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Caenorhabditis elegans as a chemical screening tool for the study of neuromuscular disorders. Manual and semi-automated methods

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ABSTRACT

We previously reported the use of the cheap and fast-growing nematode *Caenorhabditis elegans* to search for molecules, which reduce muscle degeneration in a model for Duchenne Muscular Dystrophy (DMD). We showed that Prednisone, a steroid that is generally prescribed as a palliative treatment to DMD patients, also reduced muscle degeneration in the *C. elegans* DMD model. We further showed that this strategy could lead to the discovery of new and unsuspected small molecules, which have been further validated in a mammalian model of DMD, i.e. the *mdx* mouse model. These proof-of-principles demonstrate that *C. elegans* can serve as a screening tool to search for drugs against neuromuscular disorders. Here, we report and discuss two methodologies used to screen chemical libraries for drugs against muscle disorders in *C. elegans*. We first describe a manual method used to find drugs against DMD. We further present a semi-automated method, which is currently in use for the search of drugs against the Schwartz–Jampel Syndrome (SJS). Both assays are simple to implement and can be readily transposed and/or adapted to screens against other muscle/neuromuscular diseases, which can be modeled in the worm.

Finally we discuss, with respect to our experience and knowledge, the different parameters that have to be taken into account before choosing one or the other method.

1. Introduction

Duchenne Muscular Dystrophy (DMD) or Schwartz–Jampel Syndrome (SJS) are rare inherited neuromuscular disorders. Despite the identification of the genes responsible of these diseases [1,2], their physiopathology is still poorly understood, thus hindering the development of pharmacological therapies.

The identification of chemical molecules beneficial to patients suffering from rare inherited diseases requires efficient screening strategies. The setups of traditional pharmacological *in vitro* screening systems are usually based on the binding or the action of drugs on specific target proteins [3]. Since for most rare diseases the mechanisms that lead to their establishment are unknown, it is difficult to target relevant proteins or pathways. Moreover, muscle diseases usually need the complexity of a whole organism and movement to be initiated; therefore the development of relevant high content cell culture screening systems is mostly impossible. Finally, murine or other mammalian models, which are now available for most inherited muscle diseases [4,5], are not well suited to large-scale experiments like chemical screening because of their long breeding time and high costs.

A promising alternative to traditional *in vitro* and cellular systems is to use small model organisms like the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster* or the zebrafish *Danio rerio*, which allow medium to high throughput screening of thousands of molecules at a low cost [6]. Such models can be used as first pass filters to identify molecules that can be further tested in mammalian models.

C. elegans, in particular, has many advantages with respect to the investigation of inherited neuromuscular diseases. More than 50% of human genes have counterparts in the *C. elegans* genome, among them many genes responsible for human genetic diseases [7]. In addition to this high conservation of genes, signaling pathways are in general well conserved and some of the *C. elegans* organs, most notably muscles, have a cellular physiology similar to that of vertebrates. *C. elegans* has striated and non-striated muscles. Non-striated muscles include pharyngeal, intestinal, uterine, vulval and anal muscles, while the body wall muscles are striated (Fig. 1A). Body-wall muscles are required for the movement of the worm; they are distributed in four longitudinal bands, named quadrants that run from head to tail. Each quadrant is formed by a single layer of diamond shaped muscle cells. The overall structure, composition and physiology of these striated muscle cells

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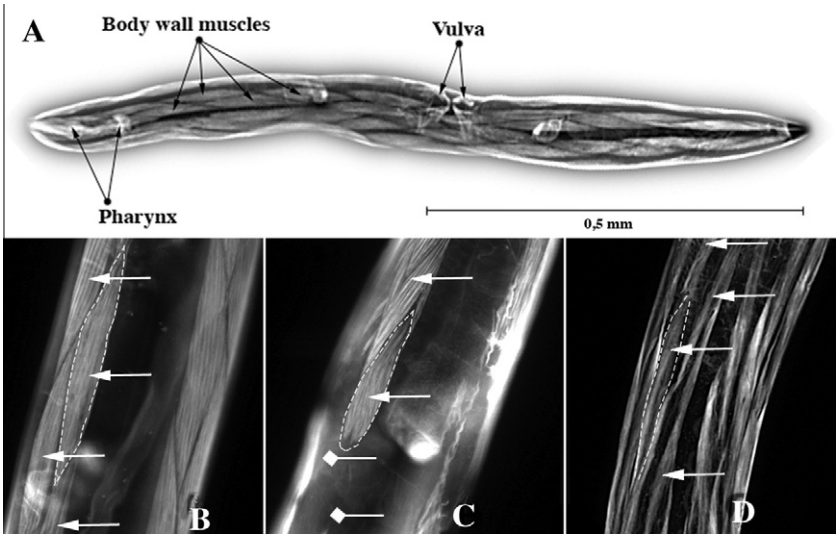

Fig. 1. Muscle cells in *C. elegans*. Images of *C. elegans* muscle cells after a phalloidin–rhodamine staining. A: Whole animal image. *C. elegans* has striated and non-striated muscles. Pharyngeal, vulval and anal muscle cells (not shown) are non-striated, while the body-wall muscle cells are striated. Body-wall muscle cells are distributed in four longitudinal bands, called quadrants that run from head to tail. Each quadrant is formed by a single layer of diamond shaped mononucleated cells. B–D: Body-wall muscle cells in wild type and *C. elegans* mutants. Muscle cells are indicated by arrows and delimited by a disrupted line. Absent cells are indicated by diamond arrows (C). In comparison to wild type muscle cells (B), muscle cells from the SJS model (D) are thinner but do not disappear as in the DMD model (C).

are close to vertebrate skeletal muscles and especially sarcomeric components are well conserved during evolution [8]. The major differences of *C. elegans* striated muscles with respect to vertebrate striated muscles are that muscle cells do not fuse and remain mono-nucleated and that *C. elegans* lacks regenerative processes.

Finally, the small size, the short life cycle and the simple and low-cost growth conditions of *Caenorhabditis elegans* allow for large scale studies such as chemical screening [6]. Indeed, *C. elegans* can be grown in multi-well plates and specific automated pipetting systems can be used at all developmental stages [6,9]. Moreover, combined with fluorescent markers, the optical transparency of the worm allows for the detection of functional and morphological abnormalities or changes in living worms. Several systems already exist to record *in vivo* fluorescence at a cellular or sub-cellular level [6].

Here, we present and discuss two screening methods used to search for small molecules against muscle disorders in *C. elegans*. First, we describe a method we used to find beneficial drugs against muscle degeneration in a *C. elegans* DMD model. This method is fully manual but really easy and cheap to set up and to perform. Secondly, we present a semi-automated experiment, which is currently in use for the screen of drugs beneficial to a *C. elegans* model of SJS. Both methods are simple to implement and can be readily transposed and/or adapted to screens for molecules on other muscular/neuromuscular diseases modeled in *C. elegans*. Depending on the phenotype to observe and the available equipment, a wide variety of readouts can be easily integrated into these procedures, such as automated imaging and automated locomotion measurements [6]. Finally we discuss the advantages and limits of each of these methods with respect to our experience.

1.1. General screening strategy

The screening strategy for the screens we have performed includes the following steps:

- Development of a pertinent *C. elegans* model,
- Set up of culture conditions and readouts sufficiently robust and in accordance with a large-scale screening campaign (time, workload),
- Screening in duplicates,
- Optional: secondary screening to confirm the first pass hits,
- Validation of hits.

The hit validation step consists in confirming the results of the screening step by reproducing the experiment with a different readout, usually a more direct and more detailed observation.

1.2. Diseases and models background information

1.2.1. Duchenne Muscular Dystrophy

Duchenne Muscular Dystrophy is a muscle wasting disease caused by the absence of dystrophin. Its physiopathology is still a matter of debate. Currently, the only pharmacological treatment proposed to DMD patients is Prednisone, a steroid, which slightly slows down muscle degeneration [10].

In order to develop more efficient pharmacological treatments, chemical screens on an appropriate model are needed. Different mammalian models of DMD exist, most notably the *mdx* mouse and the GRMD dog [11,12]. However, as mentioned above, mammalian models are not suitable for large-scale experiments like chemical screening. Muscle cell cultures are not suitable either because they do not recapitulate the muscle degeneration phenotype of DMD.

Several years ago our group identified a mutation in the *C. elegans* homolog of the dystrophin gene: *dys-1*(*cx18*), which leads to a phenotype of hyperactivity and slight muscle degeneration [13,14]. Muscle degeneration could be increased by combining the *dys-1*(*cx18*) mutation with a thermo-sensitive mutation in the *hlh-1* gene, the homolog of the myogenic factor MyoD. The *dys-1*(*cx18*); *hlh-1*(*cc561ts*) double mutants (strain LS587) become paralyzed in a time-and activity-dependant manner, due to progressive muscle degeneration resulting in muscle cells loss (Fig. 1C) [14]. It will be called here after *C. elegans* DMD model because it mimics the muscle wasting seen in DMD patients.

In a previous study, we reported that Prednisone, which is generally prescribed as palliative treatment to DMD patients, reduced muscle degeneration in *C. elegans* [15]. This was the first proof-of-principle that *C. elegans* can serve as a chemical screening tool to find candidate molecules against muscle disorders. We further showed that this strategy could lead to the discovery of unsuspected small molecules able to reduce muscle degeneration, which

we have further validated in mice [16,17]. This was the second evidence that a *C. elegans* model of a neuromuscular disease can be used as a pertinent drug-screening tool.

We describe below how we used the *C. elegans* DMD model to search for molecules able to reduce muscle degeneration.

1.2.2. Schwartz–Jampel Syndrome

The Schwartz–Jampel Syndrome is a very rare genetic disease, mostly characterized by permanent muscle stiffness (myotonia) and abnormal endochondral ossification (for review see Ref. [1]). In 2000, Nicole et al. showed that it was caused by mutations in the gene encoding perlecan, also named HSPG2 for heparan sulfate proteoglycan 2, a major protein of basement membranes [18]. Currently, the therapy for SJS consists mainly in surgical interventions to correct bones and cartilages abnormalities and physiotherapy to alleviate myotonia. A murine model of SJS was described by Stum et al. [4]. While the murine model is of great use for the study of the physiopathology of this disease, it is not suitable for pharmacological screenings.

C. elegans has a homolog of perlecan: UNC-52 [19]. UNC-52 is localized at the basement membrane adjacent to the body wall muscle cells, with an increased concentration at the bases of dense bodies (the Z-lines and costameres analogs) and M-lines. Two major classes of *unc-52* mutants have been described depending on the mutant allele [20]. Class 1 mutant alleles lead to the synthesis of a reduced quantity of nearly normal UNC-52 (as observed in patients), while class 2 alleles abolish the expression of UNC-52. Consequently, class 2 mutants show a more severe phenotype than class 1 mutants. Class 2 mutants exhibit a strong disorganization of the myofilament lattice and die at the twofold stage, while class 1 mutants show less severe sarcomere disorganization with fractured dense bodies and become paralyzed at adult stage. Unlike class 2 mutants, class 1 worms can give a viable offspring.

We describe below how we generated a *C. elegans* SJS model, which enabled us to search for molecules able to reduce the muscular phenotype.

1.3. Chemical libraries

The chemical libraries used for the screens were provided by Prestwick Chemical Inc. and the French National Chemical Library.

Molecules from the French National Chemical Library were provided in 96-well plates, at a concentration of 10 mM in dimethylsulfoxyde (DMSO) as solvent. Ninety six-well plates are composed of twelve columns and eight lines. The molecules were distributed in columns 2–11 (80 molecules per plate). Columns 1 and 12 were kept free for positive and negative controls.

Molecules from Prestwick Chemical Inc. were provided as powders. These molecules were solubilized in either water or DMSO. We decided to use only these two different solvents (although we could have used more than five different solvents) in order to limit the number of controls needed. Indeed, solvents other than water may have an effect on the growth rate of the worms, which can be difficult to handle. Furthermore, multiplying the solvents multiplies the risks for the manipulator. As most molecules are soluble in DMSO; we mainly used this solvent. We used water only when the solubility in water was known to be higher than in DMSO. All molecules were solubilized at the highest possible concentration. These saturated stock solutions were distributed in 96-well plates in columns 2–11 as for the liquid libraries.

Plates from either libraries were sealed (note that heat sealing is not recommended) and frozen at −20 °C, in airtight plastic bags to limit condensation. Before freezing we split the libraries in several aliquots sufficient for three experiments, in order to limit the number of thawing-freezing cycles.

1.4. Muscle cell markers and readouts

C. elegans has an invariant development leading to a defined number of somatic cells at the adult stage. Notably, each animal develops 95 body wall muscle cells, which do not fuse and cannot be regenerated. These muscle cells can be observed after a phalloidin–rhodamine staining that highlights actin filaments (Fig. 1A and B). Thus, muscle defects can be quantified by counting the number of abnormal muscle cells under a microscope and molecules that lower the number of affected muscle cells can be identified.

However, phalloidin–rhodamine staining is an expensive and time-consuming step. In order to increase our screening capacity, we searched for a fluorescent marker of muscle cells that could be easily observed *in vivo* and that could serve to evaluate the degree of muscle defects. For this purpose, we used the PD4251 strain, which carries the *ccIs4251* transgene [21]. This transgene drives the expression of the green fluorescent protein (GFP) in body wall muscle cells, where GFP localizes in nuclei and mitochondria. We introduced the *ccIs4251* transgene in the disease models used for the two screens described below. Since *C. elegans* is transparent during all its life cycle, muscle wasting/abnormality in these strains can be then evaluated *in vivo* under a fluorescent binocular microscope by observing the fluorescent dots (Fig. 2). We chose to work with GFP localized in nuclei rather than in the cytosol since the observation of a fluorescent nuclei or its disappearance is easily scored by both manual observation and automatic readout. Fig. 2 shows the fluorescent peaks generated by GFP labelled nuclei.

For each model, we have set two main readouts, which are further described in each corresponding chapter. For some of the readouts that were more qualitative than quantitative (like the onset of paralysis), we have set a semi-quantitative scale to be able to normalize them and to apply statistical tests. Molecules were tested in duplicates. Only the molecules showing significant effects in both duplicates were considered as hits. Molecules causing lethality or gross defects (mostly growth retardation) were retested at lower concentrations.

Depending on what type of screening method was adopted and which readout type was used (manual counting *vs.* automated analysis), different statistical tests were applied. They will be detailed further in the corresponding paragraphs below.

2. Manual screening on a *C. elegans* DMD model

2.1. The C. elegans DMD model and screening assay setup

The *C. elegans* LS587 *dys-1*(*cx18*); *hlh-1*(*cc561*) double mutant displays a progressive paralysis due to progressive muscle degeneration resulting in the absence of muscle cells. The fraction of absent muscle cells increases with time to reach 20–30% of absent muscle cells at adulthood [14]. We used standard genetic methods to introduce the *ccIs4251* transgene in different genetic contexts: the *dys-1*(*cx18*); *hlh-1*(*cc561*) double mutant and the *dys-1*(*cx18*) single mutant. The resulting strains were named respectively LS761 and LS541, both express GFP with a nuclear and mitochondrial localization in all body wall muscle cells. With respect to other parameters, LS761 worms and LS541 worms have the same phenotypes than the non transgenic strains from which they are derived.

This allowed us to assess the degree of muscle degeneration in LS761 worms by counting the number of absent muscle cell nuclei. Fluorescent muscle cell nuclei are clearly visible under a fluorescent binocular microscope (Fig. 2). Twenty worms per tested molecule were scored on a semi quantitative scale from A to C; A stood for no absent muscle cell nuclei; B: from 1 to 10 absent muscle cell nuclei; C from 10 to 30 absent muscle cell nuclei (the equivalent of non treated LS761 worms). As LS761 worms become paralyzed at adulthood we also assessed the locomotion of treated worms on

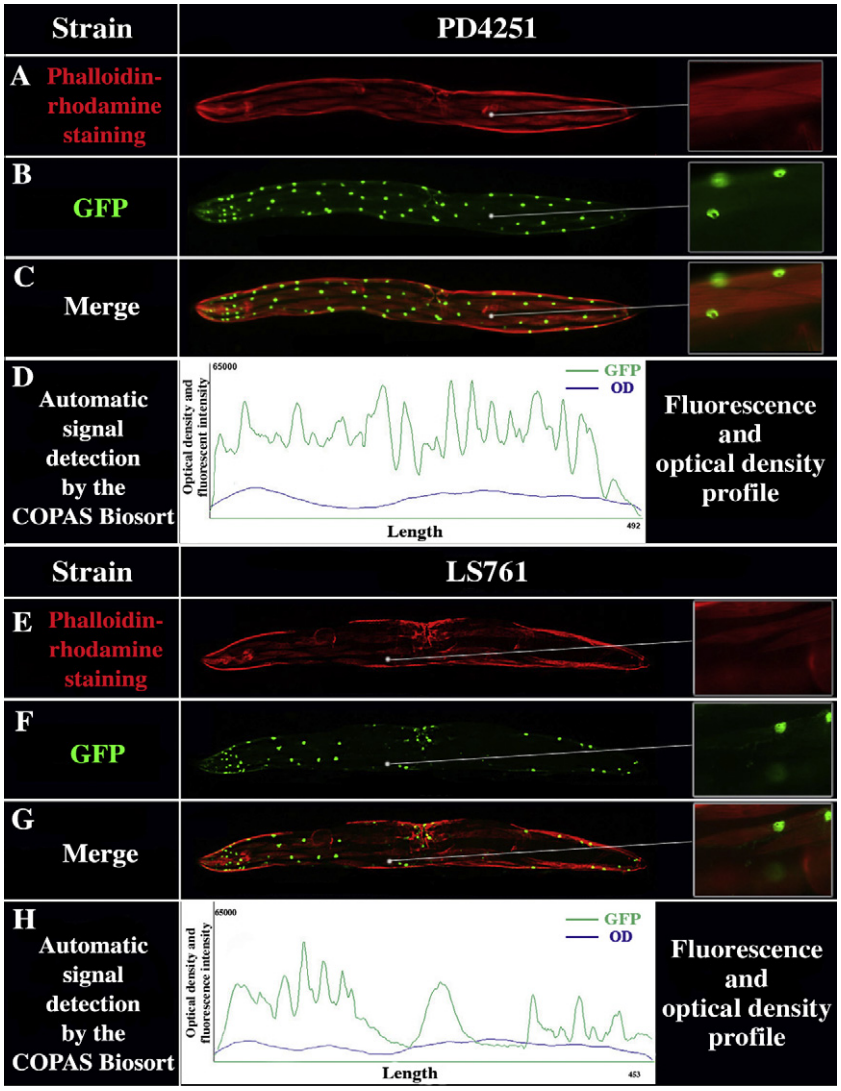

Fig. 2. Comparison of PD4251 worms and LS761 worms. LS761 worms are the *C. elegans* model of DMD. They carry the mutations *dys-1(cx18); hlh-1(cc561)* as well as the *ccls4251* transgene, which drives the expression of GFP in muscle cells. GFP localizes in nuclei and mitochondria of muscle cells. PD4251 worms carry the *ccls4251* transgene alone. Muscle cells are equally distributed all along the worm in PD4251 worms as seen by the phalloidin–rhodamine staining (A). The GFP signal from muscle cells nuclei is clearly visible (B). As muscle cells are mononucleated in *C. elegans*, one GFP dot represents one muscle cell (C). Analysis by the COPAS Biosort shows a succession of fluorescent peaks that correspond to muscle cell nuclei (D). In comparison, one can observe the absence of some muscle cells in LS761 worms on the phalloidin–rhodamine staining (E) as well as on the GFP signal image (F) or in the merge (G). The COPAS Biosort analysis shows a decrease in the number of fluorescent peaks where muscle cells are absent (H).

a qualitative scale. This qualitative scale ran from 0 to 2, 0 for a locomotion similar to that of LS761 worms, 2 for a locomotion similar to that of wild type worms and 1 for an intermediate locomotion, i.e. worms moving better than LS761 worms but still uncoordinated. The LS541 strain was used for further validation of the molecules (see Section 2.3.2).

Concerning the culture conditions, one main difference to the semi-automated method described below is that this screen was performed on solid medium because in liquid medium the *C. elegans* DMD model presents only with a weak muscle degeneration phenotype. This may be due to a reduction of muscle stress/effort in liquid medium. Performing the screening campaign on solid medium ensured to produce strong muscle degeneration in untreated animals and thus enabled us to detect even slight differences in the degree of muscle degeneration of treated animals.

2.2. Experimental screening procedure

All the steps were standardized to ensure the maximum reproducibility over the whole screening campaign. Appendix A provides the details of all the medias, reagents and equipments necessary to perform this type of screen.

2.2.1. Before screening campaigns

This step consists in preparing as much NGM, food, positive control solution and solvents as needed for one entire screening campaign.

The screen was performed in 24-well plates, each well was filled with 1 mL of Nematode Growth Medium (NGM) containing a molecule and coated with saturated OP50 bacteria cultures. The 96-well plates containing the chemicals were divided into four 24-well plates. All molecules were tested in duplicates so that each chemical plate is screened in a total of eight 24-well screening plates. Negative and positive controls were included in each plate, solvent alone as negative control and Methazolamide (0.44 mg/mL of NGM) as positive control. Methazolamide is the most active drug (more active than Prednisone), which was identified during a preliminary assay [16]. For this screen a liquid library was used where all the molecules were solubilized in DMSO at a concentration of 10 mM. In our experiments, *C. elegans* tolerated DMSO up to a final

concentration of 1% (vol/vol). Consequently, molecules were diluted 100 times, which led to a final concentration of molecules of 100 μM in the medium.

2.2.1.1. Food and media preparation/storage. For each 96-well chemical plate:

(1) Prepare 200 mL of NGM and store at 4 °C (enough for eight 24-well plates, each well filled with 1 mL of NGM).
(2) Inoculate 400 mL of LB medium with an isolated OP50 colony and incubate at 37 °C under agitation until saturation (15–20 h).
(3) Centrifuge the culture 15 min at 5000*g* and discard the supernatant. Store each OP50 pellet obtained from a 400 mL culture at −20 °C.

▲ CRITICAL STEP *Bacteria are frozen, and then considered as dead, to avoid any drug metabolization and by-products generation during the screening experiment.*

(4) Prepare 170 μL solution of methazolamide (44 mg/mL) dissolved in DMSO (positive control) and of solvent only (negative control). Store at −20 °C.

▲ CRITICAL STEP *Positive and negative controls should be placed in each 24-well screening plate to determine inter-plate variation and to normalize the data obtained at the last step.*

2.2.2. Screening

2.2.2.1. Overview. As muscle degeneration is a time-dependent process in the *C. elegans* DMD model, we had to synchronize the experiments to be able to observe age-matched worms. For this purpose, eggs were prepared from pre-synchronized cultures of the *C. elegans* LS761 *ccIs4251 dys-1*(*cx18*); *hlh-1*(*cc561*) strain (approximately 50 eggs per well).

After 8 days at 15 °C, adult worms were then directly observed under a fluorescent binocular microscope to assess their degree of muscle degeneration. Locomotion behaviors and fluorescent profiles of treated and untreated populations were evaluated. Animals that showed in both duplicates a greater number of fluorescent nuclei and/or an amelioration in locomotion rates compared to negative controls were collected along with their respective positive and negative controls. The samples were then fixed in formaldehyde and subsequently stained with phalloidin–rhodamine to be processed to the hit validation step.

All molecules that produced deleterious effects (growth retardation, lethality, morphological defects) at the initial concentration (100 μM) were retested at several lower concentrations.

2.2.2.2. Detailed protocol. The DMD screening protocol is schematized in the Supplemental Fig. 1.

Day 0 (animals amplification).

(1) For 8× 24-well screening plates, prepare 2 amplification plates by putting five LS761 worms (F0) on each plate (60 mm NGM Petri dish seeded with a thin layer of OP50 *Escherichia coli*). Incubate the plates overnight at 15 °C.

▲ CRITICAL STEP *The hlh-1(cc561) allele is a thermo-sensitive mutation. Worms carrying this mutation were always grown at the permissive temperature of 15 °C.*

Day 1 (synchronization).

(2) Withdraw the F0 LS761 worms from amplification plates. Incubate the plates at 15 °C until day 6, step11.

Day 6 (assays starting point). For 8× 24-well plates (to screen one 96-well chemical plate in duplicate):

(3) Melt 200 mL of NGM (in a microwave oven) and let it cool 2 h in a 60 °C bath.
(4) Label all 24-well screening plates and corresponding data sheets.
(5) Dispense, in duplicate, 10 μL of each drugs from a 96-well chemical plate into 4× 24-well screening plates.
(6) Add 10 μL of DMSO (or solvents used to dissolve the chemicals) in wells A1 and B1 of plate 1 and 2 and in wells A6 and B6 of plate 3 and 4 (negative controls).
(7) Add 10 μL of 44 mg/mL methazolamide stock solution in wells C1 and D1 of plate 1 and 2 and in wells C6 and D6 of plate 3 and 4 (positive controls).
(8) Dispense 1 mL of 60 °C cooled NGM in each well.
(9) Seal the plates with aluminum sealing films and vortex them 10 s with a Vortex Genie 2 on position 6. Let the NGM set for 15 min.
(10) Re-suspend a "400 ml-OP50-pellet" with 10 mL of M9. Dispense 50 μL of this 40X-OP50 solution in each well and let it dry 1 h under a horizontal flow biological hood.

▲ CRITICAL STEP *Ensure that the OP50 layer is dry enough (OP50 should stick on NGM).*

(11) Collect animals and eggs from amplification plates prepared on day 0 using 1 mL of M9 buffer.

▲ CRITICAL STEP *Ensure that no L1 larvae are visible on the plate. If L1 larvae are detectable in the F1 progeny, reduce the time of amplification (6–5 days).*

(12) Use a 37 μm mesh filter to collect the eggs in the filtered solution.
(13) Dispense 10 μL of the eggs solution in each well of the screening plates (approximately 50 eggs).

▲ CRITICAL STEP *Before adding the eggs, drop 3× 10 μL of this solution on a glass slide to evaluate the quality of filtration and eggs concentration (At least, 40–60 eggs per drop are required). If the concentration is too high, adjust the volume with M9 buffer (too many worms will deplete the food before the readout measurement). If the concentration is too low, centrifuged the eggs solution at 2000g for 2 min, remove an appropriate volume of supernatant to obtain the desired concentration.*

(14) Incubate the 24-well plates at 15 °C until day 14.

Day 14 (readout and hit selection).

(15) Observe locomotion and fluorescent patterns of adult animals under a fluorescent microscope (Stereo Lumar V12) equipped with a Lumar filter set 38 GFP.
(16) Annotate the data sheet.

■ INFORMATION *With this manual readout, additional phenotypes may be recorded, as developmental or behavioral phenotypes and reproduction defects. These observations may be useful for the establishment of a comprehensive database linking phenotype to chemical structure.*

(17) Collect hits (if any) along with respective positive and negative controls using 1 mL of PBS 1× to collect animals, and dispense them into 1.5 mL low binding tubes.
(18) Fix each collected populations 30 min by adding 30 μl of a 37% formaldehyde solution.
(19) Centrifuge each tube 2 min at 5000*g* and discard the supernatant.
(20) Wash animals with 1 mL of PBS 1× and repeat step 5.

(21) Re-suspend animals in 50 μL of PBS 1× and keep each sample tube and the respective controls at 4 °C until the validation.

2.3. Data treatment and hit validation

The molecules selected after the screen were validated by counting the number of degenerated muscle cells per phalloidin–rhodamine stained animal under a microscope. Normalization to negative controls was used to remove systematic plate-to-plate variation, making measurements comparable between different plates. Muscle degeneration measurements after phalloidin staining were then compared to their respective negative control. Degeneration was expressed as a percentage on a scale where the respective negative control of each molecule is at 100% of muscle degeneration. Hits could then be compared to each other to select the most active molecules. Sample and control data were compared by a Student's t-test to assess the significance.

2.3.1. Hit validation protocol

■ INFORMATION *In order to save time, one may wait to have a large number of hits (with respective control samples) to analyze. In our experiments, we performed the validation step once we collect more than 20 hits based on locomotion and fluorescence observation.*

(1) Centrifuge the sample tubes and their corresponding positive and negative controls, 2 min, 5000*g*; discard the supernatant.
(2) Add 1 mL of acetone (pre-cooled at −20 °C) in each tube.
(3) Incubate the tubes 2 min in a −20 °C labtop cooler and mix them gently by inverting the tubes.

▲ CRITICAL STEP *Upon acetone addition, worms may start to agglomerate. This must be avoided. If any agglomeration is visible, vortex the tube 5–10 s with Vortex Genie 2, position 8.*

(4) Centrifuge the tubes 1 min at 5000*g*; discard the supernatant.
(5) Wash animals with 1 mL of PBS 1×, and repeat step 4.
(6) Add 500 μL of PBS 1×.
(7) Add 1 μL of FluoProbes 547H-Phalloidin (1 U) and mix the tube gently.
(8) Incubate 2 h at room temperature under agitation.
(9) Centrifuge the tubes 1 min at 5000*g*; discard the supernatant.
(10) Wash animals with 1 mL of PBS 1×.
(11) Centrifuge the tubes 1 min at 5000*g*, discard the supernatant and leave 20 μL in the tubes.
(12) Add 20 μL of Dako fluorescent mounting solution and mix gently.
(13) Prepare microscope slides with this preparation.
(14) Score the number of damaged/degenerated muscle cells per animals under a microscope (Microscope AxioImager Z1, 550/570 nm)

■ INFORMATION *Sealed slides may be conserved at 4 °C for several months once they are set.*

2.3.2. Further validation

A further validation step was required to confirm that hits are active on *dys-1*(*cx18*) single mutants as well. Indeed, the *C. elegans* DMD model was created by introducing the *dys-1*(*cx18*) mutation in the *hlh-1*(*cc561*) genetic context to amplify its muscular phenotype. Thus, selected molecules were further validated in the sole *dys-1*(*cx18*) genetic context (strain LS541). For this test the same data treatment procedure was applied.

Afterwards, using the LS587 *dys-1*(*cx18*); *hlh-1*(*cc561*) double mutant, molecules were retested at several doses in order to define a dose–response curve. We also determined the optimal dose of selected molecules, meaning the most active concentration on muscle degeneration without any detectable deleterious effect.

The specificity of this screen was that the screening step and validation step were run on the same sample. Depending on personal training, this method allows for the screen of 400–800 compounds per week by one person.

Note that the record of fluorescence with an automated reader system, like the COPAS Biosort (described below), is also possible in this procedure. In this case an additional step is required, which consists in the transfer of the animals from the solid to a liquid medium prior to analysis.

3. Semi-automatic screening on a *C. elegans* SJS model

3.1. The C. elegans SJS model and screening assay setup

We chose to work with class 1 mutants, which allow for viable progeny. We tested several class 1 alleles of *unc-52*, for which strains were available from the *Caenorhabditis* Genetics Center (CGC): CB444 (*e444*), CB669 (*e669*), CB998 (*e998*) and CB1421 (*e1421*). They all exhibited muscle abnormalities but to different extents. The CB444 strain (allele *e444*) displayed the most reproductive phenotype in preliminary assays. Worms of this strain showed a progressive paralysis starting from the L4 stage, which is concomitant to a progressive detachment of muscle cells from the basement membrane (Fig. 1D). At the adult stage, CB444 worms are completely paralyzed and straight; they occasionally move their head and/or tail.

In order to be able to visualize muscle cells *in vivo*, we introduced the aforementioned *ccIs4251* transgene in CB444 worms. It resulted in the LS1095 strain, which express GFP in all body wall muscle cells (Fig. 3). With respect to other parameters, LS1095 worms have the same phenotype than the CB444 worms.

Interestingly in LS1095 worms, GFP fluorescence of muscle cells decreases progressively as the muscle cells detached from the basement membrane. We used the COPAS Biosort to exploit this readout. The COPAS Biosort can register optical density (OD) and fluorescence (different channels) all along the worms. It allows for the sorting of worms according to predefined parameters such as length of the worms, global OD and fluorescence of the worms. This device is particularly useful and efficient to sort animals of interest in screening plates at the starting point of the screening procedure [9].

In the case of the SJS model, it was also used to analyze the effect of the molecules by assessing the global fluorescence of the worms. As showed in Fig. 3, the fluorescence profile of LS1095 worms is different from the PD4251 worms, the overall fluorescence of the worms (represented by the area under the fluorescence curve) is lower, while OD remains the same. The decrease in global fluorescence is correlated with the progression of paralysis (data not shown). We observed that head, vulva and tail muscle cells do not show a dramatic decrease of fluorescence (Fig. 3), which is in accordance with histological analysis reported in previous studies [22]. The treatment of fluorescence data is described in Section 3.2.3.

It is important to note that in order to be able to use the COPAS Biosort at the endpoint of the screening, it is essential to grow the worms in liquid medium in 96-well plates as the COPAS Biosort is able to aspirate the content of each well before analysis. The experimental procedure is described below.

Finally, we aimed to find a positive control, *e.g.* a molecule used in patients that show a beneficial effect on the *C. elegans* SJS model.

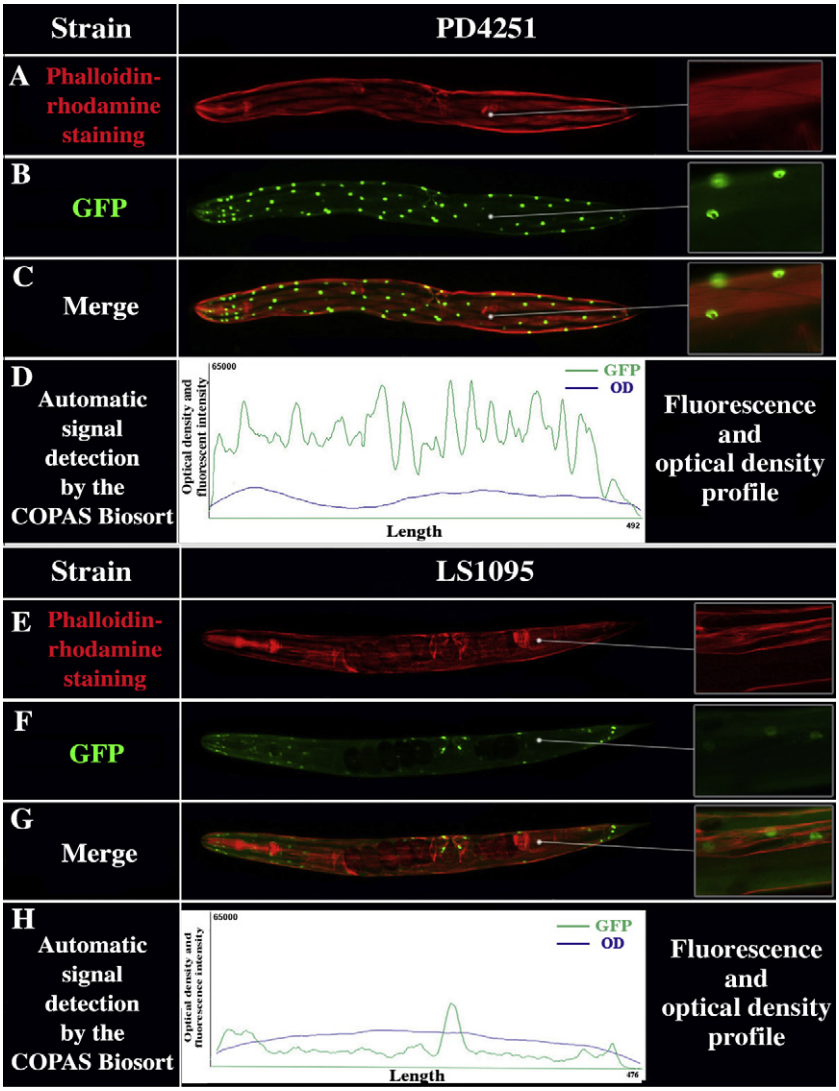

Fig. 3. Comparison of PD4251 worms and LS1095 worms. LS1095 worms are the *C. elegans* model of SJS. They carry the mutation *unc-52(e444)* as well as the *ccIs4251* transgene, which drives the expression of GFP in muscle cells. GFP localized in nuclei and mitochondria of muscle cells. PD4251 worms carry the *ccIs4251* transgene alone. Muscle cells are equally distributed all along the worm in PD4251 worms as seen by the rhodamine-phalloidin staining (A). The GFP signal from muscle cells nuclei is clearly visible (B). As muscle cells are mononucleated in *C. elegans*, one GFP dot represents one muscle cell (C). Analysis by the COPAS Biosort shows a succession of fluorescent peaks that correspond to muscle cell nuclei (D). In comparison, one can observe that phalloidin–rhodamine stained LS1095 worms have thinner muscle cells (E). The GFP signal is drastically decreased. Only vulval and anal muscle cells nuclei remain visible (F) and merge (G). The COPAS Biosort analysis shows a decrease of global fluorescence, while OD remains comparable to that of PD4251 worms (H).

Carbamazepin has been reported to improve myotonia in some SJS patients [23]. We tested its effect on the *C. elegans* SJS model. In our assays Carbamazepin failed to improve the behavioral, muscular or fluorescent phenotypes of CB444 and LS1095 worms (data not shown) at all concentration tested (1 nM–10 mM in the medium).

In the absence of a positive control, we could not be sure that the fluorescence was a good marker of the progression of the pathology, even though the decrease of fluorescence is tightly linked to the detachment of muscle cells from the basement membrane. Consequently, we decided to add a locomotion readout. The locomotion phenotype was measured along a semi-quantitative scale, where the score increased the mobility of the worms. Completely straight and paralyzed animals had a score of 0, those that were straight but could stir their head/tail had a score of 1, animals that were not straight but could not move had a score of 2, those slowly moving had a score of 3 and animals that moved normally had a score of 4. This parameter had to be measured on solid medium, as slight differences in movement or straightness are less visible in liquid. We used the PD4251 worms as positive control, both in terms of locomotion and of fluorescent profile. As negative control, LS1095 treated with solvent alone were included in each screening plate.

It is noteworthy that based on the sole fluorescence readout, this assay allowed to screen up to 2000 compounds per week with only one person. The major limitation was the throughput of the automatic readout performed by the COPAS Biosort. The additional readout on solid medium lowered the throughput of the assay to 400 molecules per week because of the time dedicated to the manipulation and observation.

3.2. Experimental procedures

Appendix B provides the details of all media, reagents and equipments necessary to perform this type of screen.

3.2.1. Before screening campaign

The molecules used for this screen were purchased as powders to allow us to test them at the highest concentration possible. We

solubilized the powders in either DMSO or water to obtain saturated stock solutions (as described in Section 2.1). Concentrations of these solutions showed an important variation between molecules, ranging from 2 to 900 mM. As described above for the manual screen, we diluted all molecules 100 times to be at a final concentration of 1% DMSO (vol/vol). In order to simplify the screening procedure and as most of the molecules were solubilized in DMSO, we applied the same dilution factor to all the molecules, whether they were in DMSO or in water. In addition, we tested a second dilution of 500 times.

The screen was performed in 96-well screening plates filled with 150 μL of OP50 bacteria solubilized in M9 buffer. One 96-well chemical plate was tested in two duplicate screening plates for each concentration (100 times dilution and 500 times dilution). Negative and positive controls were included in each plate, solvent alone on LS1095 worms in negative control wells and PD4251 worms in positive control wells.

Food and media preparation/storage.

■ INFORMATION *Each chemical plate is screened in duplicate in 2× 96-well screening plates filled with 150 μl of medium per well.*

For each 96-well chemical plates:

(1) Inoculate 400 mL of LB broth with an isolated OP50 colony.
(2) Incubate at 37 °C under agitation until saturation (15–20 h).
(3) Centrifuge the culture 15 min at 5000*g* and discard the supernatant.
(4) Store the "400 mL-OP50-pellet" at −20 °C.

▲ CRITICAL STEP *Bacteria are frozen, and then considered as dead, to avoid any drug metabolization and by-products generation during the screening experiment*.

3.2.2. Screening

The SJS screening protocol is schematized in the Supplemental Fig. 2.

Animals (either LS1095 or PD4251 worms) were collected from standard culture plates. Age-matched adult worms were then selected using the COPAS Biosort and sorted in the screening plates. Plates were sealed with porous sealing films to allow for oxygenation and placed at 23 °C under agitation.

Four days later, the worms from one of the duplicate screening plate were aspirated and dispensed on NGM plates without food. Plates were allowed to dry for 1 h and locomotion was assessed. The other duplicate screening plate was analyzed by the COPAS Biosort for the fluorescent readout.

Day 0 (animals amplification). For each 96-well chemical plates,

(1) Prepare 10 traditional NGM plates (60 mm NGM petri dish seeded with a thin layer of *E. coli*) with 10–15 LS1095 adult worms.
(2) Incubate the plates at 23 °C until day 6.
(3) Prepare 5 traditional NGM plates with 3 PD4251 adult worms.
(4) Incubate the plates at 15 °C until day 6.

Day 6 (assays starting point).

(5) Prepare the material needed for the screen of one 96-well chemical plate:
centrifuged 96-well chemical plate
flat-bottom 96-well screening plates
porous sealing film
1 300 μl Multichannel pipette
1 10 μl Multichannel pipette
P200 Adjustable-volume pipette
sterile reagent tank
48 300 μl pipette tips
96 200 μl pipette tips
96 10 μl pipette tips
1 "400 mL-OP50-pellet" re-suspended in 50 mL M9 added of 50 μL Cholesterol (5 mg/mL) (named M9(OP50) hereafter)
(6) Label or barcode the two 96-well screening plates and the data sheets.
(7) Dispense 50 mL M9(OP50) in a reagent tank.
(8) Add 300 μL of M9(OP50) in each well of one of the two screening plates.
(9) Add 3 μL of each drug in the corresponding wells (same distribution as the chemical plate columns 1 and 12 are empty of any drugs and solvents).
(10) Add 3 μL of DMSO in columns 1 and 12 (or any solvent useds in the chemical plate).
(11) With a multichannel pipette, mix gently by pipetting up and down and transfer 150 μL of each well in the second screening plate (replicate).
(12) Collect LS1095 animals from day 0 in M9 (mixed population of adults and L1–L2 larvae) and add them into the worm sorter cup (following manufacturer instruction).
(13) Use the worm sorter to add 4 young LS1095 adults (F0) into column 1–11, then wash the cup sorter (following manufacturer instructions).

▲ CRITICAL STEP *One should control/evaluate the efficiency of the sorting by observing several wells under a binocular microscope*.

(14) Collect PD4251 animals from day 0 in M9 and add them into the worm sorter cup (following manufacturer instruction).
(15) Use the worm sorter to add 2 PD4251 adults (F0) into column 12 (positive control).
(16) Seal the plate with a porous sealing film.
(17) Incubate the plates 4 days at 23 °C under 600–750 rpm agitation (Heidolf Titramax 1000).

▲ CRITICAL STEP *With liquid culture, agitation is very important to avoid worm lethality and bacteria agglomeration*.

Day 10 (readout). ▲ CRITICAL STEP *Check cultures: Vigourous F1 adult worms and L1–L2 F2 worms may be observed in control PD4251 wells (column 12). Paralyzed F1 adults and L1–L2 F2 worms may be observed in control LS1095 wells (column 1)*

(18) Check water and sheath fluid levels of the COPAS Biosort and change mesh of the Reflex module (following manufacturer instructions).
(19) Hydrate the mesh by analyzing 6 water-filled wells using a defined setting program (following manufacturer instruction).
(20) Distribute one screening plate into 2 new V-Bottom 96-well plates, where each test well is separated from the other by a washing well.

▲ CRITICAL STEP *This re-distribution is necessary to introduce a washing well after each test well. This is in order to avoid worm contamination from test well to test well during Reflex analysis*.

(21) Analyze the plates with the Reflex module of the COPAS Biosort (following manufacturer instruction).
(22) Store raw data as plateXXXX-1 and plateXXXX-2 in a specific folder.
(23) Perform further analysis with appropriate software. We analyzed our data with a program made under the R environment (available upon request).

Optional readout: Locomotion readout

(24) Transfer each well of the second screening plate on annoted food depleted traditional NGM plates.
(25) Dry the plates 1 h under horizontal flow biological hood.
(26) Qualitatively score locomotion rate of treated and non-treated F1 adult worms (use PD4251 adult worms as positive control and DMSO treated LS1095 as negative control).

3.2.3. Data treatment and hit validation

On the basis of the first screening step, we selected molecules that led to an improved locomotion and/ or fluorescence phenotype.

Locomotion data were expressed along a semi-quantitative scale (described in Section 3.1). Molecules were considered as potential hits whenever the worms had a score of 2 or more, meaning that they were not straight and/or were moving.

Fluorescence data generated by the COPAS Biosort were processed by a program made under the R environment to select the global fluorescence measurements of adult worms only (program available upon request). This program merges the data from the test wells and the washing wells for each molecule within a screening plate. Then, it determines within one well the number of adult worms based on the length of the objects analyzed (LS1095 adult worms were more than 350 OD measurements long with our settings). Finally, it summarizes the global fluorescence of each identified adult worm in a file. We used a Student's *t*-test to identify the wells where the mean global fluorescence of the worms was the most different from the negative control wells.

Finally, we compared the locomotion data and fluorescence data to select the most efficient molecules.

We then retested the molecules selected in quadruplicates along the same procedure. Molecules that reproduced a beneficial effect at least on one of the two readouts, in all quadruplicates, were selected for further validation. This last validation step consisted in staining worms grown on those molecules with phalloidin–rhodamine to observe their muscle cells. This enabled us to see whether there was any improvement in the degree of detachment of the muscle cells from the basement membrane.

4. Discussion

We, and others, have shown that *C. elegans* models of neuromuscular diseases are relevant to search for beneficial molecules [6,16,24,25]. We presented above the settings of two different screening campaigns, one manual and the other semi-automated.

Here we discuss, with respect to our experience and knowledge, the different parameters that have to be taken into account before choosing one or the other method.

4.1. Comparison of the manual and semi-automated screening methods

We used a manual method to search for molecules able to reduce muscle degeneration in a *C. elegans* model mimicking DMD. This method is cheap and easy to perform and the main equipment required is a fluorescent binocular microscope allowing for the observation of fluorescent muscle nuclei. One important advantage is that the worms are directly observed in the 24-well screening plates. Moreover, since the readouts are observed by an experimentator, quantitative as well as qualitative phenotype analysis are possible, meaning that unforeseen phenotypes or effects of the molecules can be registered. These observations may be useful for the establishment of a comprehensive database linking phenotype to chemical structure. For example, in our experiments, we detected several compounds that led to a dramatic increase of the activity of the worms. These compounds may have an effect on the nervous system and/or muscle activity.

The main limit of manual screens is the relative low screening speed compared to semi-automated approaches. In our hands the screening speed of this method ranges from 400 to 800 molecules per week (depending upon the experimentator). It is also noteworthy that only a trained experimentator can perform this type of screen to be able to detect improved phenotypes and so to identify potential hits.

In comparison, the semi-automated method we used to search for beneficial molecules on the *C. elegans* SJS model has two major advantages: 1) a higher screening speed (we could screen up to 2000 compounds per week), 2) it generates quantitative raw data that can be analyzed later on. This renders the selection of hits easier and more objective than in manual screens because traditional *in silico* data mining can be used. Moreover, this semi-automated method can be performed without being a specialist of the model or can even be realized by a screening platform.

However, although automated readouts reduce the subjectivity of the analysis, they can only detect defined parameters. The COPAS Biosort is able to detect the number of worms, their size and the optical density and fluorescence all along the worms. Although important information can be obtained from these data, it is difficult for example to differentiate egg laying defects from reduced progeny at an early stage, while those phenotypes are easy to detect by manual scoring. Moreover, the observation of qualitative phenotypes such as locomotion or behavior is impossible with this machine. Also, it has to be noted that raw data need to be processed prior to analysis, which requires minimum informatics skills.

4.2. Culture conditions and drug application

C. elegans can be grown in liquid or on solid culture media. Which kind of culture to use for a chemical screen depends on the mutant phenotype. Indeed, we showed that the DMD model requires solid media to lead to a strong phenotype, then one may be aware that the culture condition might be an issue in such screens. The work on solid culture medium hampers the use of automated pipetting systems, thus reducing the throughput. Moreover, it is difficult to reduce media volumes to less than 1 mL. Indeed, with this method, working with lower volumes would result in variations of the concentration of drugs and salts between wells, due to evaporation, and would lead to an increased heterogeneity over the 8 days of culture. The need of such an important volume of culture medium implies the use of a high amount of compounds, which is not in agreement with chemical libraries standard. Indeed, chemical plates containing 10–50 μl of 10 mM stock solution are classically used to cover several *in vitro* screening campaigns, while this entire amount may be used to perform one manual screen on *C. elegans*. Rare chemical libraries could be quickly depleted using this procedure. To limit drug consumption an alternative could be to add drugs at the top of NGM rather than mixing them into the medium.

In comparison, with liquid nematode cultures a lower amount of chemicals is needed to perform the screening campaigns, as culture volumes do not exceed 150 μl. Note that reducing the volumes down to 50 μl is also possible, but one has also to reduce the number of worms per wells, thus decreasing the statistical power of the results obtained at the end point.

It is noteworthy that in general drugs are used on *C. elegans* at higher concentrations then in other systems (such as cell culture, mammalian models and human). This can be explained (1) by the way drugs are administrated to *C. elegans*, since the molecules are added to the culture medium and (2) by the relative

impermeability of the cuticle surrounding the worm. To bypass this second point, one may use (i) DMSO as solvent as it slightly permeabilize worm cuticle, but more efficiently (ii) permeability mutants that has been recently reported (Frederick A. Partridge, personal communication).

4.3. Phenotypes and readouts

Mutations affecting muscle function can lead to embryonic or post-embryonic phenotypes. In our screens, we used *C. elegans* mutants exhibiting post-embryonic phenotypes, *e.g.* muscle degeneration in the DMD worm model and muscle cell detachment in the SJS worm model. At a cellular level, muscle phenotypes can be observed after staining of given muscular proteins with fluorescent phalloidin or antibodies or by using fluorescent transgenic markers labeling muscle proteins or compartments. At the level of the entire animal, muscle defects can result in locomotion defects, egg laying defects and also pharyngeal pumping defects. In the presented screens, we searched for molecules able to improve the mutant phenotype, either at a cellular or behavioral level. When running large-scale screens, it is crucial for the throughput to set up readouts that are fast and reliable and that need the less manipulations steps as possible. Thus, the most efficient readouts would be those that can be performed on living animals since no additional steps like fixation or staining procedures are required. In our screens we used a fluorescent marker allowing for the visualization of muscle cells and their nuclei. This marker was optimal for the quantification of muscle degeneration by manual readouts in the *C. elegans* DMD model. However, since muscle degeneration occurs only at a strong level on solid medium, the use of an automated device such as the COPAS Biosort would require additional steps, thus reducing the screening speed. On the other hand, this marker was very useful to assess muscle cells detachment from the basement membranes by the global fluorescence of the *C. elegans* SJS model. It has to be noted that some of the molecules in our libraries display a green fluorescence themselves, which sometimes interfered with our readouts. We also observed red fluorescence from other molecules. Consequently, one may choose carefully the drugs to screen to limit this kind of bias during the readout.

A large panoply of fluorescent muscular markers is available in *C. elegans* and can be used depending on the phenotype one wishes to observe and on the available equipment. It is further noteworthy, that one has to insure that the transgenes or the used co-markers do not interfere with the mutant phenotype. Actually, muscular phenotypes are often movement dependant; therefore co-injection markers that modify the movement of the worm (leading for example to a roller phenotype) are not well suited.

Finally, in order to set up the most rapid and appropriate readouts the availability of positive controls is optimal. These positive controls can be either molecular or genetic suppressors of the initial phenotype. These controls are useful to calibrate and sometimes validate the readouts.

In our screens we disposed of positive controls only for the DMD model. In the case of the SJS model, no positive control was available; we thus had to add another readout, which reduced drastically the throughput of the screen.

5. Conclusion

We have presented here two methods, one manual and the other semi-automated, which allow the screen of thousands of compounds on *C. elegans* models of muscular diseases. From our experience, the equipment available and the amount of molecules needed mainly dictate the choice between the two methods. Moreover, one has to take into account the training of the experimentator, the workload and the time dedicated to the screen and the skills that may be needed for the treatment of raw data.

Approaches using *C. elegans* models are complementary to *in vitro* and cell culture systems because the small size and the culture conditions of the worm fulfill the requirements for large-scale screens. However one has to keep in mind that the use of this model organism presents several limits in regards to drug discovery: (a) *C. elegans* is an invertebrate lacking regenerative processes, thus compounds acting through this pathway will not be identified, (b) some compounds that could be active in mammals may be missed due to a high protein divergence between nematodes and human, (c) negative results are difficult to analyze since it is arduous work to assess whether molecules enter the worm [26], and (d) it is also difficult to evaluate the active concentration in the worm in case of a positive result, and thus the range of doses to be tested in mammals is mostly unpredictable.

Despite these limitations, the relevance and utility of using *C. elegans* models in the drug discovery pipeline have already been proven, as we identified several compounds that have been further validated in a mammalian context [16,17]. Moreover, these approaches may fill the gap for diseases that cannot be modeled in cell cultures or that are not suitable for *in vitro* screening systems, which is particularly true for neuromuscular diseases.

Conflict of interest statement

The authors state that they have no conflict of interest.

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Appendix A. Material for the manual screen on a *C. elegans* DMD model

- *C. elegans* strains LS587 *dys-1*(*cx18*) I; *hlh-1*(*cc561*) II and PD4251 *ccIs4251* I are available from the CGC
- *C. elegans* strains LS541 *ccIs4251 dys-1*(*cx18*) I and LS761 *ccIs4251 dys-1*(*cx18*) I; *hlh-1*(*cc561*) II can be obtained upon request.
- The *E. coli* OP50 strain is available from the CGC
- Normal Growth Medium (NGM) agar: For 1 L, 3 g NaCl, 2.5 g bacto-peptone, 17 g bacto-agar, 25 mL of 1 M potassium phosphate buffer (pH 6.0), 1 mL of 5 mg/mL cholesterol dissolved in ethanol, water up to 1 L. Autoclave and cool down to 55 °C. Add 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$.
- Potassium phosphate buffer, pH 6.0: 108.3 g KH_2PO_4, 35.6 g K_2HPO_4, water up to 1 L. Sterilize by autoclaving.
- LB broth: For 1 L, 10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl, water up to 1 L.
- M9: For 1 L, 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL of 1 M $MgSO_4$ and water up to 1 L. Sterilize by autoclaving.
- 10× PBS: For 1 L, 2 g KCl, 2.4 g KH2PO4, 80 g NaCl, 11.45 g Na2HPO4, water up to 1 L. Sterilize by autoclaving
- 24-well plates (Greiner Bio-One), tissue culture treated, with lid, sterile (VWR catalog number 82050–892) (Screening plates)
- Aluminum Sealing films (VWR, catalog number 47734–817)
- Dimethylsulfoxyde (VWR, catalog number 23486.297)
- Methazolamide (Sigma–Aldrich, product number M4156)

- Formaldehyde 37% solution, stabilized (VWR, catalog number 8.18708.1000)
- FluoProbes 547H-Phalloidin for F-actin staining (Interchim, FP-BZ9620, 550/568 nm)
- Vortex Genie 2 with microplate support (VWR, catalog number 444-5900 and 444–5919)
- 37 μM mesh filters (Buisine, reference 03–37/24)
- Refrigerating Incubator (minimum temperature range from 10 °C to 35 °C)
- Mid bench centrifuge (Beckman GS-15R, rotor S4180)
- Binocular microscope Lumar V12 equipped with Lumar filter set 38 GFP (Carl Zeiss)
- Microscope AxioImager Z1 (Carl Zeiss)
- 60 mm Petri plates (CML, catalog number 1548553)
- Dako Fluorescent Mounting Medium (Interchim, catalog number S3023)
- Microscope slides and Cover slips (Roth, catalog numbers H868 and 1870)
- Acetone (Sigma–Aldrich, catalog number 179973)
- −20 °C Labtop cooler (Nalgene, catalog number 055411)
- Low binding microcentrifuge tubes (Sorenson Biosciences, Inc., catalog number 27210)
- Microwave oven
- Table top centrifuge
- Laminar flow hood

Appendix B. Material for a semi-automated screen on a *C. elegans* SJS model

- *C. elegans* strains CB444 *unc52*(*e444*) and PD4251 *ccIs4251* I are available from the CGC
- The *C. elegans* strain LS1095 *ccIs4251 I; unc52*(*e444*) *II* is available upon request
- The *E. coli* OP50 strain is available from the CGC
- LB broth: For 1 L, 10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl, water up to 1 L
- M9: For 1 L, 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL of 1 M $MgSO_4$, water up to 1 L. Sterilize by autoclaving
- Cholesterol (5 mg/mL) dissolved in ethanol
- Flat-Bottom 96-well plates (Greiner bio-one CELLSTAR, catalog number 655201): screening plates
- Porous sealing film (Sterile aeraseal film, Dutscher ref 760214)
- Dimethylsulfoxyde (VWR, catalog number 23486.297)
- Heidolph Titramax 1000 (VWR, catalog number 444-1391)
- Refrigerating Incubator (minimum temperature range, from 10 °C to 35 °C)
- Mid bench centrifuge (Beckman GS-15R, rotor S4180)
- COPAS BIOSORT with reflex and profiler modules (Harvard Bioscience, Boston, MA, USA)
- 60 mm Petri plates (CML, catalog number 1548553)
- Sterile reagent tanks (Dutscher, catalog number 034102)
- Acetone (Sigma–Aldrich, catalog number 179973)
- −20 °C Labtop cooler (Nalgene, catalog number 055411)
- Conical-bottom 96-well plates (Greiner Bio-one, catalog number 651101): for Reflex readout
- Table top centrifuge
- Binocular microscope
- 300 μl pipette tips
- 200 μl pipette tips
- 10 μl pipette tips
- Multichannel pipettes (300 μl and 10 μl)
- P200 Adjustable-volume pipette

Appendix C. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymeth.2011.10.010.

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