5851–5860. https://doi.org/10.1242/dev.00799

98. Li, M., Andersson-Lendahl, M., Sejersen, T., & Arner, A. (2014). Muscle dysfunction and structural defects of dystrophin-null sapje mutant zebrafish larvae are rescued by ataluren treatment. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *28*(4), 1593–1599. https://doi.org/10.1096/fj.13-240044
99. Widrick, J. J., Alexander, M. S., Sanchez, B., Gibbs, D. E., Kawahara, G., Beggs, A. H., & Kunkel, L. M. (2016). Muscle dysfunction in a zebrafish model of Duchenne muscular dystrophy. *Physiological genomics*, *48*(11), 850–860. https://doi.org/10.1152/physiolgenomics.00088.2016
100. Nagabhushana, A., & Mishra, R. K. (2016). Finding clues to the riddle of sex determination in zebrafish. *Journal of biosciences*, *41*(1), 145–155. https://doi.org/10.1007/s12038-016-9593-1
101. Berger, J., Sztal, T., & Currie, P. D. (2012). Quantification of birefringence readily measures the level of muscle damage in zebrafish. *Biochemical and biophysical research communications*, *423*(4), 785–788. https://doi.org/10.1016/j.bbrc.2012.06.040
102. Smith, L. L., Beggs, A. H., & Gupta, V. A. (2013). Analysis of skeletal muscle defects in larval zebrafish by birefringence and touch-evoke escape response assays. *Journal of visualized experiments : JoVE*, (82), e50925. https://doi.org/10.3791/50925
103. Kawahara, G., Karpf, J. A., Myers, J. A., Alexander, M. S., Guyon, J. R., & Kunkel, L. M. (2011). Drug screening in a zebrafish model of Duchenne muscular dystrophy. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(13), 5331–5336. https://doi.org/10.1073/pnas.1102116108
104. Kawahara, G., Gasperini, M. J., Myers, J. A., Widrick, J. J., Eran, A., Serafini, P. R., Alexander, M. S., Pletcher, M. T., Morris, C. A., & Kunkel, L. M. (2014). Dystrophic muscle improvement in zebrafish via increased heme oxygenase signaling. *Human molecular genetics*, *23*(7), 1869–1878. https://doi.org/10.1093/hmg/ddt579
105. Waugh, T. A., Horstick, E., Hur, J., Jackson, S. W., Davidson, A. E., Li, X., & Dowling, J. J. (2014). Fluoxetine prevents dystrophic changes in a zebrafish model of Duchenne muscular dystrophy. *Human molecular genetics*, *23*(17), 4651–4662. https://doi.org/10.1093/hmg/ddu185

106. Streisinger, G., Walker, C., Dower, N., Knauber, D., & Singer, F. (1981). Production of clones of homozygous diploid zebra fish (Brachydanio rerio). *Nature*, *291*(5813), 293–296. https://doi.org/10.1038/291293a0

107. Schier, A. F., & Talbot, W. S. (2005). Molecular genetics of axis formation in zebrafish. *Annual review of genetics*, *39*, 561–613. https://doi.org/10.1146/annurev.genet.37.110801.143752

108. Liu, J., & Stainier, D. Y. (2012). Zebrafish in the study of early cardiac development. *Circulation research*, *110*(6), 870–874. https://doi.org/10.1161/CIRCRESAHA.111.246504

109. Bakkers J. (2011). Zebrafish as a model to study cardiac development and human cardiac disease. *Cardiovascular research*, *91*(2), 279–288. https://doi.org/10.1093/cvr/cvr098

110. Jeong, J. Y., Kwon, H. B., Ahn, J. C., Kang, D., Kwon, S. H., Park, J. A., & Kim, K. W. (2008). Functional and developmental analysis of the blood-brain barrier in zebrafish. *Brain research bulletin*, *75*(5), 619–628. https://doi.org/10.1016/j.brainresbull.2007.10.043

111. Li, Z. H., Alex, D., Siu, S. O., Chu, I. K., Renn, J., Winkler, C., Lou, S., Tsui, S. K., Zhao, H. Y., Yan, W. R., Mahady, G. B., Li, G. H., Kwan, Y. W., Wang, Y. T., & Lee, S. M. (2011). Combined in vivo imaging and omics approaches reveal metabolism of icaritin and its glycosides in zebrafish larvae. *Molecular bioSystems*, *7*(7), 2128–2138. https://doi.org/10.1039/c1mb00001b

112. Jeong, J. Y., Kwon, H. B., Ahn, J. C., Kang, D., Kwon, S. H., Park, J. A., & Kim, K. W. (2008). Functional and developmental analysis of the blood-brain barrier in zebrafish. *Brain research bulletin*, *75*(5), 619–628. https://doi.org/10.1016/j.brainresbull.2007.10.043

113. Goldstone, J. V., McArthur, A. G., Kubota, A., Zanette, J., Parente, T., Jönsson, M. E., Nelson, D. R., & Stegeman, J. J. (2010). Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. *BMC genomics*, *11*, 643. https://doi.org/10.1186/1471-2164-11-643

114. Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., Koch, R., Rauch, G. J., White, S., Chow, W., … Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498–503. https://doi.org/10.1038/nature12111

115. Patten, S. A., Aggad, D., Martinez, J., Tremblay, E., Petrillo, J., Armstrong, G. A., La Fontaine, A., Maios, C., Liao, M., Ciura, S., Wen, X. Y., Rafuse, V., Ichida, J., Zinman, L., Julien, J. P., Kabashi, E., Robitaille, R., Korngut, L., Parker, J. A., & Drapeau, P. (2017). Neuroleptics as therapeutic compounds stabilizing neuromuscular transmission in amyotrophic lateral sclerosis. *JCI insight*, *2*(22), e97152. https://doi.org/10.1172/jci.insight.97152

116. MacRae, C. A., & Peterson, R. T. (2015). Zebrafish as tools for drug discovery. *Nature reviews. Drug discovery*, *14*(10), 721–731. https://doi.org/10.1038/nrd4627

117. Tiso, N., Moro, E., & Argenton, F. (2009). Zebrafish pancreas development. *Molecular and cellular endocrinology*, *312*(1-2), 24–30. https://doi.org/10.1016/j.mce.2009.04.018

118. Gut, P., Baeza-Raja, B., Andersson, O., Hasenkamp, L., Hsiao, J., Hesselson, D., Akassoglou, K., Verdin, E., Hirschey, M. D., & Stainier, D. Y. (2013). Whole-organism screening for gluconeogenesis identifies activators of fasting metabolism. *Nature chemical biology*, *9*(2), 97–104. https://doi.org/10.1038/nchembio.1136

119. Chi, N. C., Shaw, R. M., Jungblut, B., Huisken, J., Ferrer, T., Arnaout, R., Scott, I., Beis, D., Xiao, T., Baier, H., Jan, L. Y., Tristani-Firouzi, M., & Stainier, D. Y. (2008). Genetic and physiologic dissection of the vertebrate cardiac conduction system. *PLoS biology*, *6*(5), e109. https://doi.org/10.1371/journal.pbio.0060109

120. Milan, D. J., Jones, I. L., Ellinor, P. T., & MacRae, C. A. (2006). In vivo recording of adult zebrafish electrocardiogram and assessment of drug-induced QT prolongation. *American journal of physiology. Heart and circulatory physiology*, *291*(1), H269–H273. https://doi.org/10.1152/ajpheart.00960.2005

121. Milan, D. J., Peterson, T. A., Ruskin, J. N., Peterson, R. T., & MacRae, C. A. (2003). Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. *Circulation*, *107*(10), 1355–1358. https://doi.org/10.1161/01.cir.0000061912.88753.87

122. Schwerte, T., & Pelster, B. (2000). Digital motion analysis as a tool for analysing the shape and performance of the circulatory system in transparent animals. *The Journal of experimental biology*, *203*(Pt 11), 1659–1669. https://doi.org/10.1242/jeb.203.11.1659

123. Farber, S. A., Pack, M., Ho, S. Y., Johnson, I. D., Wagner, D. S., Dosch, R., Mullins, M. C., Hendrickson, H. S., Hendrickson, E. K., & Halpern, M. E. (2001). Genetic analysis of

digestive physiology using fluorescent phospholipid reporters. *Science (New York, N.Y.)*, *292*(5520), 1385–1388. https://doi.org/10.1126/science.1060418

124. Popovic, M., Zaja, R., Fent, K., & Smital, T. (2014). Interaction of environmental contaminants with zebrafish organic anion transporting polypeptide, Oatp1d1 (Slco1d1). *Toxicology and applied pharmacology*, *280*(1), 149–158. https://doi.org/10.1016/j.taap.2014.07.015
125. Park, H., Ha, J., & Park, S. B. (2019). Label-free target identification in drug discovery via phenotypic screening. *Current opinion in chemical biology*, *50*, 66–72. https://doi.org/10.1016/j.cbpa.2019.02.006
126. Eder, J., & Herrling, P. L. (2016). Trends in Modern Drug Discovery. *Handbook of experimental pharmacology*, *232*, 3–22. https://doi.org/10.1007/164_2015_20
127. Overington, J. P., Al-Lazikani, B., & Hopkins, A. L. (2006). How many drug targets are there?. *Nature reviews. Drug discovery*, *5*(12), 993–996. https://doi.org/10.1038/nrd2199
128. Swinney D. C. (2013). Phenotypic vs. target-based drug discovery for first-in-class medicines. *Clinical pharmacology and therapeutics*, *93*(4), 299–301. https://doi.org/10.1038/clpt.2012.236
129. Eder, J., Sedrani, R., & Wiesmann, C. (2014). The discovery of first-in-class drugs: origins and evolution. *Nature reviews. Drug discovery*, *13*(8), 577–587. https://doi.org/10.1038/nrd4336
130. Eder, J., & Herrling, P. L. (2016). Trends in Modern Drug Discovery. *Handbook of experimental pharmacology*, *232*, 3–22. https://doi.org/10.1007/164_2015_20
131. Park, H., Ha, J., & Park, S. B. (2019). Label-free target identification in drug discovery via phenotypic screening. *Current opinion in chemical biology*, *50*, 66–72. https://doi.org/10.1016/j.cbpa.2019.02.006
132. Eder, J., Sedrani, R., & Wiesmann, C. (2014). The discovery of first-in-class drugs: origins and evolution. *Nature reviews. Drug discovery*, *13*(8), 577–587. https://doi.org/10.1038/nrd4336
133. Swinney, D. C., & Lee, J. A. (2020). Recent advances in phenotypic drug discovery. *F1000Research*, *9*, F1000 Faculty Rev-944. https://doi.org/10.12688/f1000research.25813.1

134. Eder, J., Sedrani, R., & Wiesmann, C. (2014). The discovery of first-in-class drugs: origins and evolution. *Nature reviews. Drug discovery*, *13*(8), 577–587. https://doi.org/10.1038/nrd4336

135. Haasen, D., Schopfer, U., Antczak, C., Guy, C., Fuchs, F., & Selzer, P. (2017). How Phenotypic Screening Influenced Drug Discovery: Lessons from Five Years of Practice. *Assay and drug development technologies*, *15*(6), 239–246. https://doi.org/10.1089/adt.2017.796

136. Campagne, S., Boigner, S., Rüdisser, S., Moursy, A., Gillioz, L., Knörlein, A., Hall, J., Ratni, H., Cléry, A., & Allain, F. H. (2019). Structural basis of a small molecule targeting RNA for a specific splicing correction. *Nature chemical biology*, *15*(12), 1191–1198. https://doi.org/10.1038/s41589-019-0384-5

137. Kovarik, J. M., Schmouder, R. L., & Slade, A. J. (2004). Overview of FTY720 clinical pharmacokinetics and pharmacology. *Therapeutic drug monitoring*, *26*(6), 585–587. https://doi.org/10.1097/00007691-200412000-00001

138. Mavris, M., & Le Cam, Y. (2012). Involvement of patient organisations in research and development of orphan drugs for rare diseases in europe. *Molecular syndromology*, *3*(5), 237–243. https://doi.org/10.1159/000342758

139. Prinz, F., Schlange, T., & Asadullah, K. (2011). Believe it or not: how much can we rely on published data on potential drug targets?. *Nature reviews. Drug discovery*, *10*(9), 712. https://doi.org/10.1038/nrd3439-c1

140. Hay, M., Thomas, D. W., Craighead, J. L., Economides, C., & Rosenthal, J. (2014). Clinical development success rates for investigational drugs. *Nature biotechnology*, *32*(1), 40–51. https://doi.org/10.1038/nbt.2786

141. Vilella, A. J., Severin, J., Ureta-Vidal, A., Heng, L., Durbin, R., & Birney, E. (2009). EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. *Genome research*, *19*(2), 327–335. https://doi.org/10.1101/gr.073585.107

142. Patten, S. A., Aggad, D., Martinez, J., Tremblay, E., Petrillo, J., Armstrong, G. A., La Fontaine, A., Maios, C., Liao, M., Ciura, S., Wen, X. Y., Rafuse, V., Ichida, J., Zinman, L., Julien, J. P., Kabashi, E., Robitaille, R., Korngut, L., Parker, J. A., & Drapeau, P. (2017). Neuroleptics as therapeutic compounds stabilizing neuromuscular transmission in

amyotrophic lateral sclerosis. *JCI insight*, *2*(22), e97152. https://doi.org/10.1172/jci.insight.97152

143. Liu, G., Zhang, Y., & Zhang, T. (2019). Computational approaches for effective CRISPR guide RNA design and evaluation. *Computational and structural biotechnology journal*, *18*, 35–44. https://doi.org/10.1016/j.csbj.2019.11.006
144. *Transcript: ENSDART00000170290.2 dmd-212*. (2022). Ensembl. https://www.neurologyindia.com/article.asp?issn=0028-3886;year=2008;volume=56;issue=3;spage=231;epage=235;aulast=Kakulas
145. Smith, L. L., Beggs, A. H., & Gupta, V. A. (2013). Analysis of skeletal muscle defects in larval zebrafish by birefringence and touch-evoke escape response assays. *Journal of visualized experiments : JoVE*, (82), e50925. https://doi.org/10.3791/50925
146. Blake R. A. (2007). Target validation in drug discovery. *Methods in molecular biology (Clifton, N.J.)*, *356*, 367–377. https://doi.org/10.1385/1-59745-217-3:367
147. Kawahara, G., Karpf, J. A., Myers, J. A., Alexander, M. S., Guyon, J. R., & Kunkel, L. M. (2011). Drug screening in a zebrafish model of Duchenne muscular dystrophy. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(13), 5331–5336. https://doi.org/10.1073/pnas.1102116108
148. Guyon, J. R., Goswami, J., Jun, S. J., Thorne, M., Howell, M., Pusack, T., Kawahara, G., Steffen, L. S., Galdzicki, M., & Kunkel, L. M. (2009). Genetic isolation and characterization of a splicing mutant of zebrafish dystrophin. *Human molecular genetics*, *18*(1), 202–211. https://doi.org/10.1093/hmg/ddn337
149. Bassett, D., & Currie, P. D. (2004). Identification of a zebrafish model of muscular dystrophy. *Clinical and experimental pharmacology & physiology*, *31*(8), 537–540. https://doi.org/10.1111/j.1440-1681.2004.04030.x
150. Kunkel, L. M., Bachrach, E., Bennett, R. R., Guyon, J., & Steffen, L. (2006). Diagnosis and cell-based therapy for Duchenne muscular dystrophy in humans, mice, and zebrafish. *Journal of human genetics*, *51*(5), 397–406. https://doi.org/10.1007/s10038-006-0374-9
151. Berger, J., Sztal, T., & Currie, P. D. (2012). Quantification of birefringence readily measures the level of muscle damage in zebrafish. *Biochemical and biophysical research communications*, *423*(4), 785–788. https://doi.org/10.1016/j.bbrc.2012.06.040

152. *Phalloidin Conjugates for Actin Staining*. (2022). ThermoFisher. https://www.thermofisher.com/ca/en/home/life-science/cell-analysis/cell-structure/cytoskeleton/phalloidin-and-phalloidin-conjugates-for-staining-actin.html
153. Berger, J., Berger, S., Hall, T. E., Lieschke, G. J., & Currie, P. D. (2010). Dystrophin-deficient zebrafish feature aspects of the Duchenne muscular dystrophy pathology. *Neuromuscular disorders : NMD*, *20*(12), 826–832. https://doi.org/10.1016/j.nmd.2010.08.004
154. Mathias, J. R., Saxena, M. T., & Mumm, J. S. (2012). Advances in zebrafish chemical screening technologies. *Future medicinal chemistry*, *4*(14), 1811–1822. https://doi.org/10.4155/fmc.12.115
155. Johnson, N. M., Farr, G. H., 3rd, & Maves, L. (2013). The HDAC Inhibitor TSA Ameliorates a Zebrafish Model of Duchenne Muscular Dystrophy. *PLoS currents*, *5*, ecurrents.md.8273cf41db10e2d15dd3ab827cb4b027. https://doi.org/10.1371/currents.md.8273cf41db10e2d15dd3ab827cb4b027
156. Gibbs, E. M., Horstick, E. J., & Dowling, J. J. (2013). Swimming into prominence: the zebrafish as a valuable tool for studying human myopathies and muscular dystrophies. *The FEBS journal*, *280*(17), 4187–4197. https://doi.org/10.1111/febs.12412
157. Bassett, D. I., & Currie, P. D. (2003). The zebrafish as a model for muscular dystrophy and congenital myopathy. *Human molecular genetics*, *12 Spec No 2*, R265–R270. https://doi.org/10.1093/hmg/ddg279
158. Widrick, J. J., Kawahara, G., Alexander, M. S., Beggs, A. H., & Kunkel, L. M. (2019). Discovery of Novel Therapeutics for Muscular Dystrophies using Zebrafish Phenotypic Screens. *Journal of neuromuscular diseases*, *6*(3), 271–287. https://doi.org/10.3233/JND-190389