

1 **Consecutive signaling pathways are activated in progression of Duchenne muscular**
2 **dystrophy in *C. elegans***

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18 **Short title:** Duchenne muscular dystrophy in *C. elegans*

19

20 **One Sentence Summary:** A tissue specific transcriptome analysis of dystrophin deficient
21 muscle in *C. elegans* reveals that dystrophin has distinct, dynamic signaling roles in early
22 and late stage progression of Duchenne muscular dystrophy.

23

ABSTRACT

24 **Background:** Duchenne muscular dystrophy (DMD) is a lethal, X-linked disease
25 characterized by progressive muscle degeneration. The condition is driven by nonsense
26 and missense mutations in the dystrophin gene, but the resulting changes in muscle-
27 specific gene expression that take place in dystrophin's absence remain uncharacterized,
28 as they are potentially obscured by the chronic inflammation elicited by muscle damage in
29 humans. *C. elegans* possess a mild inflammatory response that allows for the
30 characterization of the transcriptome rearrangements affecting disease progression
31 independently of inflammation.

32 **Results:** In effort to better understand these dynamics we have isolated and sequenced
33 body muscle-specific transcriptomes from *C. elegans* lacking functional dystrophin at
34 distinct stages of disease progression. We have identified two consecutively altered gene
35 networks, which are also disrupted in the dystrophin deficient *mdx* mouse model. We
36 found an upregulation of genes involved in mitochondrial function early in disease
37 progression, and an upregulation of genes related to muscle fibre repair in later stages.
38 This suggests that dystrophin may have a signaling role early in development, and its
39 absence may activate compensatory mechanisms that counteract muscle degradation
40 caused by loss of dystrophin. We have also developed a temperature-based screening
41 method for synthetic paralysis that can be used to rapidly identify genetic partners of
42 dystrophin.

43 **Conclusions:** Our results allow for the comprehensive identification of transcriptome
44 rearrangements that potentially serve as independent drivers of disease progression and
45 may in turn allow for the identification of new therapeutic targets for the treatment of DMD.

46

BACKGROUND

47

48 Duchenne muscular dystrophy (DMD) is an X-linked, recessive disease caused by
49 out of frame mutations in the dystrophin gene [1]. The dystrophin gene codes for a
50 structural protein found beneath the sarcolemma, where it is anchored both to the
51 dystrophin glycoprotein complex (DGC) and cytoskeletal actin, thus stabilizing the protein
52 complex and the integrity of the cell membrane [2]. In humans, the absence of functional
53 dystrophin results in progressive degeneration of the skeletal and cardiac muscles. The
54 hallmark symptoms of DMD extend beyond muscle degeneration to include respiratory
55 failure, cardiomyopathy, and pseudohypertrophy. The condition remains the most
56 commonly diagnosed type of muscular dystrophy, affecting approximately 1 in 3,500 male
57 births globally.

58 While the role of dystrophin in forming a physical connection between the
59 extracellular matrix (ECM) and cytoskeleton has been well characterized [3], a
60 comprehensive molecular definition of dystrophin's function is not fully understood.
61 Vertebrate models of DMD include the *mdx* mouse [4] and the golden retriever muscular
62 dystrophy canine [5]. Both models have contributed significantly towards our
63 understanding of dystrophin function, but they face limitations when studying the cell
64 autonomous effects of dystrophin deficiencies on skeletal muscle independently of the
65 myolysis and fibrosis associated with chronic inflammation observed in mammals. In
66 addition, *mdx* mice do not breed well, making them difficult to use to study molecular
67 mechanisms using approaches like genome wide screens and other large-scale studies [6,
68 7].

69 Mitochondrial dysfunction and systemic deregulation of cellular energy homeostasis
70 have been observed in both in DMD patients and *mdx* mice [8-10]. Mitochondria are
71 important players in this disease, as a steady-state flux of ATP, Ca⁺ and other
72 components of energy metabolism are needed for muscle contraction. A recent study
73 identified a significant increase in enzymes that are major consumers of NAD⁺ in
74 dystrophic muscle tissues, and its replenishment was shown to ameliorate paralysis in
75 *mdx* mice and *C. elegans* [8]. Evidence shows that mitochondria play a pivotal role in
76 disease progression. However, it is still not clear if mitochondrial dysfunction is induced by
77 the loss of muscle fibres, which in turn induce muscle paralysis and necrosis, or if it occurs
78 in early stages of the disease when the paralysis and loss of muscle tissue has not yet
79 been initiated [11].

80 The invertebrate model *C. elegans* has the potential to address these questions and
81 serve as an informative model system for DMD. These nematodes possess a singular
82 ortholog of the dystrophin gene (*dys-1*), which is similar in protein size to the human
83 dystrophin gene, and contains similar actin binding and scaffolding regions, which are
84 used to bridge the dystrophin complex to cytoskeletal actin (**Fig. 1A**). Additionally, several
85 essential members of the DGC are conserved between humans and nematodes. The *C.*
86 *elegans* DGC is largely comparable to the human complex, and includes key proteins such
87 as dystrobrevin (*dyb-1*), sarcoglycans (*sgca-1*, *sgcb-1* and *sgn-1*), and the syntrophins
88 (*stn-1* and *stn-2*). This predicts strong selective pressure for functional conservation of the
89 DGC from *C. elegans* to humans.

90 *C. elegans* myogenesis has been extensively characterized [12], with only two main
91 types of muscles: obliquely striated and nonstriated [13]. Many structural muscle proteins

92 are also conserved between worms and humans, suggesting a maintained functional
93 mechanism of contraction (**Fig. 1B**).

94 The *C. elegans* mutant strains *dys-1(cx18)* and *dys-1(eg33)* were recently
95 introduced and represent a novel tool to study DMD *in vivo* [14, 15]. These mutant strains
96 contain different nonsense mutations leading to the expression of a truncated DYS-1,
97 which lacks the essential scaffolding portion of the C-terminus (**Fig. 1C**). *dys-1* gene
98 expression is restricted to the worm body and vulva muscles. Mimicking the human
99 condition, early reports have shown that loss of *dys-1* does not result in dramatic muscular
100 degeneration phenotypes in juvenile worms. Instead they display defects in motility that
101 are phenotypically distinct and associated with the loss of *dys-1*. This includes anomalous
102 bending of the head, hyperactivity, and slight hyper contraction of the body wall muscle in
103 early stages accompanied by an age-dependent, progressive loss of locomotor function
104 (**Fig. 1D**). In later stages, *dys-1* worm strains exhibit increased muscle cell death,
105 attenuated motility and have shorter lifespans than *wt* worms (**Additional File 1: Figure**
106 **S1**) [15].

107 Both *dys-1* worm strains display similar disease phenotypes, suggesting that both
108 mutations, although in different portions of the *dys-1* gene, affect similar pathways. The
109 introduction of human *dystrophin* cDNA in these mutant worms rescues these phenotypes
110 [15], suggesting they are indeed a highly appropriate disease model.

111 *C. elegans* possess an innate immune response; the adaptive immunity is primitive
112 and cell-mediated immunity is absent [16], leading to minimal muscle inflammation in *dys-*
113 *1(cx18)* and *dys-1(eg33)* strains. The absence of chronic inflammation, which normally
114 leads to myofiber necrosis and fibrosis in DMD patients or mouse models, causes these

115 worm strains to move somewhat similarly to *wt* worms. Paralysis is subtle, localized to
116 specific areas, and becomes most apparent in adult worms (**Fig. 1E**). Because the muscle
117 tissues in these strains are not being actively damaged by chronic inflammation as in
118 patients with DMD and *mdx* mice, this model is unique in its ability to study the cell
119 autonomous contribution of changes in gene expression in the absence of muscle
120 inflammation (**Fig. 1E**).

121 Although they were initially very promising, these two strains were not fully adopted
122 by the community along with the *mdx* mouse, as a comprehensive analysis on the extent
123 to which nematodes mimic the human version of the disease remained to be
124 characterized. Additionally, these strains lacked a quantifiable phenotype that was easy to
125 score, which initially prevented the development of large-scale genetic screens for the
126 identification of therapeutic targets.

127 Our lab has previously adapted an established technique, which optimizes tissue-
128 specific RNA extraction from intact organisms [17], allowing the identification of tissue-
129 specific transcriptomes at single-base resolution [18-20]. This method, named PAT-Seq,
130 takes advantage of the binding affinity and specificity of the cytoplasmic PolyA Binding
131 Protein (PABPC1) to polyA tails of mRNAs [21], which after UV crosslinking is
132 immunoprecipitated, released from the tissue-specific polyA+ RNAs and the resultant
133 transcripts are sequenced. *pab-1* is the worm ortholog of PABPC1, and by expressing this
134 FLAG-tagged gene using MosSCI single copy integration technique in different worm
135 tissues, our lab recently profiled *C. elegans* intestine, pharynx and body muscle tissues
136 [18]. This study allowed us to define the body muscle transcriptome. We detected 2,610
137 genes expressed in this tissue, with only 329 unique genes corresponding to 365 spliced

138 isoforms [18]. The list of top ten genes in the body muscle dataset was also enriched for
139 muscle-specific genes such as myosin and actin isoforms. This study also detected a
140 unique muscle-specific gene pool, including previously identified genes members of the
141 *dystrophin* complex in human, such as dystroglycan, syntrophin, alpha-dystrobrevin, and
142 dystrophin, the calveolin family, and type IV collagen.

143 In order to identify high quality cell-specific muscle specific transcriptome changes
144 occurring in DMD, we crossed the *dys-1(cx18)* and *dys-1(eg33)* strains with our *myo-*
145 *3p::GFP::pab-1::3xFLAG* worm strain (PAP) [18] (**Fig. 2A**), producing two novel strains
146 named DP1 and DP2 respectively, which allowed the extraction and sequencing of high
147 quality muscle-specific transcriptomes in worms lacking the dystrophin protein at different
148 stages of the disease. This approach allowed us to fully define the dynamic transcriptome
149 rearrangements occurring in the absence of inflammation at controlled time points, before
150 and after the onset of paralysis. We have also performed targeted genetic experiments to
151 study the contribution to paralysis of selected genes identified by our approach.

152 We have found that the absence of dystrophin first results in the deregulation of
153 mitochondrial function prior to muscle paralysis. This impairment leads to differential
154 expression of genes involved in muscle function and differentiation after the paralysis
155 occurs perhaps as a part of a compensatory mechanism that is able to impede dystrophin-
156 dependent muscle degeneration.

157

158

RESULTS

159

160 **Pat-Seq from muscle tissues of *C. elegans* strains lacking a functional copy of the**
161 **dystrophin gene produced high quality muscle specific RNAs**

162 In order to perform PAT-Seq in *C. elegans* dystrophin deficient muscle tissues, we
163 have taken advantage of the previously existing PAT-Seq technology used in our previous
164 studies [18, 19]. The original PAP strain expresses the gene *pab-1* fused to GFP (N-
165 terminus) and a 3xFLAG tag (C-terminus) restricted in the muscle tissue [18, 19]. *pab-1* is
166 the *C. elegans* ortholog of the human cytoplasmic polyA binding protein (PABPC1), which
167 typically binds the polyA track of mature mRNAs in the cytoplasm and is required for
168 translation [22].

169 We have crossed the worm strain *myo-3p::GFP::pab-1::3xFLAG* (PAP) with *dys-*
170 *1(cx18)* and *dys-1(eg33)* and prepared two new strains named DP1 and DP2. The two
171 new strains retain the *dys-1* mutations *cx18* and *eg33*, and express the muscle specific
172 PAP transgene, which allows the muscle specific immunoprecipitation step using our *pab-*
173 *1* based strategy. These two new strains (*dys-1(eg33)/pap* (DP1) and *dys-1(cx18)/pap*
174 (DP2) were verified using Sanger sequencing to confirm the presence of the nonsense
175 mutations in the *dys-1* gene in the crossed F1 worms that were GFP-positive in the muscle
176 tissues (**Fig. 1C**). We also used a PCR approach to confirm the genomic integration of the
177 PAP construct in the MosSCI locus [23] (**Fig. 1C**). In order to further validate our cross, we
178 subjected these worm strains to a Kaplan-Meier survival analysis (**Additional File 1:**
179 **Figure S1**). The average lifespan of N2 worms in this experiment is approximately 21
180 days, and both new strains behave very similarly to their reciprocal pre-crossed strain, with
181 their lifespan drastically declining at day 18, with an overall increase in lethality when
182 compared to *wt* worms (**Fig. S1**). Taken together these results suggest 1) we successfully

183 crossed both dystrophin deficient strains with our PAP strain, and 2) the DP1 and DP2
184 strains reflect the phenotypes already characterized in the literature before and after our
185 crosses, suggesting our PAP construct did not interfere with the *dys-1* phenotype.

186 We next performed the PAT-Seq experiments, isolating and sequencing muscle
187 specific mRNAs from DP1 and DP2 strains. We wanted to detect precise changes in gene
188 expression, not only in later stages of paralysis, but also before the paralysis was initiated,
189 to have a more comprehensive overview of the dynamic gene changes occurring during
190 the initiation and the progression of the disease. In addition, we wanted to reduce our
191 sample number to simplify the data analysis and increase the depth of our sequencing
192 results. Therefore, we decided to grow a large mixed population of our crossed worms,
193 and then performed sequential mechanical filtrations by size, which led to the isolation of
194 two pools of worms for each strain: one containing smaller and mildly phenotypic embryo-
195 L2 worms, which we labeled 'pre-symptomatic' (PRE), and another containing larger and
196 more extensively phenotypic L3-adult worms, which we labeled 'post-symptomatic' (POST)
197 **(Fig. 2)**.

198 We performed a total of 16 immunoprecipitations (see materials and methods),
199 sequencing and analyzing DP1 and DP2 mixed stage samples, and PRE and POST
200 paralysis samples in duplicate.

201 We obtained approximately 900M reads across all our sequenced samples, with
202 approximately 50M reads each dataset (**Additional File 1: Table S1**). Within these
203 samples we were able to map 42% of the reads to the *C. elegans* genome (WS250)
204 (**Additional File 1: Table S1**). Both experimental and biological replicates in each dataset
205 correlate well with each other (**Additional File 1: Figure S2**), with ~2,000 shared protein

206 coding genes within each group (**Additional File 1: Table S2**). The total and mapped
207 reads for each sample are listed in **Additional File 1: Table S3**.

208 Our sequencing efforts detected a total of 3,748 genes across all datasets
209 (**Additional File 1: Figure S3A**). Within this group 47% have been previously mapped in
210 the body muscle [19] (**Additional File 1: Figure S3A**). When we repeated this analysis
211 separately in our PRE and POST datasets, this percentage increases to ~60% similarity
212 (**Additional File 1: Figure S3A**), with >85% identity in our top 250 genes in all our
213 datasets (**Additional File 1: Figure S3B**).

214 Taken together, these results suggest that our muscle specific RNA pull down was
215 successful and able to identify *bona fide* muscle specific genes.

216

217 **Mitochondrial response is involved in the initiation of paralysis in our PRE dataset**

218 The PRE symptomatic dataset is mainly composed of embryo to L2 mildly
219 symptomatic worms. We obtained 1,932 protein coding genes expressed in this group,
220 with 176 unique genes not expressed elsewhere (**Fig. 3** and **Additional File 1: Table S3**).
221 There are very few genes that change drastically in expression level between the *wt* and
222 PRE dataset (**Fig. 3**). A GO term analysis on the top 50 genes identified an enrichment in
223 genes involved in nematode larval development and locomotion, such as *cah-4*, *dpy-30*,
224 *ran-1*, *ceh-20*, *vab-15*, *ned-8*, *rpi-1*, *uri-1*, *tfg-1*, and *bus-8*.

225 We also detect an unusual abundance of mitochondrial genes involved in ATP
226 production and regulation of apoptotic processes within the top hits identified in our PRE
227 dataset (**Fig. 3**). *ant-1.1* is an ADP/ATP translocase with a three-fold increase over the
228 median between DP1 and DP2 datasets (when compared with the *wt* PRE dataset). This

229 mitochondrial membrane receptor is responsible for transporting ATP synthesized from
230 oxidative phosphorylation into the cytoplasm and absorbs back ADP in a 1:1 molar ratio
231 [24, 25].

232 The genes Y69A2AR.18 (5-fold), F58F12.1 (3-fold), *icd-1* (2.5-fold), H28O16.1 (2-
233 fold), *atp-3* (2-fold) are all mitochondrial ATP synthase subunits involved in the synthesis
234 of ATP from ADP and inorganic phosphate.

235 Only 173 genes were uniquely detected in this stage that were absent in our *wt*
236 control, and are most likely the direct result of the initiation of the disease. Most of these
237 genes have an unknown function, but when aligned to the human proteome many of them
238 show significant matches to known genes. *smo-1* is among those significantly abundant.
239 *smo-1* is the *C. elegans* homolog of SUMO, a small ubiquitin-like signaling modifier that is
240 attached to protein dictating localization and function.

241

242 **Genes involved in myogenesis and muscle contraction are overexpressed in the** 243 **POST dataset**

244 In our POST dataset we detected 2,273 protein-coding genes, which accounts for
245 only 58% of genes in common with the PRE dataset (**Fig. 3** and **Additional File 1: Table**
246 **S3**). This discrepancy is consistent with the progressive nature of the disease and the
247 associated changes in muscle structure and overall health.

248 Essential constituents of the mitochondrial metabolism are also overexpressed at
249 this stage, although at lower levels than in our PRE dataset. They include the mitochondria
250 ATP synthase *lcd-1*, *ant-1.1*, H28O16.1, Y69A2AR.18, *atp-2* and *atp-3*, *nduo-1* (NADH-
251 ubiquinone oxidoreductase), and W09C5.8 (cytochrome c oxidase).

252 Importantly, we detected an abundance of genes involved in myogenesis; including
253 *mlc-1* and *mlc-2* (myosin light chain), *mup-2* (troponin), *act-1* (actin), *unc-27* (troponin), *lev-*
254 *11* (tropomyosin), and *unc-15* (paramyosin). This result is surprising, as an increase in
255 sarcomeric gene transcription is consistent with muscle hypertrophy or active replacement
256 of muscle, which does not occur in *C. elegans* as they lack a satellite cell equivalent. In
257 this context, the increase in transcription may suggest a hidden compensatory signaling
258 pathway activated in the absence of *dys-1* that precedes cell death.

259 Only 38 genes are uniquely present in our POST dataset, and the majority of these
260 genes have unknown functions.

261

262 **A novel synthetic screen to identify genetically linked *dys-1* targets**

263 Our approach identified specific genes differentially regulated in both dystrophin
264 deficient samples across replicates. These genes were categorized by function, and
265 among several trends, we identified an enrichment in our pre dataset of genes involved in
266 mitochondrial metabolism, and the establishment and maintenance of muscle structure
267 and function in our POST dataset.

268 In order to validate and expand the biological significance of these results, we
269 decided to perform a targeted genetic screen testing genes identified by PAT-Seq for their
270 ability to enhance muscle damage in dystrophin deficient strains when knocked down
271 through RNAi. We reasoned that if there were genetic compensatory mechanisms in place
272 to counteract paralysis, the knock down of upregulated genes detected in our screen
273 would lead to detectable impairment of muscle viability that would suggest a genetic link
274 between *dys-1* and the tested genes.

275 The *dys-1* phenotype is subtle when compared to *wt* worms. For this reason, we
276 decided to use a previously established strain that uses a background mutation to
277 enhance paralysis to create a more definitive and scorable phenotype.

278 It has been previously shown that combining dystrophin mutations with mutations
279 in the transcription factor MyoD exacerbates dystrophic phenotypes in mice, and that this
280 combination has a similar effect in *dys-1* strains [26]. The temperature sensitive strain *hlh-*
281 *1(cc561ts)* contains a hypomorphic mutation in the *C. elegans* homologue of MyoD, *hlh-1*,
282 which renders these strains viable at the permissive temperature 15°C and severely
283 uncoordinated with defects in body muscle morphology at non-permissive temperatures
284 above 20°C [27].

285 We utilized this single mutant strain *hlh-1(cc561ts)*, as well as the double mutant
286 strain *dys-1(cx18); hlh-1(cc561ts)*, and observed that at 15°C (permissive), both single and
287 double mutant strains hatch and develop to adulthood without defects. In order to
288 ameliorate the uncoordinated defects and lethality obtained at 20°C, we decided to
289 decrease this temperature to 18°C (semi-permissive). At 18°C, ~75% of the single
290 mutants, and ~50% of the double mutants are still viable (**Fig. 4A-B**), providing us with a
291 distinct and tractable range to detect changes in incidence of paralysis following the RNAi
292 experiments (**Fig. 4C**).

293 Populations were synchronized and grown at 15°C (permissive) until they reached
294 the L4 stage, and were then moved to a semi-permissive temperature of 18°C and allowed
295 to lay eggs for 24 hours. At semi-permissive temperature, the single mutant strains *hlh-1*
296 gave rise to progeny of which 26% developed with severe defects in body morphology and
297 were almost completely paralyzed (**Fig. 4B**). Under the same conditions, the double

298 mutant strains *hlh-1*; *dys-1* yielded a 21% increase in paralysis and severe defects in body
299 morphology (**Fig. 4A-B**).

300 We then selected a subset of representative genes identified by PAT-Seq as being
301 upregulated at different degrees, which are indicated with an asterisk in **Additional File 1:**
302 **Table S3**. These genes were also chosen based on function, primarily because of their
303 known roles in mitochondrial metabolism, muscle structure, and signaling function (**Fig.**
304 **4C**). We speculated that if these genes were indeed abundant in paralyzed worms as a
305 compensatory mechanism to counteract paralysis, their depletion in the double mutant
306 background would enhance paralysis when compared to the single mutant background.

307 The RNAi experiments were performed at the semi-permissive temperature of
308 18°C. Worms were scored for incidence of paralysis and morphological defects (**Fig. 4C**).
309 The results between control and experimental strains were compared with each other, and
310 then to the observed differences in semi-permissive experiments performed on single and
311 double mutant strains in the absence of RNAi (**Fig. 4B**). As a control, we performed a
312 knockdown of a known member of the nematode DGC, *dyb-1*. The knockdown of *dyb-1*
313 was able to increase incidence of paralysis in the single mutant to an extent that reflected
314 the double mutant in semi permissive control experiments (**Fig. 4D**).

315 We then tested our muscle and signaling pools. Although some of the genes were
316 synthetic lethal in combination with the *hlh-1* background mutation (*act-1*, *cmd-1*, *unc-27*
317 and *unc-15*), and could not be tested further, the majority of genes tested were able to
318 enhance paralysis, although to different degrees (**Fig. 4E**) (**Additional File 1: Table S3**).

319

320

DISCUSSION

321

322 Here we have described the use of the nematode *C. elegans* to study the cell
323 autonomous molecular events associated with the functional loss of the dystrophin gene
324 that leads to Duchenne muscular dystrophy in humans. We have performed genetic
325 crosses to establish transgenic strains that serve both as a model for DMD, and as a
326 functional tool to perform high quality, muscle specific RNA-IPs at a resolution that has not
327 yet been achieved. Using these strains, we have sequenced and analyzed muscle specific
328 transcriptomes at distinct developmental stages during disease progression, and identified
329 several differentially regulated pathways in dystrophic nematode muscle.

330 We have also developed a novel, temperature-based genetic screen and performed
331 a quantitative analysis to assess the functional significance of the identified genes in the
332 viability of muscle cells, thus confirming their contribution towards DMD initiation and
333 progression. This approach will allow a direct evaluation the role of specific genetic
334 pathways in the clinical severity of DMD, which will be fruitful in developing drug targets for
335 treating DMD patients.

336 Our dystrophin deficient strains DP1 and DP2 display phenotypes and altered
337 molecular pathways that are very similar to those observed in *mdx* mice and DMD
338 patients, strongly implying that this model system phenocopies many aspects of the
339 disease at the molecular level.

340 These new strains recapitulate phenotypes previously characterized in the literature
341 for *dys-1(cx18)* and *dys-1(eg33)*, with shortened average and maximum lifespan,
342 hyperactivity and excessive bending of the head [14, 15] (**Additional File 1: Figure S1**),
343 suggesting that introducing the PAP cassette into the *dys-1* genetic background has not

344 altered the *dys-1* phenotype. In addition, the muscle morphology of both *dys-1* strains is
345 highly similar to wild type muscle, as the muscle fibres do not present gross defects in
346 morphology.

347 Our muscle specific sequencing approach in *C. elegans* allowed us not only to
348 bypass the noise introduced by the immune response, as in *mdx* mice, but also to profile
349 muscle tissue without contamination. Isolating pure muscle tissue from mice and patients
350 is challenging, and although results are very informative and shed light on progression of
351 DMD [28, 29], these samples are potentially contaminated with myofibroblasts, connective
352 tissue, inflammatory cells, nerves and endothelial cells, and the results may be difficult to
353 reproduce or validate due to intrinsic sample variation within experiments. Our approach
354 bypassed these issues, providing the community with high-quality muscle-specific
355 transcriptomes in the absence of functional dystrophin with minimal contamination and at
356 single-base resolution.

357 Our study uncovered ~ 2,000 protein coding genes present in early and late stage
358 dystrophic muscle. Many of these genes are expressed in the muscle, and replicate what
359 we already profiled in this tissue (**Additional File 1: Figures S3A-B**). Combined with a
360 strong correlation observed between our biological replicates (**Additional File 1: Figure**
361 **S3C**), these results suggest that our approach can accurately and consistently detect
362 transcripts between biological replicates.

363 Because our approach is biochemistry-based, we were concerned about a high
364 signal-to-noise ratio, which would have prevented the identification of fine genetic changes
365 in our PRE and POST datasets. For this reason, we have applied a stringent bioinformatic
366 filter to our datasets, restricting our analysis to the top 30-40% of the total genes identified

367 in this study (**Additional File 1: Figure S3D**), which may have in turn lowered the number
368 of genes considered, but provided us with higher quality results (**Additional File 1: Figure**
369 **S3D**).

370 Our study revealed two distinct sets of genes that contribute to the DMD phenotype
371 in *C. elegans*; the first set is activated before paralysis and participates in mitochondrial
372 homeostasis, cell death and protein degradation signaling in the muscle, and the second
373 set is activated at or after the onset of paralysis and is responsible for the establishment
374 and maintenance of muscle structure.

375 Although there have already been studies in *mdx* mice that have reported the
376 involvement of both pathways in DMD progression [9, 14, 30], it was still unclear how
377 these two pathways are intertwined with each other, and in what order these molecular
378 events occur. This question is important, as impaired mitochondrial metabolism has been
379 already observed in *mdx* myoblasts in which a functional DGC has not yet been
380 assembled [31], suggesting that these two pathways may be somewhat independent.

381 More precisely, it is still unclear if the damage incurred by muscle tissue in
382 absence of dystrophin leads to an increase of extracellular Ca²⁺ accumulation in muscle,
383 which in turn causes mitochondrial membrane potential to collapse, or if the altered ATP
384 synthesis in the mitochondria in the muscle tissue before paralysis causes muscle
385 weakness, instability and degradation.

386 Our results suggest that these two pathways may not occur simultaneously; the
387 mitochondrial dysfunction is detected early in disease progression, in accordance with
388 previous findings [9], either before paralysis is initiated or while it is still mild.

389 In the PRE dataset we detected the differential regulation of many mitochondrial
390 genes involved in glycolysis and ATP/ADP transport (**Fig. 3 and Additional File 1: Table**
391 **S5**). Proteomics studies in *mdx* mice support these results [9]. Muscle contraction is
392 dependent upon ATP production and usage, and its abundance is tightly regulated in
393 healthy muscles.

394 Aberrant mitochondrial activity has been previously reported in patients and animal
395 models with DMD [8, 32-36], even prior to dystrophin assembly at the sarcolemma [31]. In
396 this context, our results validate these studies and highlight an important signaling role for
397 *dys-1*, which evidently functions outside of its accepted role as a scaffolding protein in the
398 DGC.

399 After the onset of paralysis in post-symptomatic samples, while the mitochondrial
400 dysfunction is still present, we detect a second pathway, which perhaps tries to actively
401 compensate for the loss of muscle structure by overexpressing genes involved in muscle
402 formation. We do not know if these transcripts are indeed carrying out a compensatory
403 mechanism, and more experiments need to be performed to further validate this finding. Of
404 note, many of them were able to enhance paralysis in our RNAi experiments in **Fig. 4C**,
405 suggesting that they are functionally translated.

406 Our RNAi experiments in **Fig. 4** most effectively screen for genes that play a role in
407 the muscle and work cooperatively with dystrophin beginning early in development. The
408 effects of RNAi knockdown are scored in the first hours after L1 animals hatch. In this light,
409 the results of our semi permissive control experiments show that the absence of
410 dystrophin is able to affect the very early development of the embryos that ultimately hatch
411 with severe muscle defects. This underscores our sequencing results in PRE datasets that

412 suggest dystrophin may play an early role, both in development and in regulating
413 mitochondrial function, and is not simply a structural gene whose absence can impair
414 developed muscle after continuous contraction throughout an organism's lifespan.

415 The changes in gene expression observed in our POST dataset suggest that many
416 of the genes acting as structural units within the sarcomere are involved in the progression
417 of dystrophin-induced muscle damage (**Fig. 3**). Our RNAi experiments in **Fig. 4** confirmed
418 the involvement of these genes in DMD progression and verify their role in a
419 compensatory mechanism that may allow *C. elegans* to increase transcription of muscle-
420 structure related genes in response to muscle damage.

421 *mup-2*, a gene that codes for the ortholog of muscle protein troponin, was able to
422 increase incidence of muscle defects and paralysis in the dystrophin deficient strain *dys-*
423 *1(cx18)*; *hlh-1(cc561ts)*, without inducing the same effect on the control strain *hlh-*
424 *1(cc561ts)*. In the same manner, the interference of *ttn-1*, an ortholog of the muscle protein
425 titin, and *mhc-1*, an ortholog of myosin regulatory light chain, was able to increase
426 incidence of muscle defects and paralysis in the dystrophin deficient strain *dys-1(cx18)*;
427 *hlh-1(cc561ts)*, without inducing the same effect on the control strain *hlh-1(cc561ts)* (**Fig.**
428 **4C**).

429 Taken together these results suggest that *mup-2*, *ttn-1*, and *mhc-1* are all genetically
430 connected to *dys-1*, and are not only overexpressed as transcripts when paralysis occurs,
431 but are also expressed as proteins, as their dosage is necessary to increase paralysis in a
432 *dys-1* dependent manner.

433 Outside this group of genes selected because of their role in muscle structure, we
434 also studied upregulated genes essential in several signaling pathways. *icd-1* is the β

435 subunit of the nascent-polypeptide associated complex. It was significantly upregulated in
436 POST symptomatic data sets, it mediates proteins transport to mitochondria [37], and it is
437 necessary and sufficient to suppress apoptosis [38]. *icd-1* knockdown induces a two-fold
438 increase of incidence of muscle defect and paralysis in *dys-1* dependent manner (**Fig. 4B**),
439 suggesting that these two genes are genetically connected.

440 An observed limitation in using our approach for RNAi screens was the incidence of
441 synthetic lethality. When combined with background mutations in *hlh-1*, several genes
442 involved in development of muscle proved to be embryonic lethal when knocked down.
443 Because this synthetic lethality achieved the same penetrance in single and double mutant
444 strains, it prevented the scoring of differences between the two strains. It is important to
445 note that this did not occur in the bulk of our experiments that knocked down muscle
446 specific genes, meaning it is still feasible to use the method to screen the majority of
447 genes in the genome, both muscle specific and ubiquitously expressed. This screening
448 method is also now mature for high throughput, genome wide RNAi screens. The system
449 can easily be adapted to 96 well, liquid screens. This can in turn provide a platform for
450 rapidly identifying potential therapeutic targets that can be first validated in mammalian
451 model systems, and eventually considered as part of a treatment plan for human DMD
452 patients.

453

454

CONCLUSIONS

455 We have definitely characterized that the nematode *C. elegans* fully mimics the
456 majority of human DMD symptoms. The mitochondrial dysfunction previously observed in
457 humans and vertebrate DMD muscle is also present in DMD worms, but is not induced by

458 muscle fiber lesions as previously found in *mdx* mice, instead it precedes the observed
459 muscle damage and paralysis in late stage disease phenotypes. In response to
460 mitochondrial dysfunction, *C. elegans* activate a compensatory pathway to upregulate the
461 expression of muscle structure related genes to stave off paralysis, which could explain
462 their lack of a strong paralysis as observed in DMD patients.

463

464

METHODS

465

466 Preparation of the nematode strains

467 Body muscle specific PolyA-Pull expressing transgenic lines were obtained for
468 previous crosses [18]. Young adult *C. elegans* worms were isolated on nematode growth
469 media (NGM) agar plates seeded with OP50-1 and incubated at 31°C for 3.5 hours. Plates
470 were then incubated for four days at 20°C and males were isolated from populations.
471 Groups of five males were paired with 10 L4 hermaphrodites and incubated for 3 days at
472 20°C. Crosses between PAP and *dys-1(cx18)* and *dys-1(eg33)* strains were screened for
473 GFP fluorescence using a Leica DM13000B microscope, and crosses between *dys-*
474 *1(cx18)* and *dys-1(eg33)* strains and VS21/DM8005 strains were screened for mCherry
475 fluorescence using a Leica MZ10F microscope. Strains positive for fluorescent markers
476 were subjected to Sanger sequencing for the verification of mutations in the dystrophin
477 gene using the following primers: *dys-1(cx18)*_F: GGCTTAATATGAGCTGGACGAAG,
478 *dys-1(cx18)*_R: CGCTGTCCATCTTCTTGTGG, *dys-1(eg33)*_F:
479 GGACGGTCATGCGACCC, *dys-1(eg33)*_R: TTTGCACACGTTGCATTTGG. In order to

480 simplify the nomenclature, we have renamed the crossed strain *dys-1(eg33)/PAP* to DP1
481 and the crossed strain *dys-1(cx18)/PAP* to DP2 throughout this manuscript.

482

483 **Preparation of PRE and POST symptomatic *C. elegans* PAT-Seq Strains**

484 *C. elegans* strains were divided into pre-symptomatic (PRE) and post-symptomatic
485 (POST) pools using mechanical filtration using pluriStrainer cell strainers (pluriSelect).

486 Mixed stage populations were harvested from nematode growth media (NGM) agar plates
487 seeded with OP50-1 and pelleted at 1,500 rpm. Solid pellets of approximately 2ml were
488 sequentially pipetted through 40 μ m and 20 μ m nylon cell strainers to isolate an
489 L3/L4/Adult population and an embryo/L1/L2 population, with the 40 μ m strainer retaining
490 adult enriched populations, which were combined with the L3/L4 populations retained by
491 the 20 μ m strainer.

492

493 **RNA Immunoprecipitation**

494 *C. elegans* strains used for RNA immunoprecipitations were maintained at 20°C on
495 nematode growth media (NGM) agar plates seeded with OP50-1. Populations were
496 passaged until a solid 1 ml pellet for mixed stage IPs and a solid 2 ml pellet for split stage
497 IPs was obtained after centrifugation at 1,500 rpm. Harvested worms were then
498 suspended and crosslinked in 0.5% paraformaldehyde solution for one hour at 4°C.
499 Worms were pelleted at 1,500 rpm, washed with M9 buffer, and flash frozen in an ethanol-
500 dry ice bath. Pellets are thawed on ice and suspended in 2 ml of lysis buffer (150 mM
501 NaCl, 25 mM HEPES, pH 7.5, 0.2 mM dithiothreitol (DTT), 30% glycerol, 0.0625%
502 RNAsin, 1% Triton X-100) [18]. Lysate was then subjected to sonication for five minutes at

503 4°C (amplitude 20%, 10 sec pulses, 50 sec rest between pulses) using a sonicator (Fisher
504 Scientific), and centrifuged at 21,000 x g for 15 min at 4°C. 1 ml of supernatant was added
505 per 100 µl of Anti-FLAG® M2 Magnetic Beads (Sigma-Aldrich) and incubated overnight at
506 4°C in a tube rotisserie rotator (Barnstead international). mRNA immunoprecipitations
507 were carried out as previously described [18, 19]. Total precipitated RNA was extracted
508 using Direct-zol RNA Miniprep Plus kit (R2070, Zymo Research), suspended in nuclease
509 free water and quantified with a Nanodrop® 2000c spectrophotometer (Thermo-Fisher
510 Scientific).

511

512 **cDNA Library Preparation and Sequencing**

513 We prepared a total of 16 cDNA libraries from *dys-1(eg33)* mixed stages, DP1
514 mixed stages , DP1 PRE1, DP1 PRE2, DP1 POST1, DP1 POST2, DP2 mixed stages,
515 DP2 PRE1, DP2 PRE2, DP2 POST1, DP2 POST2, PAP mixed stages, PAP PRE1, PAP
516 PRE2, PAP POST1 and PAP POST2. Each cDNA library was prepared using 100 ng of
517 precipitated RNAs. cDNA library preparation was performed using the SPIA (Single Primer
518 Isothermal Amplification) technology (IntegenX and NuGEN, San Carlos, CA) as
519 previously described [18, 19]. cDNA was then sheared using a Covaris Covaris S220
520 system (Covaris, Woburn, MA), and sample-specific barcodes were sequenced using the
521 HiSeq platform (Illumina, San Diego, CA). We obtained ~60-90M mappable reads (1x75)
522 each dataset.

523

524 **Bioinformatics Analysis**

525 Raw Reads Mapping: The raw reads were demultiplexed using their unique tissue-specific
526 barcodes and converted to FASTQ files. Unique datasets were then mapped to the *C.*
527 *elegans* gene model WS250 using the Bowtie 2 algorithm) [39] with the following
528 parameters: --local -D 20 -R 3 -L 11 -N 1 -p 40 --gbar 1 -mp 3. Mapped reads were further
529 converted into a bam format and sorted using SAMtools software using generic
530 parameters [40].

531 Cufflinks/Cuffdiff Analysis: Expression levels of individual transcripts were estimated from
532 the bam files by using Cufflinks software [41]. We calculated the fragment per kilobase per
533 million base (FPKM) number obtained in each experiments and performed a pairwise with
534 other tissues using the Cuffdiff algorithm [41]. We then used the median FPKM value ≥ 1
535 between each replicate as a threshold to define positive gene expression levels. The
536 results are shown in **Additional File 1: Tables S1-S3. Additional File 1: Table S4** was
537 compiled using scores produced by the Cuffdiff algorithm [41] and plot using the
538 CummeRbund package.

539

540 **Survival Curves**

541 Survival curves were performed as previously described [42]. Briefly, we prepared special
542 NGM plates each containing 330 μ l of 150 mM FUDR (Sigma Life Sciences, Darmstadt,
543 Germany). These plates were seeded with OP50-1 UV inactivated prior to plating worms
544 to minimize contamination. We plated 25 L4 worms from each strain per plate, each
545 across 3 replicate plates, and kept these plates in 18C incubators for the duration of the
546 experiment. For each time point, the plates were recovered, and worms were visually
547 inspected and counted directly in the plate using a dissection stereomicroscope (Leica

548 S6E) and a common cell counter. The strains were scored for survival every 48 hours, with
549 survival being defined as pharyngeal pumping or the ability to move the head in response
550 to prodding by a worm pick.

551

552 **RNAi screens at semi-permissive temperatures**

553 RNAi screens were performed using the temperature sensitive strains PD4605 (*hlh-*
554 *1(cc561)*) and LS587 (*hlh-1(cc561); dys-1(cx18)*). Strains were synchronized with bleach
555 as previously described [43] and eggs were incubated at permissive temperature (15°C)
556 until populations reached young adult stage. Young adults were then plated on NGM
557 plates seeded with OP50-1 with 5 worms per plate and incubated at semi-permissive
558 temperature (18°C) for 24 hours to allow young adults to lay eggs. Plates were then
559 recovered, and adult worms were removed, and plates containing eggs were returned to
560 semi-permissive temperature to incubate for 24 hours. Plates were then scored for gross
561 defects in body morphology and paralysis. We scored approximately 100 worms per plate
562 across 5 replicate plates for each strain and each gene tested [44].

563

564

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566

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572 **Competing interests:** The authors declare that they have no competing interests.

573

574 DATA AND MATERIALS AVAILABILITY

575 Raw reads were submitted to the NCBI Sequence Read Archive
576 (<http://trace.ncbi.nlm.nih.gov/Traces/sra/>). The results of our analyses are available in
577 Excel format as **Additional File 1: Table S3**.

578

579

580

581 MAIN FIGURES

582 **Fig. 1: *C. elegans* as a model system for human DMD.** (A) Diagram of functional protein
583 domains in human dystrophin and the *C. elegans* ortholog *dys-1*. The functional domains
584 of the human dystrophin protein are conserved in *C. elegans*. Mutational hotspot for DMD
585 mutations in humans is labeled in red. The mutant strains *dys-1(cx18)* and *dys-1(eg33)*
586 have nonsense mutations in the DMD ortholog *dys-1* in the actin binding and C-terminal
587 scaffolding domains respectively. (B) A diagram of a human sarcomere highlights the
588 human genes and associated *C. elegans* ortholog of several of the muscle structure
589 related genes that were selected for RNAi screens. (C) DP1 and DP2 strains retain *cx18*
590 and *eg33* mutations after being crossed with our PAP strain. Top panel: PCR analysis
591 from genomic DNA extracted from *dys-1(eg33)*, *dys-1(cx18)*, DP1, DP2 and PAP strains.

592 “+” denotes primers pairs that confirm the presence of a single copy integrated construct,
593 and “-” denotes primers pairs that confirm the absence of an integrated construct. Lower
594 panel: Trace files produced from the sequencing of genomic PCR of the *dys-1* locus
595 confirm the presence of the nonsense mutation in our DP1 and DP2 strains. After crosses
596 were performed, DP1 and DP2 strains retain both the associated nonsense mutations in
597 the dystrophin gene, and the presence of our integrated PolyA Pull construct. (D) *dys-1*
598 strains *dys-1(cx18)* and *dys-1(eg33)* exhibit a head bending phenotype. *wt* head bending
599 coincides with direction of movement (black arrows), while *dys-1* head bending opposes
600 direction of movement. (E) Confocal images of *wt* (DM8005, [myo-3p::GFP::myo-3 + rol-
601 6(su1006)]) and dystrophin deficient strains ([myo-3p::GFP::myo-3 + rol-6(su1006)];DP1)
602 for myosin heavy chain fibres. Right panels show an enlargement of the head muscles in
603 both strains. There are no major changes in muscle structure between *wt* and dystrophic
604 muscle in *C. elegans*.

605

606 **Fig. 2: Pipeline used to isolate PRE and POST paralysis muscle specific**

607 **transcriptomes.** PAP expression construct places a FLAG-tagged *pab-1* under the control
608 of a body muscle specific promotor (*Pmyo-3*) to isolate tissue-specific mRNA. PAP-
609 expressing worms are crossed with *dys-1(cx18)* and *dys-1(eg33)* strains to establish new
610 transgenic strains (DP1 and DP2) used in our muscle specific RNA-IPs. Worms were
611 grown in solid culture on NGM plates and are sequenced directly for mixed stage IPs. The
612 PRE and POST symptomatic datasets are obtained through sequential mechanical
613 filtration. Muscle specific RNA-IPs are performed and sequenced in duplicate.

614

615 **Fig. 3: Summary of the muscle specific PAT-Seq results.** The heat map summarizes
616 the average changes in gene expression of both dystrophin deficient strains (DP1 and
617 DP2) as compared to the *wt* strain. White boxes in the heat map mark genes that are
618 upregulated in DP1 and DP2 strains in PRE and POST datasets. An expansion of each of
619 these groups details relative change in expression level for genes selected based on
620 function and rank in our datasets. Below heat maps the change in expression level of
621 several genes are graphed linearly between PRE and POST symptomatic stages (green:
622 median between DP1 and DP2; orange: *wt*). GO term analysis of the top 50 genes
623 uniquely present either in our PRE or POST datasets shows an enrichment in genes
624 involved in mitochondria metabolism (PRE) and muscle biogenesis (POST).

625

626 **Fig. 4: A synthetic screen to identify genetically linked *dys-1* targets** (A) Single (*hlh-1*)
627 or double (*hlh-1; dys-1*) mutant sample populations scored for paralysis at semi-permissive
628 temperature after 48 hours after parent progeny are plated. Red arrows indicate worms
629 that were scored as paralyzed based on defects in morphology and resulting lack of
630 movement (B) Quantification and comparison of average incidence of paralysis for single
631 and double mutant strains at semi-permissive temperature. At this temperature *dys-1* is
632 responsible for a 21% increase in paralysis in the presence of an *hlh-1* sensitized genetic
633 background. Average % paralysis of 10 replicate plates summarized above each strain. A
634 total of 543 worms were scored for semi-permissive experiments without RNAi. (C)
635 Overview of semi permissive RNAi screen protocol describing which steps occur at
636 permissive and semi-permissive temperatures. (D) Percentage of paralyzed worms
637 following RNAi for *dyb-1*, with average % paralysis of 5 replicate plates summarized

638 above each strain. (E) Synthetic paralysis RNAi assay. Each gene is displayed as the
639 difference in paralysis observed between the *hlh-1* and *hlh-1;dys-1(cx18)* mutants and
640 normalized to the difference of paralysis obtained from semi-permissive control screens
641 (21%). Red dotted line indicates point of normalization for change in paralysis. Genes
642 were grouped according to function (muscle structure and signaling). Percent increase
643 change is shown on top of each bar chart.

644

645 DESCRIPTION OF ADDITIONAL DATA FILES

646

647 **Additional File 1: Figure 1:** Kaplan-Meier Survival Analysis. **Figure S2 :** Sequencing
648 results. **Figure S3:** Validation and comparison with other studies. Differential gene
649 expression analysis in PRE- and POST- symptomatic strains. **Figure S5:** Spearman
650 correlation of read counts in DP1 (cx18) and DP2 (eg33) PRE and POST datasets. **Table**
651 **S1:** Summary of results after deep sequencing. **Table S2:** Summary of sequencing results
652 after mapping genes to WS250.

653

654

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Fig. 1

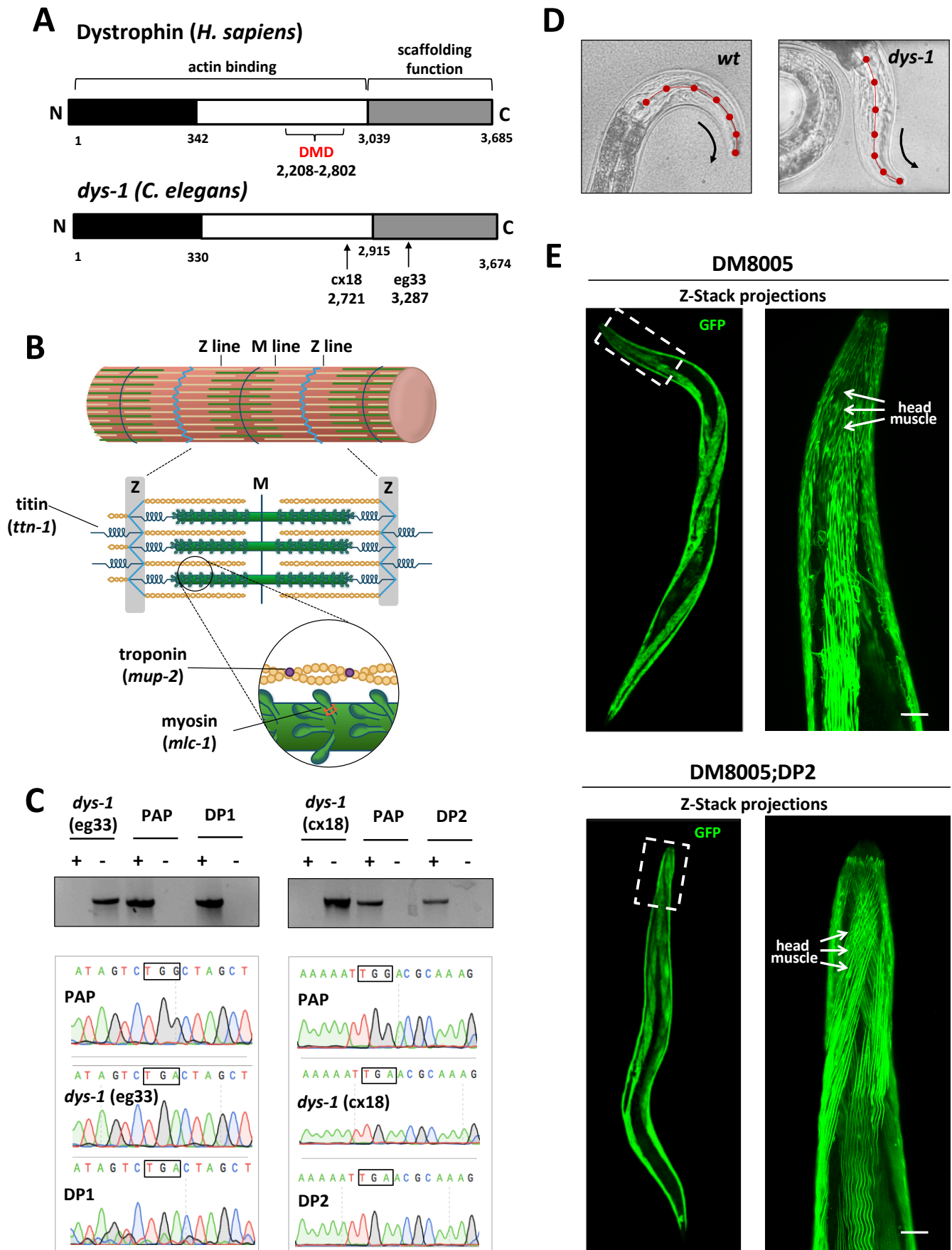


Fig. 2

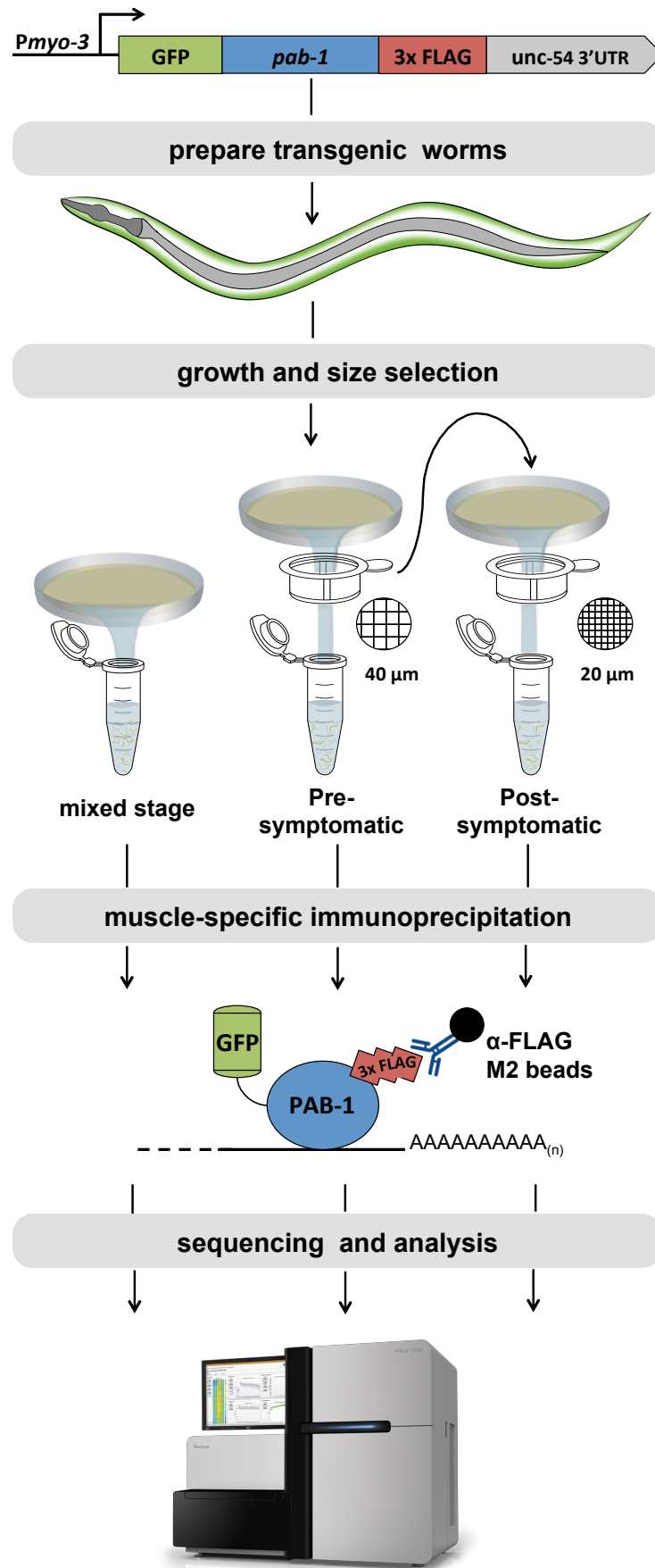
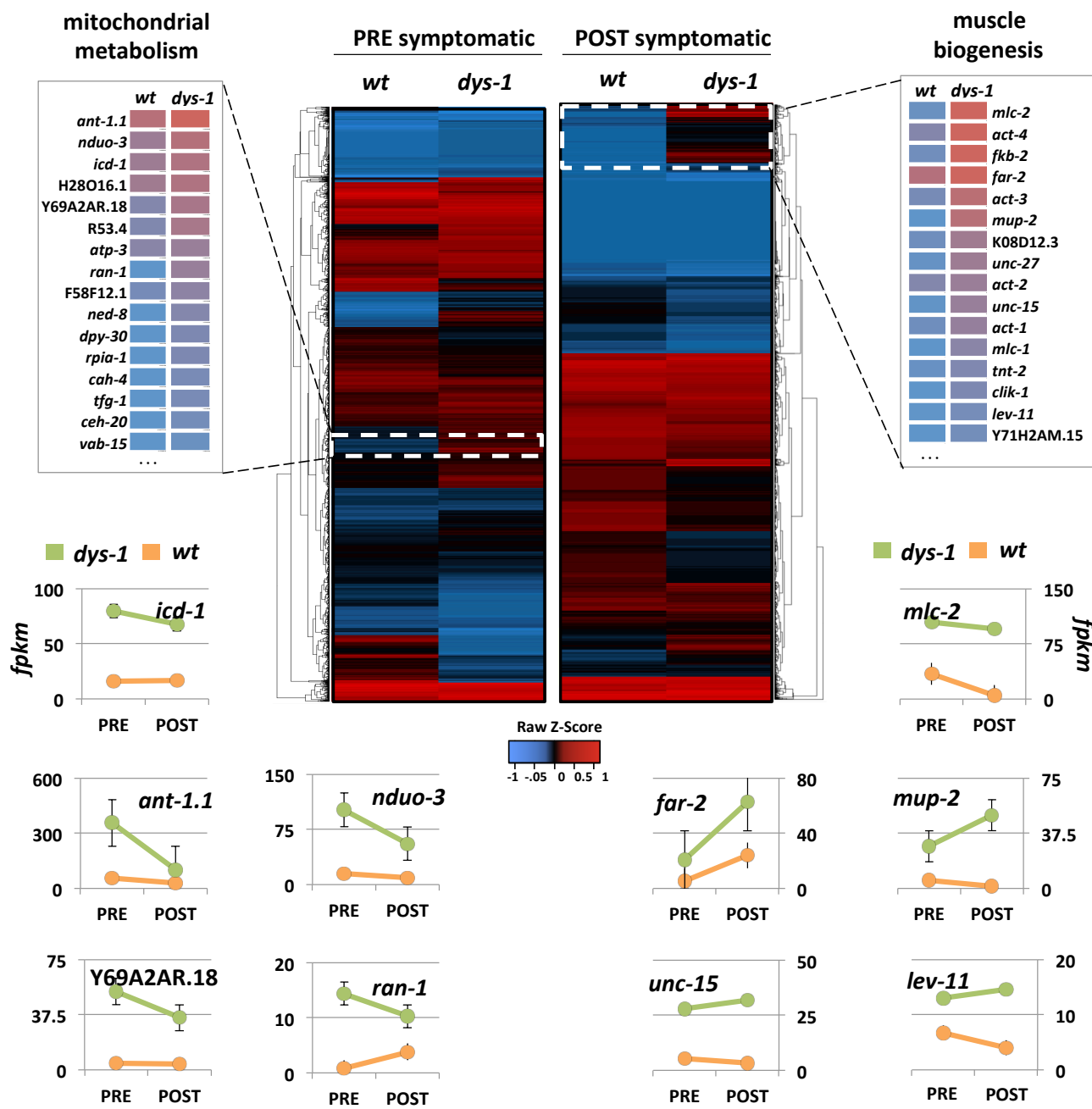


Fig. 3



PRE symptomatic

id	description	q-value	hits	total
GO:0005739	mitochondrion	6.96E-10	20	136
GO:0015992	proton transport	1.85E-03	5	11
GO:0044281	small molecule metabolic process	2.24E-03	16	229
GO:0006818	hydrogen transport	2.24E-03	5	12
GO:0003012	muscle system process	5.76E-03	7	42
GO:0006937	regulation of muscle contraction	1.05E-02	5	20
GO:0010941	regulation of cell death	1.44E-02	7	56
GO:0046034	ATP metabolic process	1.51E-02	5	24
GO:0015672	monovalent inorganic cation transport	1.51E-02	6	39
GO:0042981	regulation of apoptotic process	2.69E-02	5	29

POST symptomatic

id	description	q-value	hits	total
GO:0005856	cytoskeleton	1.29E-07	18	153
GO:0043292	contractile fiber	2.50E-05	5	11
GO:0046034	ATP metabolic process	4.90E-05	5	20
GO:0046034	ATP metabolic process	4.90E-05	7	24
GO:0005739	mitochondrion	1.27E-04	7	56
GO:0030239	myofibril assembly	1.67E-04	16	229
GO:0055001	muscle cell development	2.07E-04	5	12
GO:0051146	striated muscle cell differentiation	2.07E-04	13	145
GO:0061061	muscle structure development	3.34E-04	14	178
GO:0042692	muscle cell differentiation	4.44E-04	13	158

Fig. 4

