Research article

Itaconate and its derivatives repress C2C12 myogenesis via succinate dehydrogenase inhibition

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Abstract

A Krebs cycle intermediate metabolite, itaconate, has gained attention as a potential antimicrobial and autoimmune disease treatment due to its anti-inflammatory effects. While itaconate and its derivatives pose an attractive therapeutic option for the treatment of inflammatory diseases, the effects outside the immune system still remain limited, particularly in the muscle. Therefore, we endeavored to determine if itaconate signaling impacts muscle differentiation. Utilizing the well-established C2C12 model of *in vitro* myogenesis, we evaluated the effects of itaconate and its derivatives on transcriptional and protein markers of muscle differentiation as well as mitochondrial function. We found itaconate and the derivatives dimethyl itaconate and 4-octyl itaconate disrupt differentiation media-induced myogenesis. A primary biological effect of itaconate is a succinate dehydrogenase (SDH) inhibitor. We find the SDH inhibitors dimethyl malonate and harzianopyridone phenocopies the anti-myogenic effects of itaconate. Furthermore, we find treatment with exogenous succinate results in blunted myogenesis. Together our data indicate itaconate and its derivatives interfere with *in vitro* myogenesis, potentially through inhibition of SDH and subsequent succinate accumulation. More importantly, our findings suggest the therapeutic potential of itaconate and its derivatives could be limited due to deleterious effects on myogenesis.

Keywords: C2C12, myogenesis, itaconate, succinate, succinate dehydrogenase

44 **Introduction**

45 It is well appreciated that dramatic metabolic reprogramming occurs in response
46 to inflammatory challenges. One of the most robust metabolic adaptations made in
47 myeloid cells in response to inflammatory cues is the diversion of cis-aconitate away from
48 α-ketoglutarate towards the synthesis of itaconate (1,2). One primary function itaconate
49 serves is to reduce inflammation via the inhibition of succinate dehydrogenase (SDH)
50 thereby reducing accumulation of reactive oxygen species (ROS) (1). Due to its potent
51 anti-inflammatory properties, itaconate and its derivatives such as dimethyl itaconate
52 (DMI) and 4-octyl itaconate (4-OI) have gained attention as potential treatments for
53 autoimmune conditions such as psoriasis (3), multiple sclerosis (4), rheumatoid arthritis
54 (5), and systemic lupus erythematosus (6).

55 While the signaling attributes of itaconate with regards to immune function are well
56 delineated, not much is known on the actions of itaconate in non-immune organs. What
57 is known is that itaconate possesses anti-inflammatory, anti-fibrotic and pro-survival
58 attributes in organs such as the liver (7) and kidney (8). However, the effects of itaconate
59 regulation of muscle function is yet unknown.

60 Given that chronic inflammation is hypothesized to incur sustained itaconate
61 production, coupled to its potential therapeutic use for the treatment of a variety of
62 diseases, it is of the upmost importance to understand potential off target tissues and
63 effects in the face of elevated itaconate levels. To address this critical gap in knowledge
64 we utilized the well-established C2C12 murine myoblast model system for *in vitro*
65 myogenesis and studied the effects of itaconate and its derivatives on altering myoblast
66 differentiation.

Results

Dimethyl itaconate inhibits myogenesis in C2C12 cells

Past research has shown that Krebs cycle metabolites serve as signal transducers in the immune response within macrophages (9). We endeavored to evaluate how the presence of itaconate affects myogenic differentiation within C2C12 cells. Given its recent focus as a potential treatment for autoimmune conditions, the function of dimethyl itaconate (DMI) was first investigated. Gene expression markers for myogenesis (i.e. *Myh1*, *Myh2*, *Myh7*, *Tnnt1*, and *Tnnt3*) were measured in the presence of DMI versus vehicle over a 4-day differentiation protocol. As expected, vehicle group cells had a time-dependent increase in the transcription of myogenic genes (Fig. 1a). However, treatment with DMI significantly inhibited the induction of these myogenic markers during our differentiation protocol (Fig. 1a).

To further determine how DMI impairs myogenesis, we next assessed the expression of the essential myogenic transcription factors *Myog* and *Myod.* The expression of *Myog* mRNA was significantly impaired by DMI throughout day 1 to day 4 of differentiation compared to the control (Fig. 1b). Protein levels of MYOD were not different in the DMI groups compared to the control in response to differentiation media (DM). However, MYOG expression was almost completely abolished by DMI (Fig. 1c). Consequently, DMI-treated C2C12 myoblasts failed to differentiate into myotubes. DMI-treated groups showed no trace of MYHC in response to DM cues, while vehicle-treated cells displayed a time-dependent increase in MYHC protein (Fig. 1c). MYHC was visualized by immunofluorescence at day 4 of differentiation (Fig. 1d) and myogenic index was significantly impaired by DMI (Fig. 1e).

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91 **4-octyl itaconate inhibits myogenesis in C2C12 cells**

92 We next tested another analog of itaconate since dimethyl itaconate cannot be
93 endogenously converted back to intracellular itaconate (10). 4-OI was subsequently used
94 because it has been suggested as a possible treatment for autoimmune conditions, but
95 unlike DMI, it can be endogenously converted to itaconate (10,11). We analyzed C2C12
96 cells treated with 4-OI over 4 days in the presence of DM. 4-OI significantly inhibited the
97 induction of myogenic gene transcription (Fig. 2a). Transcription of *Myog* was also blunted
98 in response to 4-OI exposure (Fig. 2b). 4-OI significantly blocked MYOG and MYHC
99 expression levels although it did not change MYOD levels (Fig. 2c). Subsequently, 4-OI
100 led to significant decreases in both MYHC staining (Fig. 2d) and myogenic index (Fig.
101 2e).

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103 **Physiological itaconate inhibits myogenesis**

104 To investigate a role of physiological itaconate, C2C12 myoblast cells were then
105 treated with DM only or DM plus itaconate and then examined over 4 days. It was reported
106 that exogenous itaconate readily enters cells (9). Itaconate was used to portray
107 physiological mechanisms more accurately. Transcription levels of *Myh2* and *Myh7* were
108 not significantly impacted by itaconate exposure. However, the blunted induction of *Myh1*,
109 *Tnnt1*, and *Tnnt3* was significant on the 4th day of exposure (Fig. 3a). Significant inhibition
110 of *Myog* transcription on days 2 and 4 was also observed in itaconate groups (Fig. 3b).

111 MYOG and MYHC expression were also inhibited in itaconate groups (Fig 3c). Itaconate
112 treatment reduced MYHC staining (Fig. 3d) and myogenic index (Fig. 3e).

113 Overall DMI shows the strongest myogenic inhibitory response and itaconate has
114 the weakest amount of myogenic suppression among itaconate and derivatives tested.
115 Taken together, these results suggest that itaconate and its derivatives are sufficient to
116 inhibit myogenesis in C2C12 cells, modulating myogenic regulating factors at the
117 transcription and protein levels.

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119 **Malonate mimics itaconate-like inhibition of myogenesis**

120 Given that both itaconate and malonate are Krebs cycle metabolites that are
121 shown to inhibit SDH (1,12), our next step was to determine whether malonate exposure
122 could also inhibit C2C12 myogenesis. Malonate was administrated instead of itaconate
123 under similar parameters to those discussed in Figures 1 through 3. Malonate inhibited
124 the induction of myogenic markers during myogenesis (Fig. 4a). This inhibition was more
125 severe than itaconate but not as extreme as the inhibition caused by itaconate derivatives.
126 Malonate lowered *Myog* levels compared to the control at all the time points (Fig. 4b).
127 MYOD levels were similar in malonate-treated cells during differentiation but malonate
128 led to a reduction in MYOG levels, followed by a significant decrease in MYHC levels
129 (Fig. 4c). Malonate showed similar extent of reduction in MYHC staining (Fig. 4d) and
130 myogenic index (Fig. 4e) as itaconate. Collectively, these data suggest another SDH
131 inhibitor, phenocopies the inhibition of myogenesis.

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133 **Pharmacological succinate dehydrogenase inhibitor obstructs myogenesis**

134 Krebs cycle metabolites which inhibit SDH have been corroborated to suppress
135 myogenesis. Our next course of action was to determine whether this specific shared
136 inhibition was the primary inducer of myogenesis repression. Harzianopyridone (Harz) is
137 a potent and specific inhibitor of SDH that is used as an antibiotic and antifungal treatment
138 (13). We examined C2C12 cells exposed to Harz using the same methods to elucidate
139 whether SDH inhibition independent of the Krebs cycle might also elicit a dramatic
140 obstruction of myogenesis. Harz treatment resulted in significant suppression of
141 myogenic markers (Fig. 5a). Consistent suppression of *Myog* expression was also
142 observed following Harz treatment throughout the time course (Fig. 5b). MYOG and
143 MYHC protein expression levels were also significantly reduced (Fig. 5c). Harz displayed
144 the most robust inhibition of myogenesis shown by MYHC staining (Fig. 5d) and myogenic
145 index (Fig. 5e). These data show pharmacological inhibition of SDH blocks myotube
146 formation. The degree of suppression caused by Harz was most comparable to the
147 amount of inhibition by DMI. In tandem, these findings support that SDH activity affects
148 myogenesis in C2C12 cells.

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150 **Myogenesis is inhibited by exogenous succinate**

151 Inhibition of SDH activity can lead to an increase in intracellular succinate levels
152 (14). Given that SDH inhibition alone is able to significantly inhibit myogenesis, we next
153 asked if an abundance of succinate could be the connecting factor leading to myogenic
154 inhibition. Thus, we tested if exogenous succinate can cause myogenic failure in C2C12

155 cells treated with DM. All the data with succinate represented a very similar trend to
156 previous findings. Myogenic gene expression (Fig. 6a), *Myog* (Fig. 6b), and myogenic
157 proteins (Fig. 6c) were all inhibited by the exogenous succinate treatment. Reduced
158 MYHC expression (Fig. 6d) and myogenic index (Fig. 6e) by succinate indicate its
159 inhibitory potency of myogenesis. The degree of suppression was most similar to those
160 results observed in groups treated with itaconate or malonate. These results support a
161 possibility that inhibition of myogenesis by itaconate or malonate is at least partly due to
162 accumulated succinate caused by SDH inhibition.

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176 **Discussion**

177 Itaconate has become the epitome of metabolites that also have
178 immunomodulation properties. It is a decarboxylated cis-aconitate which is primarily
179 known for its SDH inhibiting activity in the TCA cycle. While initially used as a polymer for
180 industrial purposes, it has been researched to have both antimicrobial and anti-
181 inflammatory properties. Some bacteria (e.g. *Yersinia pestis*) have itaconate degrading
182 enzymes and itaconate itself has been observed to restrict the growth of bacteria by
183 inhibiting isocitrate lyase activity (15). It also regulates host mechanisms. Its anti-
184 inflammatory role has gained the most attention recently. Itaconate is observed to
185 significantly increase in response to macrophage activation (12). It acts to reduce
186 inflammation by inhibiting the release of proinflammatory cytokines through a plethora of
187 mechanisms, inhibiting ROS production through suppression of SDH activity (1),
188 activating the regulator of antioxidant expression NRF2 (16), and activating the anti-
189 inflammatory signaling transcription factor ATF3 (3). These effects in tandem have
190 attracted scientists to get approval for itaconate derivatives as potential treatments to limit
191 microbial pathogenicity and to attenuate autoimmune disease symptoms.

192 DMI and 4-OI have been researched to have promising effects on psoriasis,
193 rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus due to their
194 ability to suppress IL-17 signaling and reduce proinflammatory cytokine production (3,6).
195 While seemingly propitious as a potential treatment, the fact is that knowledge is very
196 limited on the effects of itaconate and its derivates outside of those relating to the
197 immunomodulatory axis. A consequence of itaconate derivative use may be one or more

198 harmful side effects which could lessen enthusiasm for their utilization as treatments.
199 Blocking itaconate signaling, which has yet to be proposed, might also be used to
200 attenuate symptoms of other conditions. The focus of our research is to showcase the
201 additional effects of itaconate on muscle regulation. Chronic inflammation has been linked
202 to muscle wasting. Prolonged itaconate exposure resulting from chronic inflammation
203 may cause or exacerbate muscle wasting.

204 Our hypothesis is that itaconate obstructs myogenesis via inhibition of SDH. This
205 hypothesis arose from research done on the effects of succinate abundance on muscle
206 wasting. Succinate is a substrate for SDH, so its intracellular concentration is directly
207 linked to SDH activity. Succinate abundance has been proposed as a causative agent of
208 muscle wasting. Treatments to elevate intracellular succinate concentration has been
209 observed to decrease total muscle protein expression by ~25%, inhibit in vitro C2C12
210 myoblast cell myogenesis, decrease myofiber diameter in murine models, inhibit
211 myogenesis in vivo following barium chloride injury, reduce respiration capacity by ~35%
212 in myoblasts, and also disrupt a number of other metabolic processes within muscle cells
213 (17). SDH has also been shown to have significantly reduced activity in sarcopenic
214 muscle (18). Given that anti-inflammatory activity of itaconate is incurred via SDH
215 inhibition, we endeavored to determine if itaconate treatment led to similar effects seen
216 in succinate abundant cells.

217 We first tested the proposed treatment forms of itaconate, DMI, and 4-OI. We then
218 examined itaconate alone followed by malonate, a pharmacological inhibitor of SDH, and
219 then lastly diethyl succinate. Treatment incurred transcriptional modulation of *Myh1,*
220 *Myh2, Myh7, Tnnt1, and Tnnt3* along with alteration of myogenic transcription factor

221 (mainly MYOG) and myogenesis marker MYHC protein expression were examined. Our
222 results showcased substantial inhibition of myogenesis in C2C12 cells by DMI. 4-OI
223 treatment also exhibited significant inhibition, but not as aggressive as DMI. Itaconate
224 treatment also resulted in significant myogenesis inhibition, but not to the degree seen in
225 either DMI or 4-OI treated groups. It is possible that inability of DMI conversion to
226 itaconate leads to a strong inhibition of myogenesis exhibiting sustained activity because
227 4-OI can be converted to itaconate, but DMI cannot (10). It is established that itaconate
228 can be converted to itaconyl-CoA by succinate-CoA ligase (19). A possible mechanism
229 to explain this tiered order of inhibition severity may involve itaconate conversion to
230 itaconyl-CoA. Regardless of potency, all itaconate treatments inhibited *in vitro*
231 myogenesis.

232 We show that SDH suppression and subsequent succinate accumulation
233 significantly inhibit myogenesis via myogenic transcription suppression. Given that
234 MYOG expression is dependent upon MYOD activity, it is hypothesized that MYOD
235 activity was also suppressed in the treatment groups, but via post translational
236 modification that leads to changes in MYOD activity such as phosphorylation and
237 acetylation (20,21) rather than transcriptional inhibition. Inactive MYOD in response to
238 SDH inhibition and succinate causes blunted induction of *Myog* thereby suppressing the
239 transcription of myogenic genes. Together, our results support that itaconate may
240 contribute to impairment of myogenesis via its effects on SDH which modulates myogenic
241 transcription mechanisms.

242 Sarcopenia patients have been observed to have inhibited mitochondrial function
243 via transcriptional downregulation of key genes involved in oxidative phosphorylation and

244 mitochondrial proteostasis (18). 4-OI has been shown to increase aerobic respiration by
245 reducing glycolytic function through inhibition of GAPDH (11). This data may implicate
246 that in addition to subsequent muscle wasting induced by increased succinate
247 concentration, prolonged itaconate activity may exacerbate issues relating to
248 mitochondrial function. What demands further study is the determination of whether
249 itaconate and its derivatives induce mitochondrial activity suppression which may cause
250 subsequent inhibition of the oxidative phosphorylation pathway and thus reduce energy
251 demand accommodation capability.

252 In this study, we find that itaconate and derivatives contribute to suppression of
253 myogenesis. What remains to be researched is how itaconate affects muscle regulation
254 outside of succinate accumulation. Our results showcase functions of itaconate outside
255 of immunomodulation and implicate that derivative use to treat autoimmune diseases or
256 microbial infections should be cautioned due to a potential for deleterious effects on
257 myogenesis. Further elucidation of these functions may lead to the development of
258 treatments to attenuate muscle wasting via possible itaconate signaling inhibition.

Materials and methods

Antibodies and chemical reagents

Primary antibodies used in this study are as follows: GAPDH (Santa Cruz Biotechnology, sc-32233), MYHC (Sigma, 05-716), MYOD (Santa Cruz Biotechnology, sc-377460), and MYOG (Santa Cruz Biotechnology, sc-12732). Reagents added to the media are as follows: Diethyl succinate (Sigma, 112402), Dimethyl itaconate (Sigma, 592498), Dimethyl malonate (Sigma 136441), Harzianopyridone (Santa Cruz Biotechnology, sc-280769), Itaconic acid (Sigma, I29204), and 4-octyl itaconate (Tocris Bioscience, 6662).

Cell culture and differentiation

The mouse myogenic C2C12 myoblasts were maintained on plastic cell culture plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified incubator kept at 37 °C and 5% CO_2. Cells were used up until passage 9. For differentiation, cells at 90% confluency were serum restricted with differentiation medium (DMEM, 2% horse serum, 1% penicillin-streptomycin) for up to 4 days. Growth or differentiation medium was replenished every day.

RNA isolation and RT-qPCR

279 RNA isolation was performed using the commercially available Aurum RNA
280 miniprep kit (Bio-rad, 732-6820). Gene expression analysis was conducted with 50 ng
281 RNA using iTaq Universal One-Step RT-qPCR Kit (Bio-rad, 1725140). The reaction was
282 carried out according to the manufacturer's instructions using CFX Connect Real-Time
283 PCR Detection System (Bio-rad, 1855200). Probes used for TaqMan® Gene Expression
284 Assays (ThermoFisher) were as follows: *Myog* (Mm00446194_m1), *Myh1*
285 (Mm01332489_m1), *Myh2* (Mm01332564_m1), *Myh7* (Mm00600555_m1), *Ppib*
286 (Mm00478295_m1), *Tnnt1* (Mm00449089_m1), and *Tnnt3* (Mm01137842_g1).

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288 **Western blot**

289 Cells were lysed in RIPA buffer (ThermoFisher, 89901) in the presence of Halt™
290 Protease and Phosphatase Inhibitor Cocktail (ThermoFisher, 78440), and protein
291 concentration was measured using Pierce™ BCA Protein Assay Kit (ThermoFisher,
292 23227). Lysates were boiled for 5 min with 4x Laemmli Sample Buffer (Bio-Rad, 1610747)
293 and 2-mercaptoethanol (Bio-Rad, 1610710). Lysates were resolved on SDS
294 polyacrylamide gels and blotted onto PVDF membranes using Trans-Blot Turbo RTA Midi
295 0.45 µm LF PVDF Transfer Kit (Bio-Rad, 1704275). Transfer was run in Trans-Blot Turbo
296 Transfer System (Bio-Rad, 1704150) using Mixed MW (1.3A-25V-7M) protocol. The
297 membranes were blocked with Intercept™ (TBS) Blocking Buffer (LI-COR, 927-60001)
298 for 1 h at room temperature and incubated with appropriate primary antibodies at 4 °C
299 overnight. After 3 washes with 0.05% tween20 (Bio-Rad) in TBS buffer (Bio-Rad), the
300 membrane was incubated with IRDye® 800CW Goat anti-Mouse IgG (LI-COR, 926-
301 32210) and IRDye® 680RD Goat anti-Rabbit IgG (LI-COR, 926-68071) secondary

302 antibodies for 1 h at room temperature. Band images were visualized using ChemiDoc™
303 MP Imaging System (Bio-Rad, 12003154).

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305 **Immunofluorescence and calculation of myogenic index**

306 Cells were seeded in a 24-well cell culture plate and followed by above-mentioned
307 differentiation protocol. At day 4 of differentiation, cells were rinsed with PBS and fixed
308 with 4% paraformaldehyde for 10 min at room temperature. Then, cells were
309 permeablized with 0.5% Triton™ X-100 (Fisher, BP151-100) in PBS for 10 min at room
310 temperature. Next, cells were submerged with Intercept® (PBS) Blocking Buffer (LI-COR,
311 927-70001) for 1 h at room temperature. MYHC primary antibody was diluted to 1:100 in
312 blocking buffer and incubated overnight at 4 °C with the fixed cells. Next day, after 3
313 washes with 0.05% tween20 in PBS, Alexa Fluor 594 secondary antibody (Fisher,
314 A11005) was diluted to 1:500 in blocking buffer and incubated with the cells for 1 h at
315 room temperature. Followed by further washes, cells were mounted with VECTASHIELD®
316 Antifade Mounting Medium with DAPI (Vectalabs, H-1200) and covered with circular
317 cover glass. Images were taken using ZOE™ Fluorescent Cell Imager (Bio-rad,
318 1450031). Myogenic index was calculated as the percentage of nuclei in fused myotubes
319 (MYHC positive) out of the total nuclei in given images. Distribution of nuclei in myoblasts
320 and myotubes was measured by counting over 100 nuclei at 4 distinct locations. The
321 number of nuclei was counted using imageJ.

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323 **Statistical analysis**

Unpaired t-test or two-way ANOVA with multiple comparisons was performed to determine significant relationships between groups in the experiments. Data are represented as mean ± SEM.

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410 **Figure legend**

411 **Fig. 1. Dimethyl itaconate (DMI) represses C2C12 myogenesis by modulating**
412 **myogenic transcription mechanisms.** (a) 4-day time course showcasing fold change
413 differences of myogenic gene transcription levels detected via RT-qPCR when exposed
414 to differential media with or without DMI relative to the untreated group. (b) Fold change
415 of *Myog* mRNA expression at various time points comparing DM control groups to DM
416 and DMI groups relative to expression without either treatment. (c) Western blotting data
417 showing differences in myogenic transcription factor expression between DM treated
418 groups and groups treated with both DM and DMI over a 4-day time course as well as
419 groups which were not exposed to either treatment for any period. (d) MYHC staining at
420 day 4 of differentiation. Bar: 100 μm. (e) Relative myogenic index (percentage of total
421 nuclei associated with myotubes). ** denotes $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ based
422 on two-way ANOVA.

423

424 **Fig. 2. 4-octyl itaconate (4-OI) represses C2C12 myogenesis by modulating**
425 **myogenic transcription mechanisms.** (a) 4-day time course showcasing fold change
426 differences of myogenic gene transcription levels detected via RT-qPCR when exposed
427 to differential media with or without 4-OI relative to the untreated group. (b) Fold change
428 of *Myog* mRNA expression at various time points comparing DM control groups to DM
429 and 4-OI groups relative to expression without either treatment. (c) Western blotting data
430 showing differences in myogenic transcription factor expression between DM treated
431 groups and groups treated with both DM and 4-OI over a 4 day time course as well as
432 groups which were not exposed to either treatment for any period. (d) MYHC staining at

433 day 4 of differentiation. Bar: 100 μm. (e) Relative myogenic index (percentage of total
434 nuclei associated with myotubes). *** denotes $p < 0.001$, ****$p < 0.0001$ based on two-
435 way ANOVA.

436

437 **Fig. 3. Itaconate represses C2C12 myogenesis by modulating myogenic**
438 **transcription mechanisms.** (a) 4-day time course showcasing fold change differences
439 of myogenic gene transcription levels detected via RT-qPCR when exposed to differential
440 media with or without itaconate relative to the untreated group. (b) Fold change of *Myog*
441 mRNA expression at various time points comparing DM control groups to DM and
442 itaconate groups relative to expression without either treatment. (c) Western blotting data
443 showing differences in myogenic transcription factor expression between DM treated
444 groups and groups treated with both DM and itaconate over a 4 day time course as well
445 as groups which were not exposed to either treatment for any period. (d) MYHC staining
446 at day 4 of differentiation. Bar: 100 μm. (e) Relative myogenic index (percentage of total
447 nuclei associated with myotubes). * denotes $p < 0.05$, **$p < 0.01$, ***$p < 0.001$,
448 ****$p < 0.0001$ based on two-way ANOVA.

449

450 **Fig. 4. Malonate exposure represses C2C12 myogenesis via similar mechanism of**
451 **action to that of itaconate and its derivatives.** (a) 4-day time course showcasing fold
452 change differences of myogenic gene transcription levels detected via RT-qPCR when
453 exposed to differential media with or without DMM relative to the untreated group. (b) Fold
454 change of *Myog* expression at various time points comparing DM control groups to DM

455 and malonate groups relative to expression without either treatment. (c) Western blotting
456 data showing differences in myogenic transcription factor expression between DM treated
457 groups and groups treated with both DM and malonate over a 4 day time course as well
458 as groups which were not exposed to either treatment for any period. (d) MYHC staining
459 at day 4 of differentiation. Bar: 100 μm. (e) Relative myogenic index (percentage of total
460 nuclei associated with myotubes). * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,
461 **** $p < 0.0001$ based on two-way ANOVA.

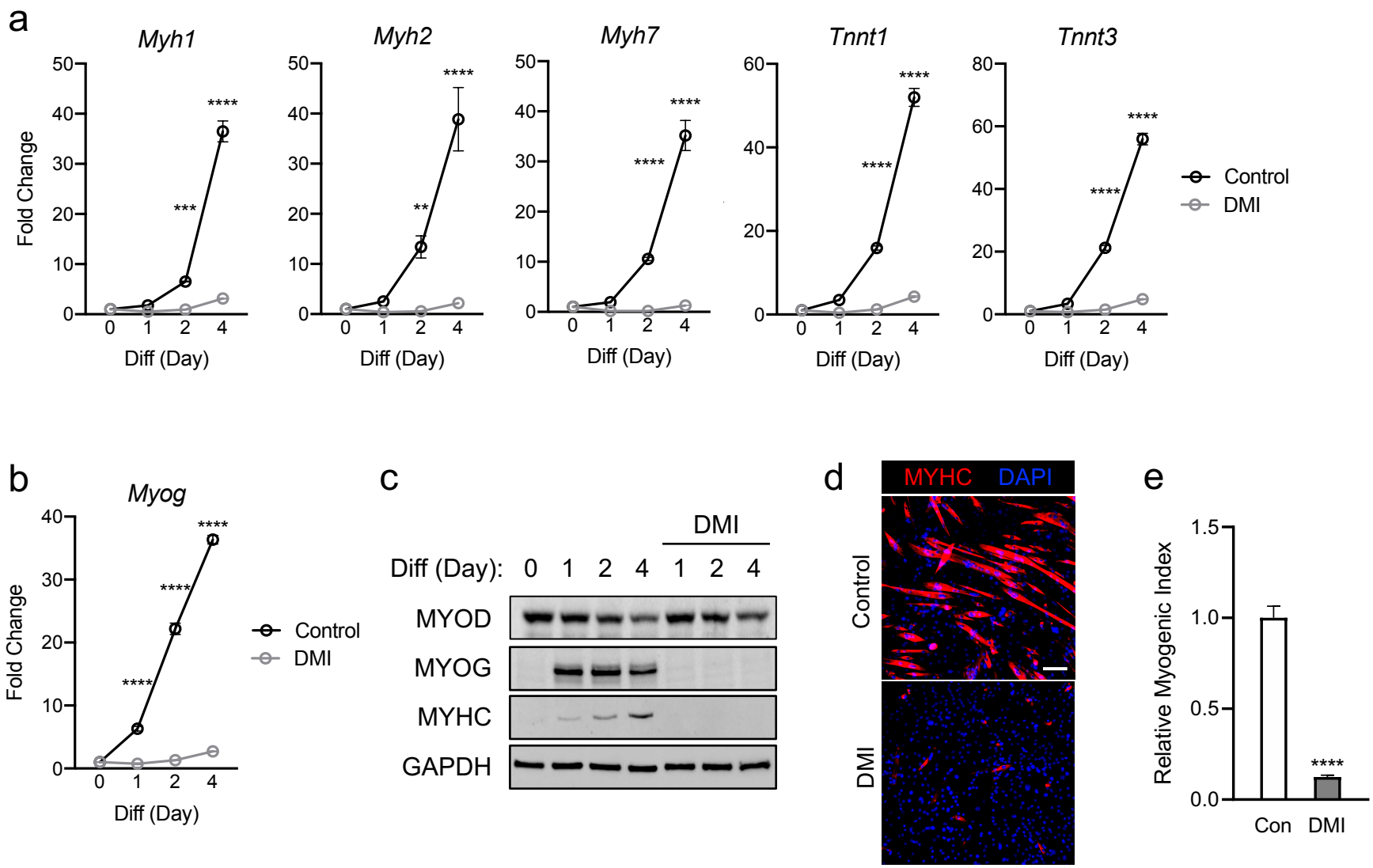
462

463 **Fig. 5. Exposure to pharmacological inhibitor of succinate dehydrogenase**
464 **harzianopyridone (Harz) obstructs C2C12 myogenesis via mechanisms similar to**
465 **that of those observed due to itaconate and malonate exposure.** (a) 4-day time
466 course showcasing fold change differences of myogenic gene transcription levels
467 detected via RT-qPCR when exposed to differential media with or without Harz relative
468 to the untreated group. (b) Fold change of *Myog* mRNA expression at various time points
469 comparing DM control groups to DM and Harz groups relative to expression without either
470 treatment. (c) Western blotting data showing differences in myogenic transcription factor
471 expression between DM treated groups and groups treated with both DM and Harz over
472 a 4 day time course as well as groups which were not exposed to either treatment for any
473 period. (d) MYHC staining at day 4 of differentiation. Bar: 100 μm. (e) Relative myogenic
474 index (percentage of total nuclei associated with myotubes). * denotes $p < 0.05$,
475 ** $p < 0.01$, **** $p < 0.0001$ based on two-way ANOVA.

476

477 **Fig. 6. Elevated exposure to succinate (diethyl succinate: DES) elicits inhibition of**
478 **C2C12 myogenesis via mechanism similar to those observed when exposing cells**
479 **to itaconate and malonate.** (a) 4-day time course showcasing fold change differences
480 of myogenic gene transcription levels detected via RT-qPCR when exposed to differential
481 media with or without DES relative to the untreated group. (b) Fold change of *Myog*
482 expression at various time points comparing DM control groups to DM and DES groups
483 relative to expression without either treatment. (c) Western blotting data showing
484 differences in myogenic transcription factor expression between DM treated groups and
485 groups treated with both DM and DES over a 4 day time course as well as groups which
486 were not exposed to either treatment for any period. (d) MYHC staining at day 4 of
487 differentiation. Bar: 100 μm. (e) Relative myogenic index (percentage of total nuclei
488 associated with myotubes). * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$
489 based on two-way ANOVA.

Fig. 1.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Myh7
Tnnt1
Tnnt3
Fold Change
Diff (Day)
Control
DMI
b
Myog
c
DMI
Diff (Day): 0 1 2 4 1 2 4
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MYOG
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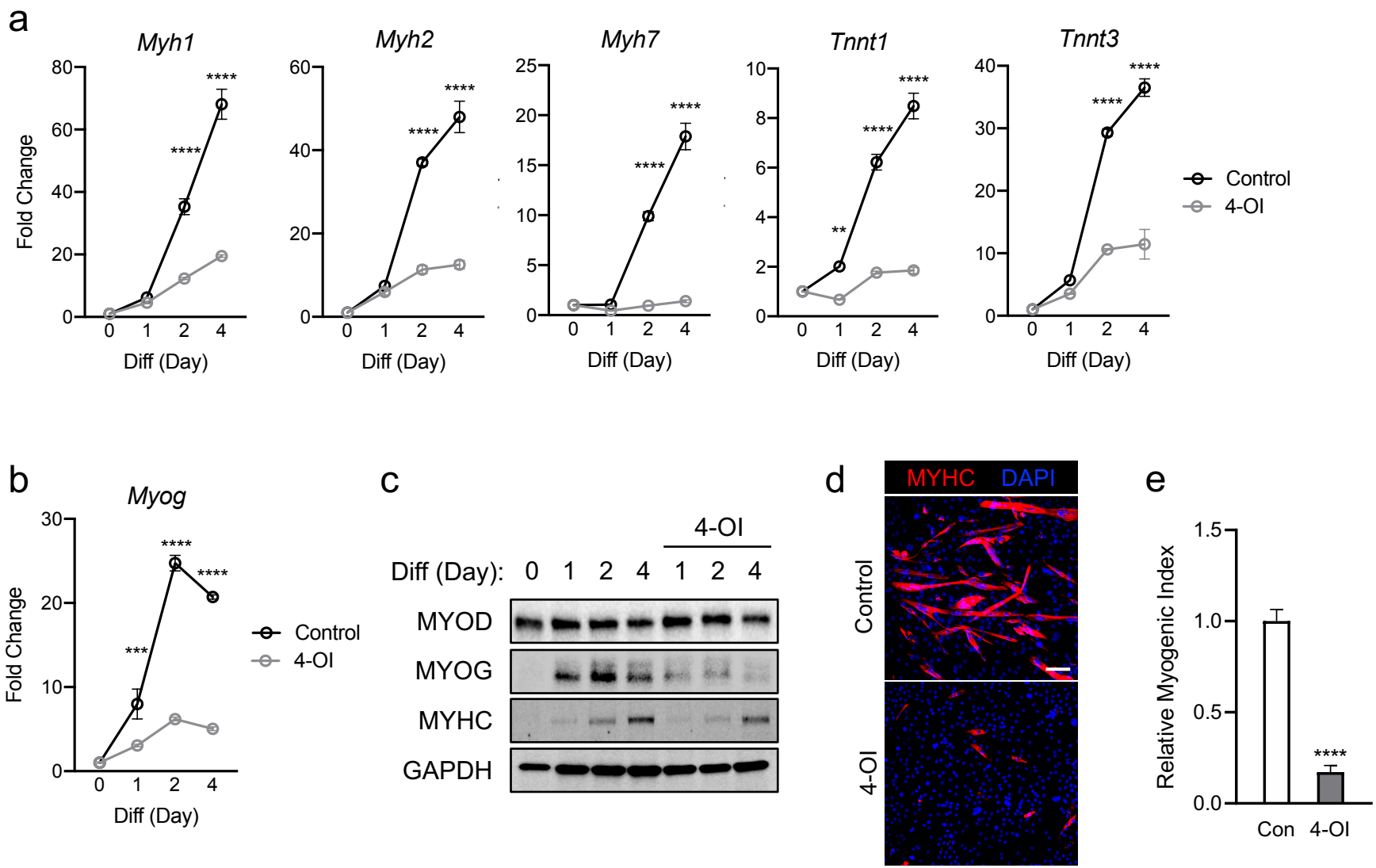
GAPDH
d
MYHC DAPI
Control
DMI
e
Relative Myogenic Index
Con
DMI

Fig. 2.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Diff (Day)
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Myog
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4-OI
Diff (Day): 0 1 2 4 1 2 4
MYOD
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MYHC
GAPDH
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MYHC DAPI
Control
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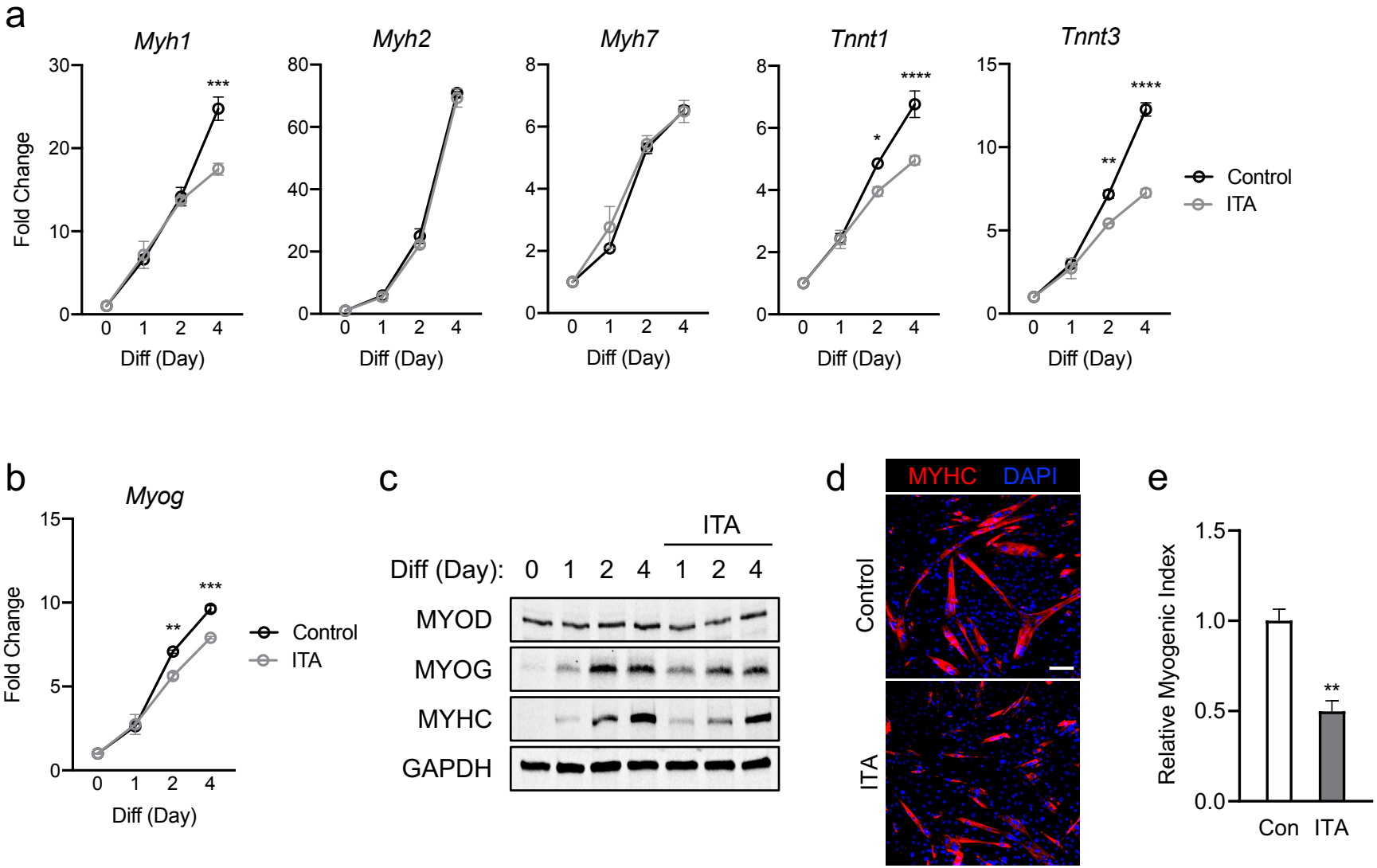
e
Relative Myogenic Index
Con 4-OI

Fig. 3.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Tnnt1
Tnnt3
Fold Change
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GAPDH
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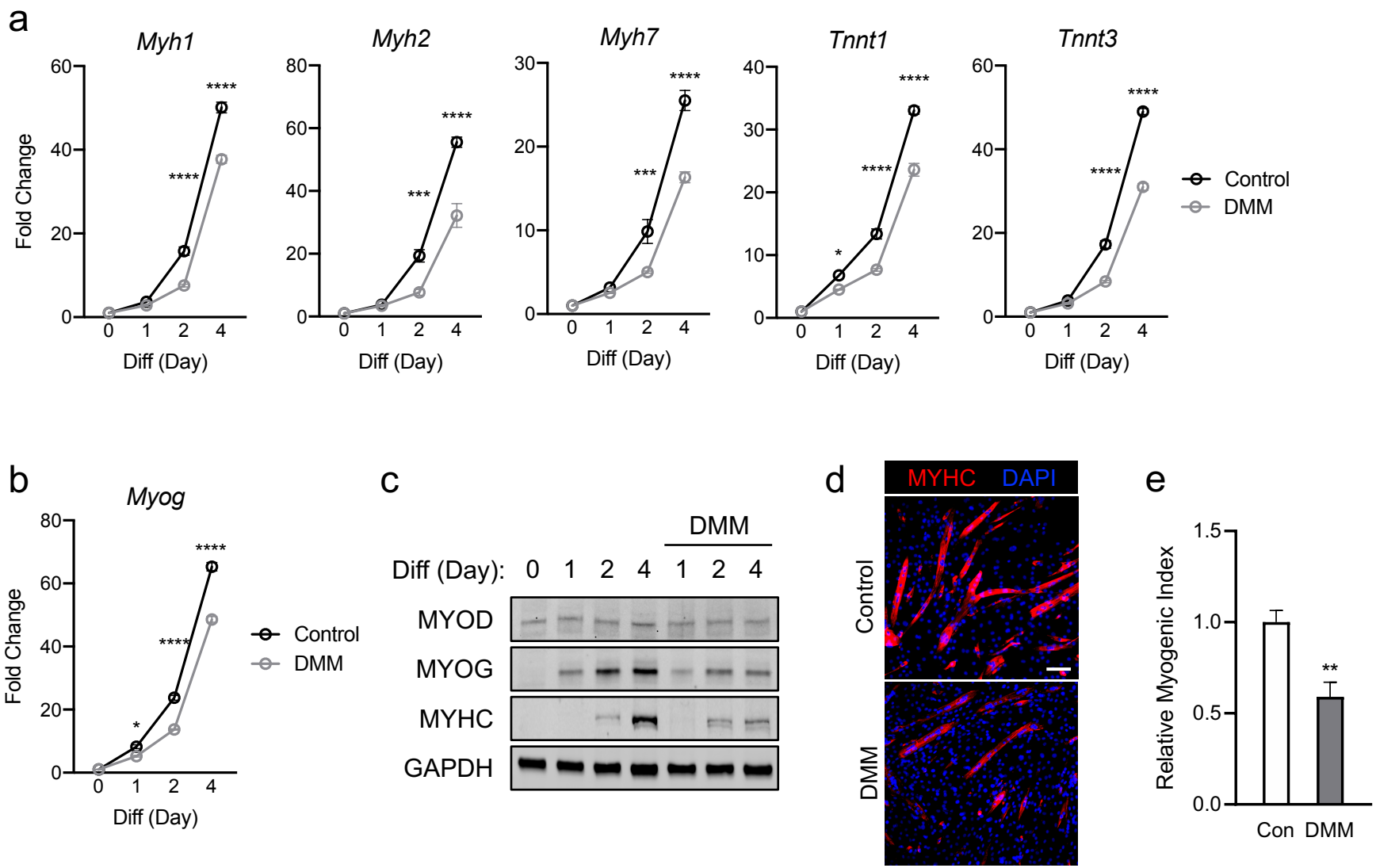
Fig. 4.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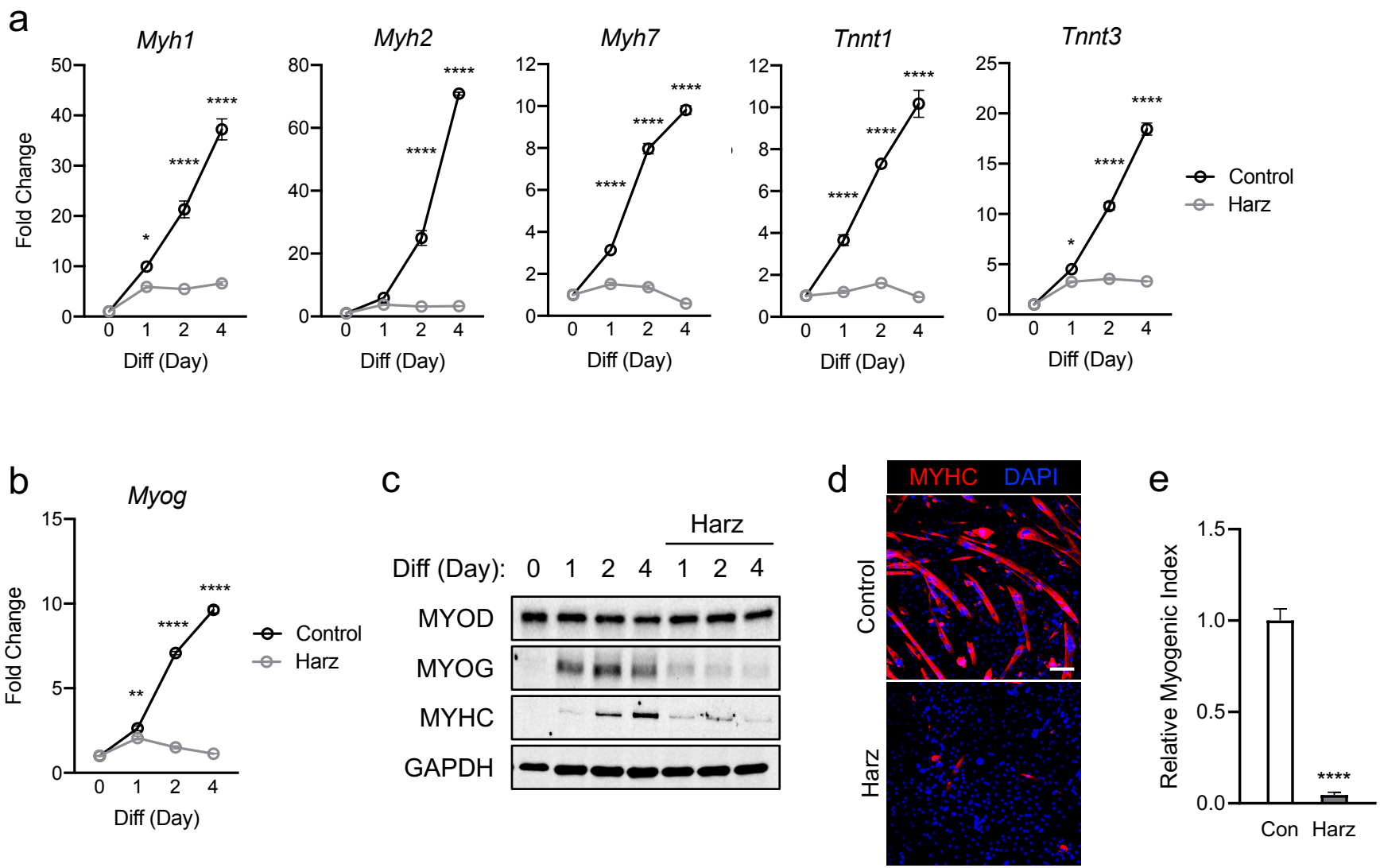
Myh1
Myh2
Myh7
Tnnt1
Tnnt3
Fold Change
Diff (Day)
Control
DMM
b
Myog
c
DMM
Diff (Day): 0 1 2 4 1 2 4
MYOD
MYOG
MYHC
GAPDH
d
MYHC DAPI
Control
DMM
e
Relative Myogenic Index
Con DMM

Fig. 5.


a
Myh1
Myh2
Myh7
Tnnt1
Tnnt3
Fold Change
Diff (Day)
Control
Harz
b
Myog
c
Harz
Diff (Day): 0 1 2 4 1 2 4
MYOD
MYOG
MYHC
GAPDH
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MYHC DAPI
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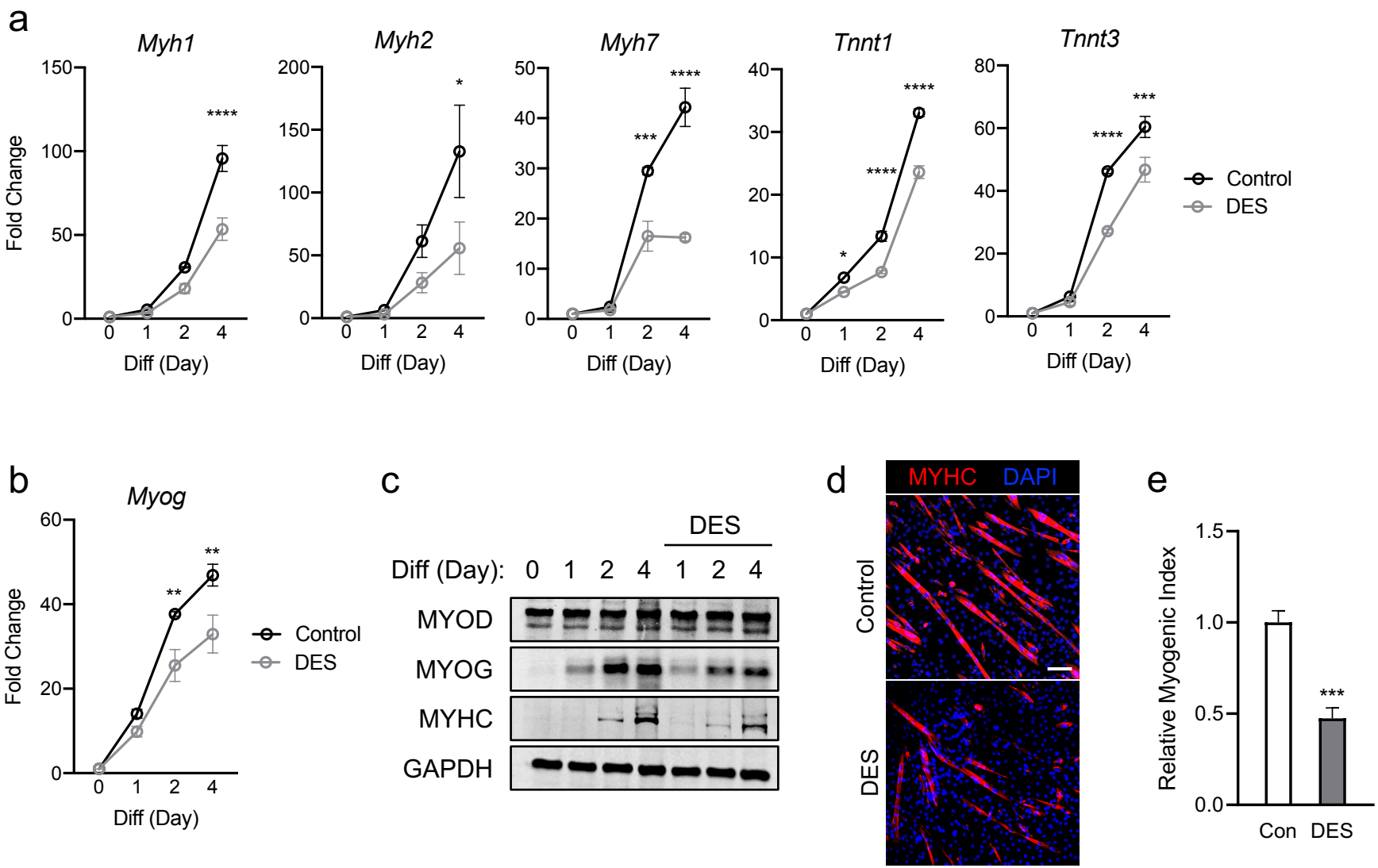
e
Relative Myogenic Index
Con Harz

Fig. 6.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Myh1
Myh2
Myh7
Tnnt1
Tnnt3
Fold Change
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DES
b
Myog
c
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MYOD
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GAPDH
d
MYHC DAPI
Control
DES
e
Relative Myogenic Index
Con DES