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► To cite this version:

Florence Delort, Bertrand-David Segard, Coralie Hakibilen, Fany Bourgois-Rocha, Eva Cabet, et al.. Alterations of redox dynamics and desmin post-translational modifications in skeletal muscle models of desminopathies. *Experimental Cell Research*, 2019, 383 (2), pp.111539. 10.1016/j.yexcr.2019.111539 . hal-02374328

HAL Id: hal-02374328

<https://hal.science/hal-02374328>

Submitted on 21 Nov 2019

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1 **Research Article**

2 **Alterations of redox dynamics and desmin post-translational**
3 **modifications in skeletal muscle models of desminopathies**

4 **Florence Delort^a, Bertrand-David Segard^a, Coralie Hakibilen^a, Fany Bourgois-**
5 **Rocha^a, Eva Cabet^a, Patrick Vicart^a, Meng-Er Huang^b, Guilhem Clary ^c, Alain**
6 **Lilienbaum^a, Onnik Agbulut^d, and Sabrina Batonnet-Pichon^a**

7 ^aUniversité de Paris, Unité de Biologie Fonctionnelle et Adaptative, CNRS UMR
8 8251, F-75013 Paris, FRANCE.

9

10 ^bInstitut Curie, PSL Research University, CNRS UMR3348, Université Paris-Sud,
11 Université Paris-Saclay, Orsay, 91405, France.

12

13 ^cInserm U1016, Institut Cochin, CNRS UMR8104, Université Paris-Descartes,
14 Sorbonne Paris Cité, Plateforme Protéomique 3P5, Paris, France.

15

16 ^dSorbonne Université, Institut de Biologie Paris-Seine (IBPS), CNRS UMR 8256,
17 Inserm ERL U1164, Biological Adaptation and Ageing, 75005, Paris, France.

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21 **Abbreviations:** IF, intermediate filament; ROS, reactive oxygen species; WT,
22 wildtype; PTM, post-translational modifications; NAC, N-acetyl-L-cysteine; GSSG,
23 oxidized glutathione; GSH, reduced glutathione; AAV, adeno-associated-virus; TA,
24 tibialis anterior; DMEM, Dulbecco's Modified Eagle's medium; HE, hematoxylin/eosin;
25 SDH, succinate dehydrogenase

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27
28

Abstract:

29 Desminopathies are a type of myofibrillar myopathy resulting from mutations in *DES*,
30 encoding the intermediate filament protein desmin. They display heterogeneous
31 phenotypes, suggesting environment influences. Patient muscle proteins show
32 oxidative features linking oxidative stress, protein aggregation, and abnormal protein
33 deposition. To improve understanding of redox balance in desminopathies, we further
34 developed cellular models of four pathological mutants localized in 2B helical domain
35 (the most important region for desmin polymerization) to explore desmin behavior
36 upon oxidative stress. We show that the mutations desQ389P and desD399Y share
37 common stress-induced aggregates, desR406W presents more scattered
38 cytoplasmic aggregative pattern, and pretreatment with N-acetyl-L-cysteine (NAC),
39 an antioxidant molecule, prevents all type of aggregation. Mutants desD399Y and
40 desR406W had delayed oxidation kinetics following H₂O₂ stress prevented by N-
41 acetyl-L-cysteine pretreatment. Further, we used AAV-injected mouse models to
42 confirm *in vivo* effects of N-acetyl-L-cysteine. AAV-desD399Y-injected muscles
43 displayed similar physio-pathological characteristics as observed in patients.
44 However, after 2 months of N-acetyl-L-cysteine treatment, they did not have reduced
45 aggregates. Finally, in both models, stress induced some post-translational
46 modifications changing Isoelectric Point, such as potential hyperphosphorylations,
47 and/or molecular weight of human desmin by proteolysis. However, each mutant
48 presented its own pattern that seemed to be post-aggregative. In conclusion, our
49 results indicate that individual desmin mutations have unique pathological molecular
50 mechanisms partly linked to alteration of redox homeostasis. Integrating these
51 mutant-specific behaviors will be important when considering future therapeutics.

52

53 **Highlights**

54 ► Desmin mutations trigger variable aggregative patterns and NAC pretreatment
55 avoids it.

56 ► Desmin mutations induce delayed oxidation kinetic with H₂O₂ stress, which are
57 prevented by NAC.

58 ► Stresses induce own post-aggregative post-translational modifications, which are
59 prevented by NAC.

60 ► Distinct pathological molecular mechanisms of desmin mutations weigh on
61 therapeutics.

62 **Keywords:** desmin, oxidative stress, aggregation, N-acetyl-L-cysteine, myopathies,
63 intermediate filaments.

64 **Introduction:**

65 Desmin is a muscle-specific type III intermediate filament (IF). All IFs share a
66 common tripartite organization characterized by a central alpha-helical coiled coil-
67 forming region (rod-domain) and two non-alpha-helical regions (“head” and “tail”) with
68 variable lengths and sequences (1) as represented for desmin in figure 1A. This
69 structure allows protein monomers to assemble (2,3) and ultimately form networks
70 within cells. Thus, desmin participates in cytoskeleton formation, crosslinking
71 myofibrils and connecting them to mitochondria (4), nuclei, membrane desmosomal
72 proteins in cardiac cells (2,3), or costamers in skeletal muscle (4,5).

73 Currently, ~70 mutations within the human desmin gene, *DES* (HUGO Gene
74 Nomenclature Committee database #2770), lead to desminopathies, a subcategory

75 of myofibrillar myopathies that mostly arise between the second and fifth decade of
76 life (6,7). Individuals with desminopathies present skeletal or cardiac muscle defects
77 (8) that exhibit heterogeneous features, such as progressive skeletal myopathy (9),
78 different types of cardiomyopathy (10), or both (11–14). The same point mutation can
79 produce different phenotypes within a family, suggesting that genetic factors as well
80 as environmental (such as intensive sport practice, alimentation, anxiety...) influence
81 disease progression (15).

82 Most desmin mutations are missense and affect the 2B end of the protein rod central
83 dimerization domain, leading to desmin network disorganization (16). The main
84 histological characteristics are myofibrillar disorganization, sarcomere misalignment,
85 and desmin-containing protein aggregates in the sarcoplasm. Many studies have
86 investigated how desmin mutations affect the capacity of recombinant proteins to
87 self-assemble in filaments *in vitro*, as well as in transient cell experiments with or
88 without another IF (e.g., vimentin) (17). Indeed, several mutant monomers
89 polymerize normally *in vitro*, while some present irregular-diameter filaments, such as
90 desD399Y (17,18). For other mutants, the assembly process stops at intermediate
91 steps, such as tetramer, unit-length filament, filament elongation, or IF maturation
92 (17).

93 Thus, desmin assembly and/or desmin aggregation seem to depend on sequence,
94 without obvious links to mutation position. Moreover, some post-translational
95 modifications (PTMs) of desmin or other IFs have been linked to
96 assembly/disassembly (17). Modifications of PTMs pattern have also recently been
97 highlighted in myofibrillar (19,20) or cardiac pathology (21) context.

98 In addition, mouse *Des*^{-/-} muscle cell mitochondria exhibit an increase in size and
99 number, loss of correct positioning, and pronounced degeneration after work
100 overload (22,23). Further, desL345P transgenic (24) or desR349P knock-in (KI) mice
101 also present mitochondria mislocalization or enzyme dysfunction (25). These
102 alterations indicate a link between oxidative metabolism and desmin.

103 Skeletal muscle fiber metabolism constantly generates reactive oxygen species
104 (ROS) with level depending to exercise intensity and regularity (26). Redox
105 homeostasis continually neutralizes ROS with antioxidant protective molecules that
106 belong to three main types: enzymes that can metabolize ROS and environmental
107 oxygen peroxide (H₂O₂), endogenous non-enzymatic compounds, and exogenous
108 non-enzymatic compounds (e.g., provided by food). Among endogenous
109 antioxidants, glutathione appears to be most important in the context of muscular
110 fatigue—the ratio between oxidized glutathione [disulfide glutathione (GSSG)] and
111 reduced glutathione (GSH) is an indicator of redox status in cells or tissues (27).
112 Among exogenous antioxidants, N-acetyl-L-cysteine (NAC) is known to counteract
113 the increased levels of ROS and then to provide effects within muscle cells. In fact,
114 this precursor of endogenous antioxidant, increases levels of the reduced glutathione
115 before, during, and after exercise (28).

116 Imbalanced redox homeostasis is a pathogenic mechanism and thus potential
117 therapeutic target in myopathies. Indeed, SEPN1-related myopathy, RYR1-related
118 myopathy, or Duchenne muscular dystrophy have direct defects in redox regulation
119 systems or have secondary redox abnormalities (29). Previously, pretreatment with
120 NAC before H₂O₂ exposure significantly improved cell survival in SelN-devoid
121 myoblasts, as well as in controls, whereas other food antioxidants did not (30).

122 Further, a link between oxidative stress and desmin protein aggregation has been
123 shown in patient biopsies. Indeed, main glycooxidation marker (advanced glycation
124 end product, N-carboxy-methyl-lysine, or N-carboxy-ethyl-lysine) levels are increased
125 in muscle samples from individuals with desminopathies (31). Moreover, desmin is a
126 major target of these oxidation and nitration modifications (32).

127 As patient cells are rarely available, we previously constructed isogenic cell lines
128 expressing inducible wildtype (desWT) or mutant desmin—with mutations located in
129 the head, rod, and tail domains (desS46Y, desD399Y, and desS460I, respectively)—
130 to explore protein aggregation mechanisms. Only desD399Y is sensitive to stresses,
131 such as heat shock or redox environmental changes (H₂O₂ or cadmium treatment). In
132 addition, some antioxidant molecules, such as N-acetyl-L-cysteine (NAC) (33) and
133 alpha-tocopherol (34), can prevent stress-induced aggregation.

134 Here, we first further expanded our inducible cell lines to other 2B mutations. Indeed,
135 mutations in the end of the 2B domain of desmin are predominant (~66%) in human
136 patients with desminopathies and lead to musculoskeletal and cardiac phenotypes of
137 variable severity (35). This area also contains a stutter region, a conserved sequence
138 important for filament assembly and stabilization (36). Thus, the 2B region seems
139 essential for polymerization of the desmin network, perhaps making it susceptible to
140 stress. Moreover A357P, Q389P, and R406W mutations share with D399Y a
141 common skeletal muscle phenotype in patients (distal and proximal myopathy).
142 Patients also mainly present respiratory insufficiency (except for Q389P) and cardiac
143 defects (except for A357P) (for review see 17). R406W leads to the most severe
144 phenotype and presents particular subsarcolemmal desmin accumulation in skeletal
145 muscle.

146 Mutations introducing proline in the C-terminal part of the 2B domain are the most
147 frequent (12 out of 24) (35). They lead to desmin aggregation and affect cellular
148 assembly of IFs (37). However, not all mutations are dominant—A360P is recessive
149 (13). We chosed to study at least two mutations carrying a proline: A357P and
150 Q389P. These two mutations present a phenotype, especially on skeletal muscles, in
151 agreement with our model of myoblasts. In addition to these two mutations, R406W
152 substitution affects the YRKLLLEGEE region, another extremely conserved and
153 important domain in the regulation of network assembly (38–40).

154 Thus, we investigated whether the aggregative behaviors in stress conditions and the
155 response to NAC pretreatment depend on the 2B location or on the specific mutation.
156 Furthermore, we also analyzed the link between 2B mutations presenting stress
157 induced aggregation and redox dynamics. To confirm the effect of NAC in a more
158 physiological context, we performed experiments with injection of adeno-associated-
159 virus (AAV), expressing WT or mutated desmin, in mouse tibialis anterior (TA)
160 muscle, with or without antioxidant treatment. Finally, to understand these behaviors,
161 we analyzed post-translational modifications (PTMs) of desmin in cell or in muscle.
162 Indeed, as described for other intermediate filaments, some PTMs, such as
163 phosphorylation, ubiquitination, sumoylation, glycosylation, and/or ADP ribosylation,
164 are known to modify their behavior (41). Thus, they could also regulate desmin in
165 response to stress.

166 Altogether, desmin cytoplasmic redox dynamic and PTMs alterations in our models
167 provide new information on the variability and importance of oxidative redox balance
168 in skeletal muscular cells containing a pathological variant of desmin.

169

170 **Materials and methods:**

171 **Molecular model and secondary structure predictions**

172 The crystal structure of human vimentin coil 2B fragment (CYS2) [residues 328
173 (Cys328)–411; PDB entry 1GK4] was used to construct desmin 2B end domain
174 models (WT and mutant) with PYMOL software (DeLano Scientific LLC, Palo Alto,
175 CA, United States). For energy minimization, computations were done in vacuo with
176 GROMOS96 43B1 parameters set, without reaction field, from Swiss-PdbViewer
177 4.0.1 (<http://spdbv.vitalit.ch/download.html>). We used Pubmed Blast
178 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for sequence alignment of CYS2 (residues
179 328–411) and desmin (residues 333–416).

180 **Cell lines and culture**

181 New stable cell lines from murine C2C12 myoblasts (ATCC, NY, USA) were
182 generated as previously described (33). Briefly, mutagenesis was performed on
183 pPuro-Myc-human-Desmin vector to introduce human mutations desA357P,
184 desQ389P, and desR406W, following manufacturer's instructions (Quick Change-XL
185 mutagenesis kit, Stratagene, San Diego, CA, United States). Plasmids were
186 electroporated (Genepulser II, BioRad, Hercules, CA, United States; 500 μ F, 350 V,
187 with 10 μ g of DNA) into clone 21 (tet-on) cells (33). Stable clones were selected after
188 one week of puromycin selection. Doxycycline induction of human desmin variants
189 was verified by western blotting (using c-Myc epitope antibody (1/1000, 9E10, Santa
190 Cruz Biotechnologies, Dallas, TX, United States)) and immunostaining. Double stable
191 cell lines were grown at 37°C and 5% CO₂ in Dulbecco's Modified Eagle's medium
192 (DMEM, Life Technologies, Carlsbad, CA, United States) supplemented with 20%

193 fetal calf serum (Eurobio, Les Ulis, France), 1% penicillin/streptomycin (Life
194 Technologies, Carlsbad, CA, United States), 1 mg/mL G418 (Euromedex,
195 Souffelweyersheim, France), and 2 µg/mL puromycin (Euromedex,
196 Souffelweyersheim, France).

197 Satellite cells were extracted from 2 gastrocnemius and 2 plantaris muscles of 1-
198 month-old C56Bl6(N) knock-in mice for the R405W mutation (murine homologue of
199 human desmin R406W; named after KI-R405W) (12). Following previous work (42),
200 muscles were incubated 4 x 10 min in a solution of DMEM-10 HamF12/Glutamax
201 (Life Technologies, Carlsbad, CA, United States) containing 1.5 mg/mL of protease
202 XIV (Sigma-Aldrich, Saint-Louis, MO, United States) and 1/500 primocin (Invitrogen,
203 Carlsbad, CA, United States) at 37°C under regular agitation. After each incubation,
204 tubes were centrifuged at 400 g for 30 seconds at room temperature. Floating cells
205 were eliminated, and 3 other fractions containing satellite cells were diluted in 20%
206 FBS medium and sieved with a 40-µm filter. Filtrate containing satellite cells was
207 centrifuged for 5 min at 1400 g at room temperature. Cells in DMEM-
208 HamF12/Glutamax containing 20% FBS, 2% Ultrosor (Pall Life Sciences,
209 Portsmouth, United Kingdom), 8.6 ng/mL of FGF2 (Life Technologies, Carlsbad, CA,
210 United States), 1/100 N2 (Life Technologies, Carlsbad, CA, United States), and
211 1/500 primocin were seeded onto 1/20 Matrigel-coated 6-well plates (Corning,
212 Corning, NY, United States) at 1000 cells/well. After activation and proliferation,
213 satellite cells were maintained or analyzed by seeding at 1000 cells/cm² on plastic
214 covered with 1/20 Matrigel.

215 **Oxidative and heat stress procedures**

216 As previously described, stable cell lines were seeded at 3×10^3 cells/cm² in 6-well
217 plates and induced with doxycycline (10 µg/mL, Sigma-Aldrich, Saint-Louis, MO,
218 United States) after 24 h (28). Hydrogen peroxide and heat stresses were performed
219 48 h after induction as follows: 0.2 mM H₂O₂ (Sigma-Aldrich, Saint-Louis, MO, United
220 States) was added to the cell culture for 2 h or cells were heat shocked at 42°C for 2
221 h. Cells were immediately analyzed (T0 time point) or media was replaced and cells
222 were treated 24 h later (T24 time point). For pre-treatment, NAC (10 mM, #A7250
223 Sigma-Aldrich, Saint-Louis, MO, United States) was added to cells 16 h before
224 stress.

225 **Western blotting**

226 Myoblast proteins were extracted by scraping cells in RIPA buffer without SDS [50
227 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP40, 1 mM Na₃VO₄, 10 mM NaF,
228 1 mM PMSF, protease inhibitor cocktail 1X (complete mini, EDTA free, Roche, Bâle,
229 Switzerland)] for 1D SDS-PAGE or urea buffer (8 M urea, 2 M thiourea, 4% CHAPS,
230 50 mM DTT) for 2D SDS-PAGE. After migration, proteins were transferred to
231 nitrocellulose membranes (0.45 µm, Macherey Nagel, Düren, Germany), which were
232 saturated with 5% non-fat milk in 0.5% Tween/PBS. Membranes were incubated with
233 the primary antibody at the appropriate dilution for 1 h at room temperature or 16 h at
234 4°C. Primary antibodies used were: 1- mouse monoclonal anti-c-Myc (1/1000, 9E10,
235 Santa Cruz Biotechnologies, Dallas, TX, United States); 2- rabbit polyclonal anti-
236 desmin (1/1000, # C3956, Sigma-Aldrich, Saint-Louis, MO, United States); 3- mouse
237 monoclonal anti-alpha-actin (1/2000, #MAB1501R, Millipore, Burlington, MA, United
238 States); or 4- rabbit polyclonal anti-GFP (1/2500, A11122, Life Technologies,
239 Carlsbad, CA, United States). Isotype-specific anti-mouse or anti-rabbit secondary

240 antibody coupled with horseradish peroxidase (1/10000, #31430 or #31460, Pierce,
241 Thermo Scientific, Waltham, MA, United States) was detected by incubating with
242 Clarity Western ECL (BioRad, Hercules, CA, United States) and visualized with a
243 CCD camera (FUJI Las 4000 or Ai600, GE Healthcare, Chicago, IL, United States).

244 **Cell immunofluorescence**

245 Cells were fixed with 2% paraformaldehyde (Santa Cruz Biotechnologies, Dallas, TX,
246 United States) for 15 min at room temperature, permeabilized 5 min with 0.5% Triton
247 X-100, and incubated with mouse monoclonal anti-c-Myc primary antibody (1/100,
248 9E10 #sc40 Santa Cruz, Biotechnologies, Dallas, TX, United States) for 1 h at room
249 temperature. After 3 washes with PBS, cells were incubated 45 min with isotype-
250 specific anti-mouse secondary antibody labeled with Alexa-488 (#A11001, Molecular
251 Probes, Eugene, OR, United States). DNA was stained with Hoechst 33258 (1
252 µg/mL, Sigma-Aldrich, Saint-Louis, MO, United States) for 10 min. Finally, cells were
253 washed in PBS and mounted in Fluoromount medium (Interchim, San Diego, CA,
254 United States). Images were acquired with confocal microscopy (LSM700 Zeiss,
255 Jena, Germany) at the imaging facility of the Functional and Adaptive Biology (BFA)
256 unit.

257 For aggregation measurements, images were taken as 5x5 tile scans on 3 random
258 nuclear fields chosen in Hoechst-stained areas. Total cell number was calculated by
259 counting nuclei with an ImageJ software in-house macro. Cells with aggregates were
260 visually counted on the images. Aggregation percentage and ratio between stressed
261 versus unstressed aggregated cells were calculated. Experiment was repeated at
262 least 3 times.

263 **Redox dynamics**

264 Mammalian cell vector expressing cytoplasm-targeted HyPer (cyto-HyPer) was
265 constructed by amplifying HyPer sequence (Evrogen, Moscow, Russia, (43)) and
266 introducing a Nuclear Export Signal (NES). The PCR product, fusing NES in frame to
267 the 3' end of HyPer, was then cloned in pCMV/myc/cyto vector (Life Technologies,
268 Carlsbad, CA, United States). Vector expressing cytoplasm-targeted Grx1-roGFP2
269 (cyto-Grx1-roGFP2) was constructed in a similar way using pEIGW-Grx1-roGFP2
270 (Addgene, Cambridge, MA, United States (44)) as template. Expression vector
271 encoding HyPer targeted to mitochondrial matrix (mito-HyPer) was purchased from
272 Evrogen (Moscow, Russia). Stable cell lines or satellite cells were seeded at 11,500
273 cells/cm² or 22,000 cells/cm², respectively, in 6-well plates. After 24 h, cells were
274 transfected with cyto-HyPer, mito-HyPer, or cyto-Grx1-roGFP2 in DMEM medium
275 without antibiotics using JetPRIME (Polyplus-transfection, Illkirch-Graffenstaden,
276 France) according to manufacturer's instructions. Eight hours after transfection,
277 antibiotics were added, including doxycycline for desmin induction. Transfection
278 efficiency was evaluated on an inverted fluorescence microscope with a FITC filter
279 (Zeiss, Jena, Germany) at the imaging facility of the BFA unit.

280 Oxidative kinetics were started by adding 0.1 mM H₂O₂ to cells in complete media
281 and incubating for 5, 15, 30, 60, or 120 min. Medium was aspirated and cells were
282 washed with PBS and then overlaid with 15% trichloroacetic acid. After 30 min, cells
283 were scraped and pelleted in 1.5-mL microtubes. After two washes with ice-cold
284 acetone and air drying, pellets were resuspended in TES buffer (100 mM Tris pH 8.8,
285 10 mM EDTA, 1% SDS). Loading buffer without reducing agent was added to
286 samples. Equal volumes were loaded on 12% SDS acrylamide gels and results were
287 analyzed by western blotting.

288 **Intramuscular injection of AAV vectors**

289 Plasmids encoding c-Myc-tagged human desmin (desWT and desD399Y) under the
290 regulation of the CMV promoter were used to prepare AAV vectors.
291 AAV2/2.CMV.Myc-desWT (AAV-desWT) or Myc-desD399Y (AAV-desD399Y) were
292 provided by the Vector Production Center of the Therapeutic Research Institute at
293 the Health Research Institute of the University of Nantes (UMR1089, Nantes,
294 France). Final viral preparations were split in 50- μ L aliquots in D-PBS (with Ca^{2+} and
295 Mg^{2+}) and stored at -80°C . Number of viral genomes (vg) was titrated by polyA
296 quantitative PCR and was $5.2 \times 10^{12} \cdot \text{mL}^{-1}$ for AAV-desWT and $5 \times 10^{12} \cdot \text{mL}^{-1}$ for AAV-
297 desD399Y.

298 The injection procedure was previously described by Joanne et al. (45) Briefly, for
299 intramuscular injections, 11 week-old male C57BL/6J mice (Janvier Labs, Le Genest-
300 Saint-Isle, France) were anesthetized by intraperitoneal injection of an analgesic and
301 sedative drug mix [$100 \text{ mg} \cdot \text{kg}^{-1}$ ketamine (Merial, Lyon, France) and 10 mg/kg
302 xylazine (Bayer, Leverkusen, Germany)]. Each TA muscle was injected with $70 \mu\text{L}$ of
303 AAV diluted in PBS ($\sim 3 \times 10^{11} \text{ vg}/70 \mu\text{L}$) using an insulin syringe. Animals were split
304 into 4 groups of $n=5$ mice: AAV-desWT with NAC, AAV-desWT without NAC, AAV-
305 desD399Y with NAC, and AAV-desD399Y without NAC. NAC was diluted to 20 mM
306 in drinking water. Bottles were changed every 2–3 days and weighed before and
307 after replacement to calculate the absorption volume of NAC by mice.

308 Animal experiments were conducted with respect to animal health and well-being,
309 and all procedures were approved by our institutional ethics committee (authorization
310 APAFIS#5441 -20 16052314036983 v7, for AAV-injected mice; CEB-16-2016, for KI-
311 R405 mice). All procedures were also conducted according to French and European

312 laws, directives, and regulations on animal care (European Commission Directive
313 86/609/EEC; APAFIS#5441 -20 16052314036983 v7). For AAV experiments, the
314 animal facility (Centre d'expérimentation Fonctionnelle (CEF), Pitié Salpêtrière
315 Hospital, Paris) is fully licensed by French competent authorities and has animal
316 welfare insurance.

317 **Preparation of TA muscles**

318 TA muscles were removed 1 or 2 months following injection after euthanizing mice by
319 cervical dislocation. For immunohistochemical analysis, muscles were embedded in
320 tragacanth gum (Fisher Scientific, Hampton, NH, United States) and frozen by
321 plunging in isopentane precooled in liquid nitrogen for at least 1 minute. For western
322 blotting, muscles were directly frozen in liquid nitrogen in microtubes and later
323 pulverized in a cryogenic mortar (Dutscher, Issy-les-Moulineaux, France) and
324 resuspended in urea buffer for 2D gels.

325 **Muscle sections and histological staining**

326 Serial sections of 6 μm thickness were sliced using a CM1950 cryostat, (Leica,
327 Wetzlar, Germany) recovered on Superfrost Plus microscope slides (Thermo
328 Scientific, Waltham, MA, United States), and stored at -80°C . Muscle sections were
329 kept at room temperature for 20 min before staining with either hematoxylin/eosin
330 (HE), succinate dehydrogenase (SDH), or fluorescent antibodies as follows.

331 HE: Transverse muscle sections were incubated with 0.7% hematoxylin solution
332 (Harris modified, Sigma-Aldrich, Saint-Louis, MO, United States) for 6 min at room
333 temperature, washed 5X with water, and stained with 0.5% eosin in acidified 90%
334 ethanol (Sigma-Aldrich, Saint-Louis, MO, United States) for 1 min at room

335 temperature. Muscle sections were dehydrated by incubating in successive baths of
336 gradually increasing ethanol concentrations (30%, 50%, 70%, 85%, 95%, and 100%,
337 repeated twice) for 2 min each, followed by two baths in clearing solution (Histo-
338 Clear, National Diagnostics, Atlanta, GA, United States) for 10 min and 15 min, and
339 rapidly mounted in VectaMount medium (Clinisciences, Nanterre, France). Images
340 were acquired on a stereomicroscope (Leica, Wetzlar, Germany) at the BFA unit.

341 SDH: Transverse muscle sections were stained by immersion in SDH buffer (0.2 M
342 phosphate buffer containing 5.4% sodium succinate and 0.1% nitro blue tetrazolium)
343 for 2 h at 37°C and then washed with water. Finally, muscle sections were either
344 mounted directly in VectaMount medium (Clinisciences, Nanterre, France) or
345 processed for further immunofluorescence. Images were captured on a
346 stereomicroscope (Leica, Wetzlar, Germany) at the BFA unit.

347 Immunostaining: Warmed muscle sections were fixed with 4% paraformaldehyde for
348 10 min at room temperature. Endogenous fluorescence was prevented with 50 mM
349 NH₄Cl for 30 min. Sections were permeabilized in 0.5% triton/PBS for 10 min,
350 blocked in 1% bovine serum albumin/PBS, and incubated with rabbit anti-Myc
351 (1/1000, # C3956, Sigma-Aldrich, Saint-Louis, MO, United States) or rabbit anti-
352 laminin (1/100, #L9393, Sigma-Aldrich, Saint-Louis, MO, United States) primary
353 antibodies for 1 h at room temperature or overnight at 4°C. Primary antibodies were
354 detected by incubating sections with suitable secondary antibodies for 45 min.

355 DNA was stained with 1 µg.mL⁻¹ Hoechst 33258 (Sigma-Aldrich, Saint-Louis, MO,
356 United States) for 10 min. Cells were washed in PBS and mounted in Fluoromount
357 medium (Interchim, San Diego, CA, United States). All images were captured using a

358 digital camera mounted to a confocal laser scanning microscope (LSM700 Zeiss,
359 lena, Germany) at the imaging facility of the BFA unit.

360 **Analysis of TA muscle characteristics**

361 TA fiber amount and diameter (minimum Feret values) were calculated on whole
362 muscle transverse sections stained with laminin. Images were analyzed with ImageJ
363 software. Percent transduction was calculated by measuring the number of fibers that
364 were positively stained for Myc compared to the total number of fibers in the section.
365 For aggregation rate, number of fibers containing aggregates was reported in relation
366 to total transduced fibers.

367 **2D gel electrophoresis**

368 2D gel electrophoresis was performed at the proteomics platform at the Cochin
369 Institute (Paris, France). Briefly, protein separation was performed by
370 isoelectrofocusing on 7-cm pH 4–7 strips in the first dimension and SDS-PAGE on 10
371 or 12% polyacrylamide gels in the second dimension. Proteins were transferred to
372 nitrocellulose membranes and analyzed by western blotting. Calculation of theoretical
373 isoelectric points (pI) for Myc-labeled desWT and mutant desmin proteins provided
374 different values according to the website used (Table 1).

375

Isoelectric point (pI) calculation	ExPASy	Protein Calculator v3.4	Protein isoelectric point calculator	Scansite
WT murine desmin	5.21	5.28	5.05	5.21
WT human desmin	5.21	5.28	5.06	5.21
WT Myc-desmin	5.11	5.17	5.07	5.11
D399Y Myc-desmin	5.15	5.20	5.03	5.15

377

378

Table 1: Algorithmic isoelectric point (pI) calculations from different web sites:

379 ExPASy (https://web.expasy.org/compute_pi); Protein Calculator v3.4 (<http://protcalc.sourceforge.net>);380 Protein isoelectric point calculator (<http://isoelectric.org>);381 Scansite (<https://scansite4.mit.edu/4.0/#calcMolWeight>).

382

383 One site also indicates phosphorylated peptide molecular weights and

384 phosphorylation that regulate desmin network assembly. For tagged desWT, the

385 obtained values are detailed in Table 2.

Phosphates	0	1	2	3	4	5	6	7	8
Molecular weight (kD)	54.7	54.8	54.9	54.9	55.0	55.1	55.2	55.3	55.3
Isoelectric point	5.11	5.07	5.04	5.00	4.97	4.94	4.91	4.88	4.85

386 **Table 2:** Predictions of molecular weight evolutions and isoelectric points according to the number of387 phosphorylation of desWT (<https://scansite4.mit.edu/4.0/#calcMolWeight>).388 **Statistical analysis**

389 Box plots were created with GraphPadPrism software or R free software, and results

390 were statistically analyzed with non-parametric tests—"nparcomp" function with

391 Dunnet comparison to WT, or Tukey comparison for others. Curves, scatter plots,
392 and histograms as well as corresponding graphic analyses were done with
393 GraphPadPrism software—either 2-way ANOVA corrected by Bonferroni non-
394 parametric function, or Kruskal-Wallis tests. Significant differences were considered
395 at $p < 0.05$.

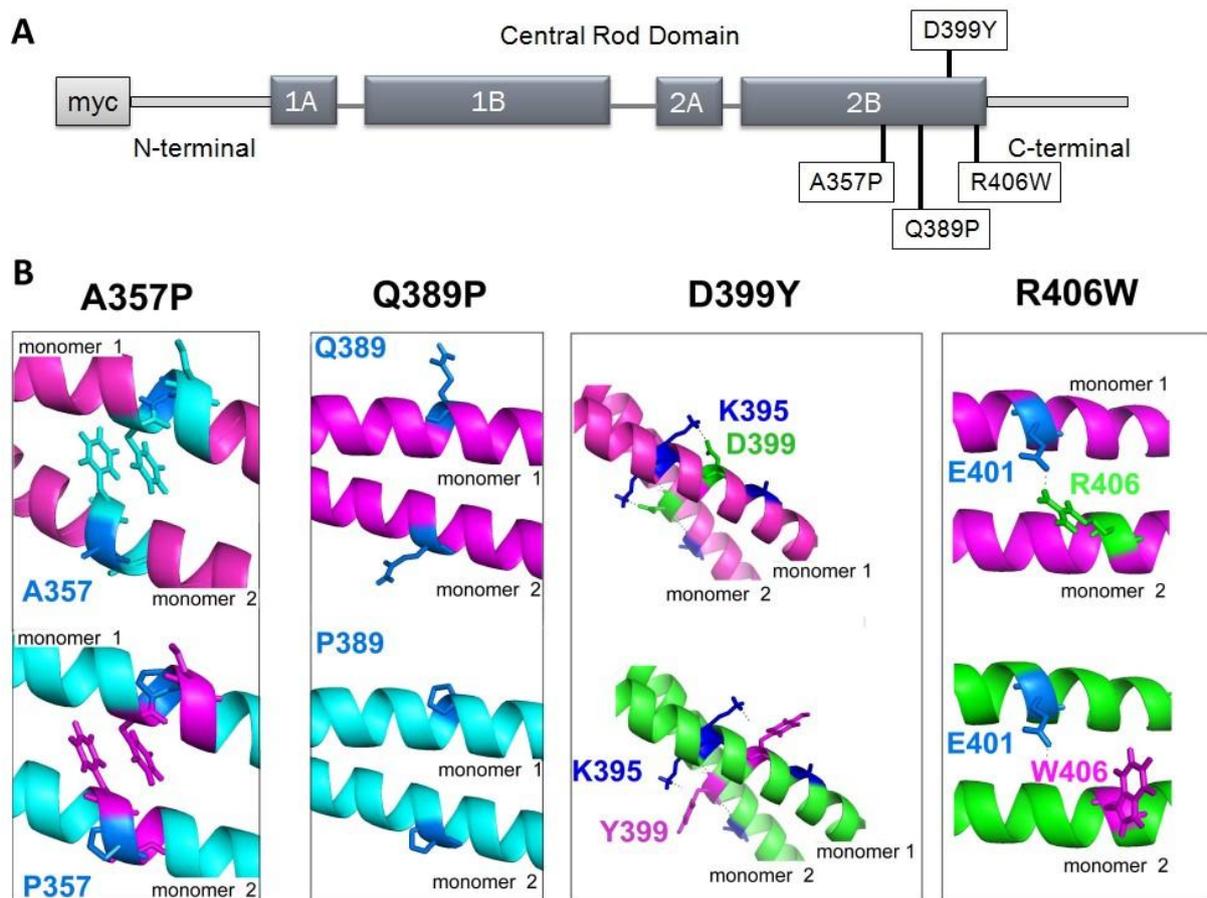
396 **Results:**

397 **DesA357P, desQ389P, desD399Y, and desR406W mutants located in the 2B** 398 **desmin rod domain exhibit *in silico* distinctive structural interactions.**

399 DesD399Y is located distally in the 2B desmin rod domain. We previously
400 demonstrated that it exhibits stress-induced aggregation, whereas mutations located
401 in the head (desS46Y) or tail (desS460I) do not display this aggregation (33). To
402 further investigate mechanisms leading to stress-induced aggregation, we chose
403 three other pathological mutations—desA357P, desQ389P, and desR406W (Figure
404 1A)—also located in the end of this domain between stutter and C-terminal regions,
405 which is a notorious mutation hot spot (35).

406 First, we constructed desmin 2B end domain mutant and WT models with PYMOL
407 software and compared *in silico* the impact of each substitution (Figure 1B). A357P is
408 located in the stutter region that is considered critically important for proper filament
409 assembly. Further, it is in the "e" position in the heptad (internal residue repeats
410 conserved in IF, as described by Strelkov et al.(46). On our structural representation,
411 it is well inside the dimer and phenylalanine lateral chains face in both directions. We
412 can see the position of alanine in the helix/dimer and its replacement with proline.
413 Representation of structural modifications occurring with Q389P substitution shows

414 loss of a large negative side chain residue and gain of a small neutral one. D399Y
 415 and R406W substitutions provide insight about ionic bridging over two disrupted
 416 domains—in both, ionic residues are replaced with aromatic amino acids.
 417

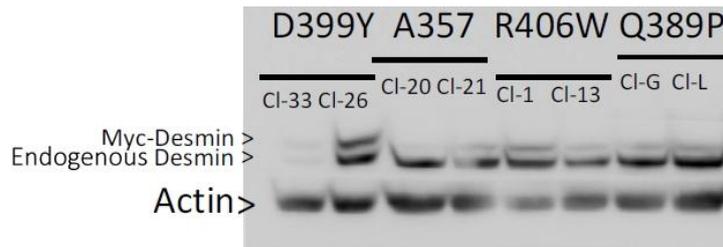


418

419 **Figure 1:** Mutation locations within desmin structure and in silico modeling of mutation effects on
 420 structure of desmin coiled coil 2B domain. (A) Overview of desmin structure showing locations of
 421 individual mutations in the 2B domain and myc tag. (B) Graphic representation of tripartite desmin,
 422 based on vimentin crystal structure (1gk4), with mutations in central rod 2B domain of each monomer,
 423 with amino acids of interest represented by molecular structures. WT helical regions are magenta and
 424 mutants are cyan or green. A357P and Q389P mutations are dark blue. A357P is located in the stutter
 425 region, so conserved phenylalanine is also in molecular structure. D399 and R406 interactions (salt-
 426 bridge) with side chain residue K395 or E401 are in dark blue and are represented with dotted lines.

427 **New stable cell lines expressing desmin mutations exhibit the same expression**
 428 **level as desD399Y-expressing cells.**

429 Second, we generated six new inducible stable cell lines (2 clones per mutation)
430 expressing desA357P, desQ389P, and desR406W according to the same protocol
431 that we used for desD399Y and desWT (25). Expression in all clones was analyzed
432 by western blot to confirm the level of exogenous human desmin compared to
433 desD399Y (Figure 2).



434

435 **Figure 2: Steady-state desmin expression levels.** Western blot of endogenous and human Myc-
436 desmin in inducible stable cell lines 72 h after induction (T24 without stress). Actin represents a
437 loading control. "Cl-X" represents name of the selected clone for each desmin mutation.

438 For each mutation, one construct expressed low levels of desmin and another
439 expressed higher levels. At steady state, endogenous desmin was always more
440 highly expressed than exogenous protein (maximum ratio exogenous/endogenous of
441 1:1 for desWT clone 29 and 0.8:1 for des D399Y clone 26; Table 3). Overall, clones
442 had a similar ratio of ~0.4:1, permitting us to compare new inducible clones to each
443 other in basal conditions or upon stress. Moreover, as demonstrated in table 3, the
444 basal protein aggregation in these cells remained below 3.5% (Table 3).

445

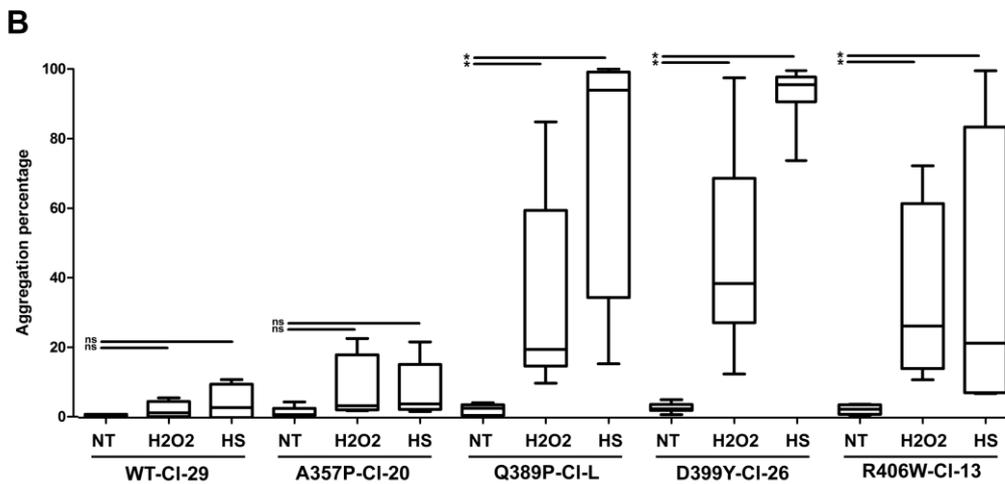
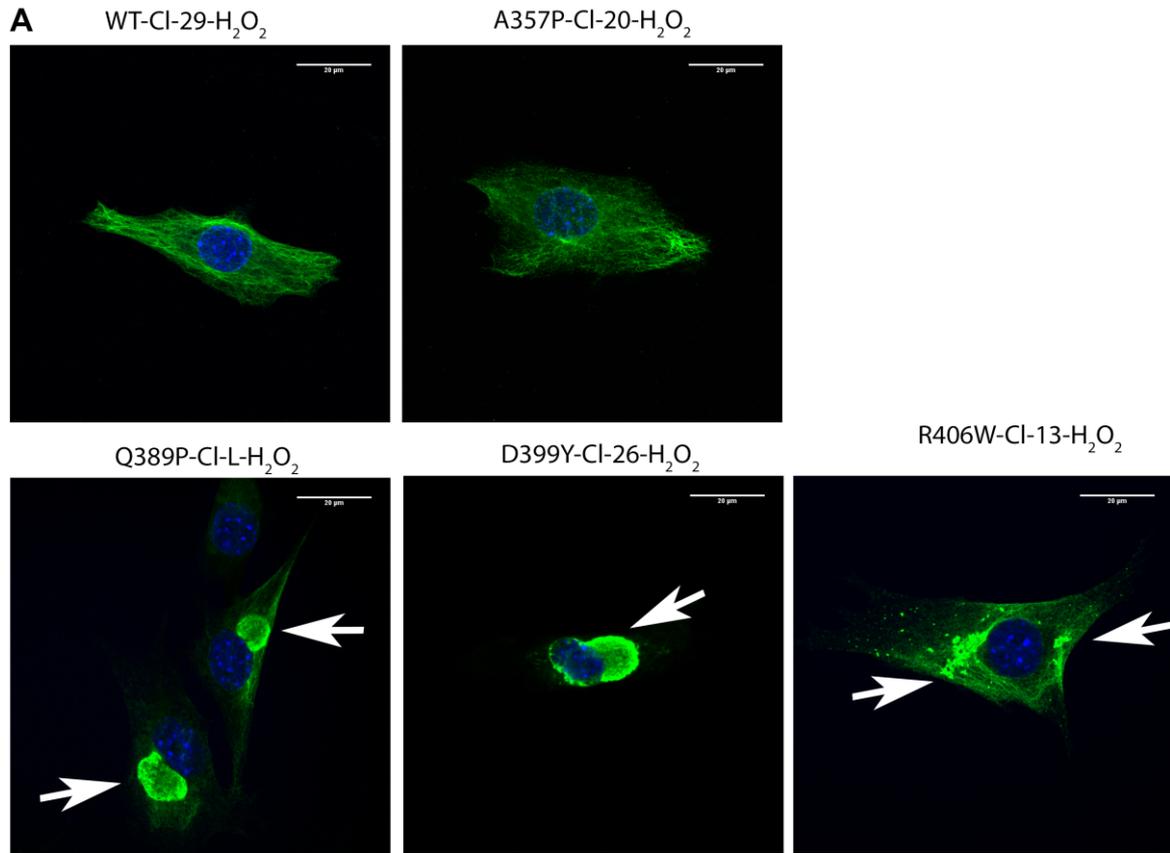
Clone	Desmin expression ratio (exogenous/endogenous)	Basal aggregation (%)
desWT-CI-16	0.4	0.6
desWT-CI-29	1.0	
desD399Y-CI-26	0.8	2.9
desD399Y-CI-33	0.8	2.1
desA357P-CI-20	0.1	0.5
desA357P-CI-21	0.3	
desR406W-CI-1	0.5	1.4
desR406W-CI-13	0.4	3.5
desQ389P-CI-G	0.2	2.0
desQ389P-CI-L	0.3	3.0

447

448 **Table 3: Steady-state desmin expression levels and aggregation percentages.** Western blot
449 analysis of endogenous and human Myc-desmin in inducible stable cell lines 72 h (T24) after
450 induction. Quantification was realized with ImageJ software. Protein amounts were normalized with
451 anti-alpha-actin antibody (Actin). Basal aggregation percentage was calculated as described in
452 material and methods.

453 **Stresses induce protein aggregation only in desQ389P, desD399Y, and**
454 **desR406W, with varying aggregate morphologies.**

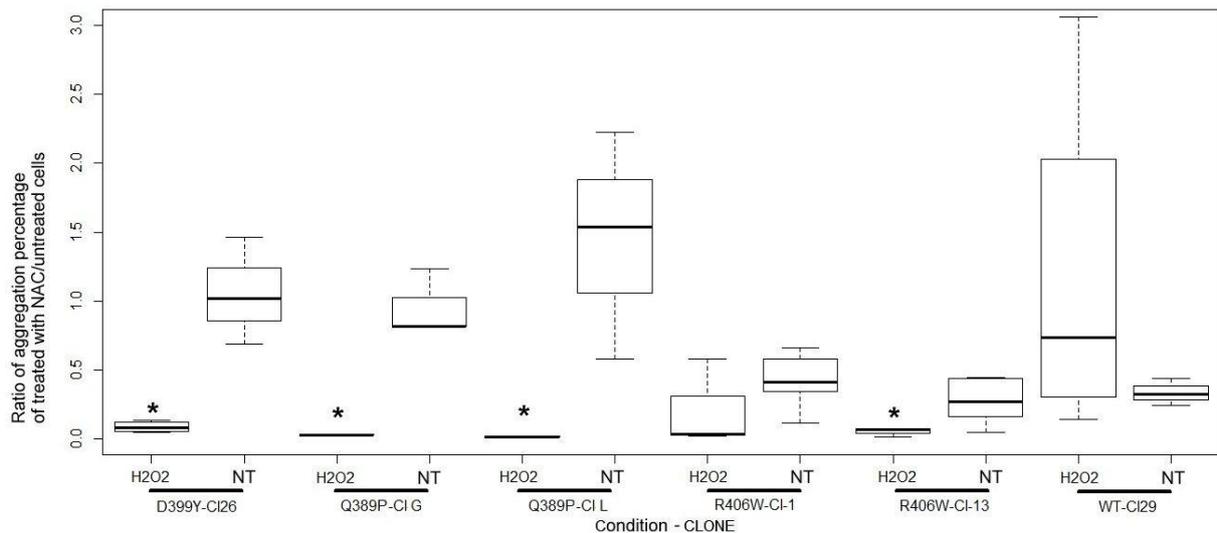
455 We measured desmin aggregation in all stable cell lines in response to oxidative or
456 heat shock stresses after 24h. In response to stress, a normal desmin network was
457 present in desWT- or desA357P-expressing cell lines (Figure 3A, first row).



458

459 **Figure 3: Increased aggregation upon stresses in desD399Y, desQ389P, and R406W cells, but**
 460 **not desA357P cells.** (A) Representative confocal micrographs showing immunostaining at 24 h after
 461 oxidative stress (T24) for Myc-desmin (green) and Hoechst dye (blue). DesWT and desA357P form
 462 normal networks. DesQ389- and desD399Y-expressing cells form large aggregates near the nucleus
 463 (arrows, left and middle panels). DesR406W-expressing cells form smaller accumulations distributed
 464 throughout the cytoplasm (arrows, right panel). White bar = 20 μ m, 40x magnification. (B) Box plot
 465 representing the aggregation percentages 24 h after stress (T24) compared to untreated cells. Three
 466 different treatments are shown: heat shock (HS), oxidative stress (H₂O₂), and no stress (NT). Cells
 467 were counted in at least 3 independent experiments (>100 cells each) (ns: not significant; **p* < 0.05,
 468 compared to unstressed).

469 As previously described, stress of desD399Y-expressing cells induced large
470 aggregates very close to the nucleus (Figure 3A, middle in second row). DesQ389P
471 displayed similar characteristics (Figure 3A, left in second row). DesR406W also
472 exhibited stress-induced, variably-shaped aggregates, although they had weaker
473 staining, maybe due to small size. These aggregates were also more numerous and
474 more dispersed in the cytoplasm (Figure 3A, right in second row). We quantified
475 aggregation rates for each stable clone, representing cells with any aggregates, as
476 compared to the total cell population (Figure 3B, Supp. Data 1). DesA357P-
477 expressing cells presented the same pattern as desWT, with no modification of
478 aggregation rate, indicating that desA357P cells are not sensitive to oxidative or heat
479 shock stress. In contrast, heat shock significantly induced a high aggregation rate for
480 desQ389P and desD399Y (~20–40-fold increase), while oxidative stress led to
481 significant, but less, aggregation (~10-fold increase). DesR406W cells also had
482 significant aggregation in response to stress, although with more dispersed values.
483 The anti-aggregative effect of NAC in the oxidative condition was confirmed for
484 desQ389P, desD399Y, and desR406W (Figure 4). Indeed, ratios of aggregate
485 percentages comparing NAC-treated and untreated cells showed a noticeable
486 decrease. For all cell lines sensitive to oxidative stress, NAC pretreatment
487 significantly reduced stress-induced aggregation.

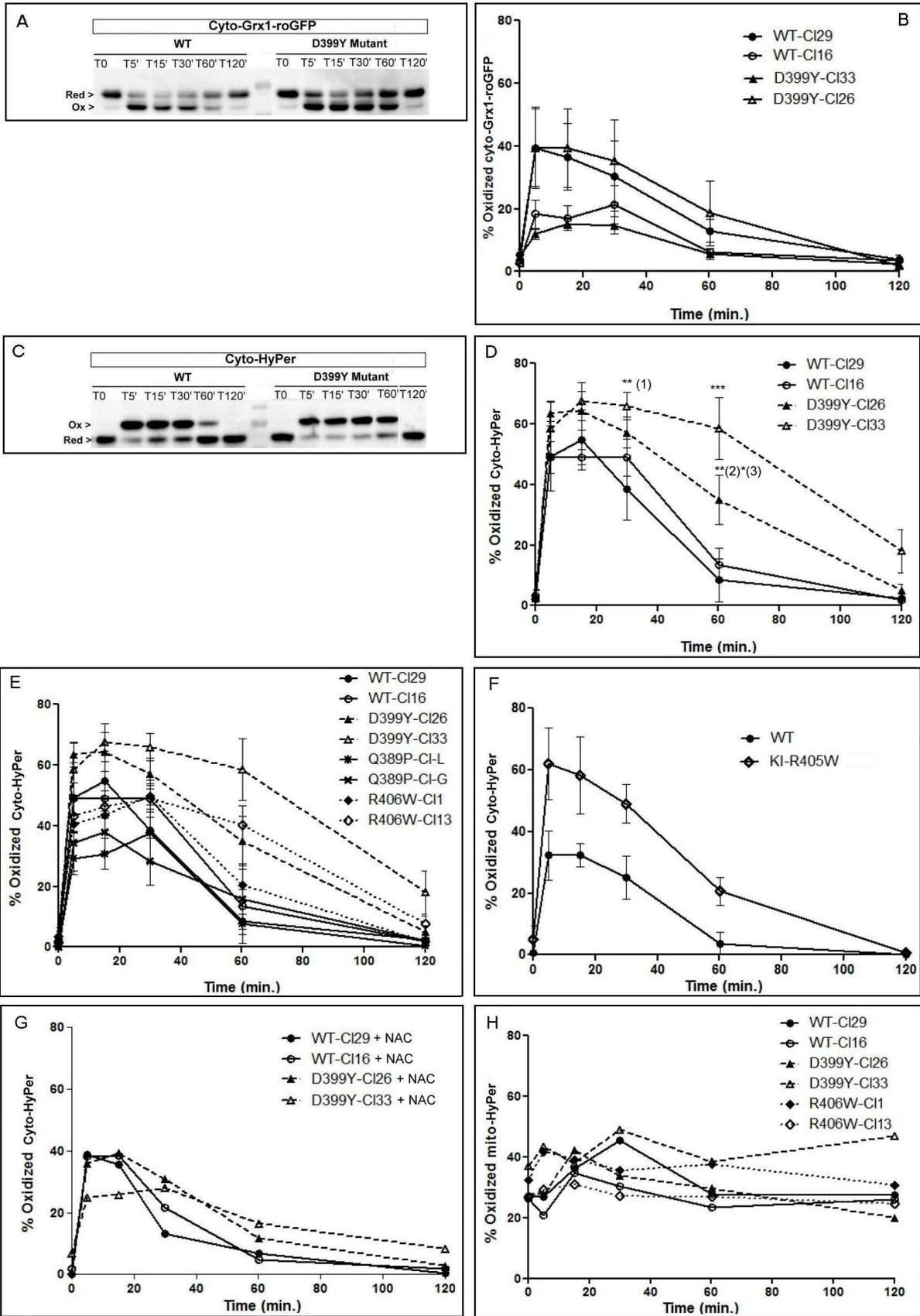


488

489 **Figure 4: Effect of NAC as an anti-aggregative molecule on stable cell lines expressing**
 490 **desQ389P, desD399Y, and desR406W.** First, aggregate-containing cells were quantified, untreated
 491 or pretreated with NAC, 24 h after stress or control conditions (NT). Then, box plots represent the ratio
 492 of percentage of cells with aggregates in the presence of NAC, reported to untreated cells. Cell
 493 counting was performed on three independent experiments (~100 cells each). For each clone,
 494 compared to its control NT, * $p < 0.05$, as calculated with Kruskal-Wallis non-parametric test.

495 **Cytoplasmic redox dynamic in desmin mutant cell lines exhibits various**
 496 **behaviors.**

497 To further investigate how stress induces aggregation in all responsive cell lines and
 498 how NAC can prevent this effect, specific redox reporters were used to monitor redox
 499 balance in cells expressing desmin mutants following oxidative stress. We first used
 500 the cyto-Grx1-roGFP2 to monitor cytoplasmic redox dynamic. This reporter
 501 specifically reacts with GSSG/GSH in the targeted compartment, so the percentage
 502 of the oxidized form reflects the redox state of glutathione (44) (Figure 5A and 5B). A
 503 second probe, HyPer, reacts specifically with H_2O_2 and is targeted to the cytoplasm
 504 (cyto-HyPer). Therefore, the percentage of the oxidized form of cyto-HyPer reflects
 505 the quantity of cytoplasmic H_2O_2 over time (Figure 5C and 5D).



506

507 **Figure 5: desD399Y and desR406W trigger alteration of redox balance during stress.**

508 (A) Representative western blots of the oxidized and reduced forms of cyto-Grx1-roGFP2 reporter
509 following oxidative stress in stable cell lines expressing desWT or desD399Y (B) Quantification of
510 oxidation kinetics of the cyto-Grx1-roGFP reporter over time. (C) Representative western blots of the
511 oxidized and reduced forms of cyto-HyPer reporter following oxidative stress in stable cell lines
512 expressing desWT or desD399Y with the cyto-HyPer reporter. (D) Quantification of oxidation kinetics
513 of the cyto-HyPer reporter over time. (E) Percentage of oxidized cyto-HyPer in stable cell lines
514 expressing desWT, desD399Y, desQ389P, or desR406W, without pretreatment. (F) Percentage of
515 oxidized cyto-HyPer in satellite cells from KI-R405W mice. (G) Percentage of oxidized cyto-HyPer in
516 stable cell lines expressing desWT or desD399Y after NAC pretreatment (10 mM). (H) Percentage of
517 oxidized mito-HyPer in stable cell lines expressing desWT, desD399Y, desQ389P, or desR406W,
518 without pretreatment. Each point represents the mean of three independent experiments, with bars
519 indicating SEM. Prism software was used for 2-way ANOVA, corrected by Bonferroni non-parametric
520 function (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

521 Both cyto-Grx1-roGFP2 and cyto-HyPer probes were first compared in experiments
522 with desWT and desD399Y clones. There were no significant differences in cells
523 expressing desWT or desD399Y with Cyto-Grx1-roGFP (Figure 5B). Using cyto-
524 HyPer, however, we detected significantly delayed oxidation kinetics in the cytoplasm
525 of desD399Y clones (Figure 5D). Indeed, more H₂O₂ remained in desD399Y clones
526 at 60 min after the beginning of oxidative stress compared to desWT clones,
527 although both constructs reached near-zero values after 120 min.

528 We thus expanded cyto-HyPer probes analysis to the two other mutants with stress-
529 induced aggregation, desR406W and desQ389P. Similar to desD399Y, both clones
530 expressing desR406W had similar significant differences in reporter oxidation
531 depending on the duration of oxidative stress (Figure 5E). We confirmed these
532 results using the more physiological model of satellite cells extracted from our new
533 KI-R405W animal model. These cells showed very comparable delayed recovery of
534 basal reporter reduction (Figure 5F). In contrast, unexpectedly considering the
535 aggregative pattern seen in Figure 3A, desQ389P cell lines have a similar behavior
536 to desWT (Figure 5E).

537 To better understand the effect of NAC pretreatment on the evolution of cytoplasmic
538 content during H₂O₂ incubation, we made the same measurements in the presence

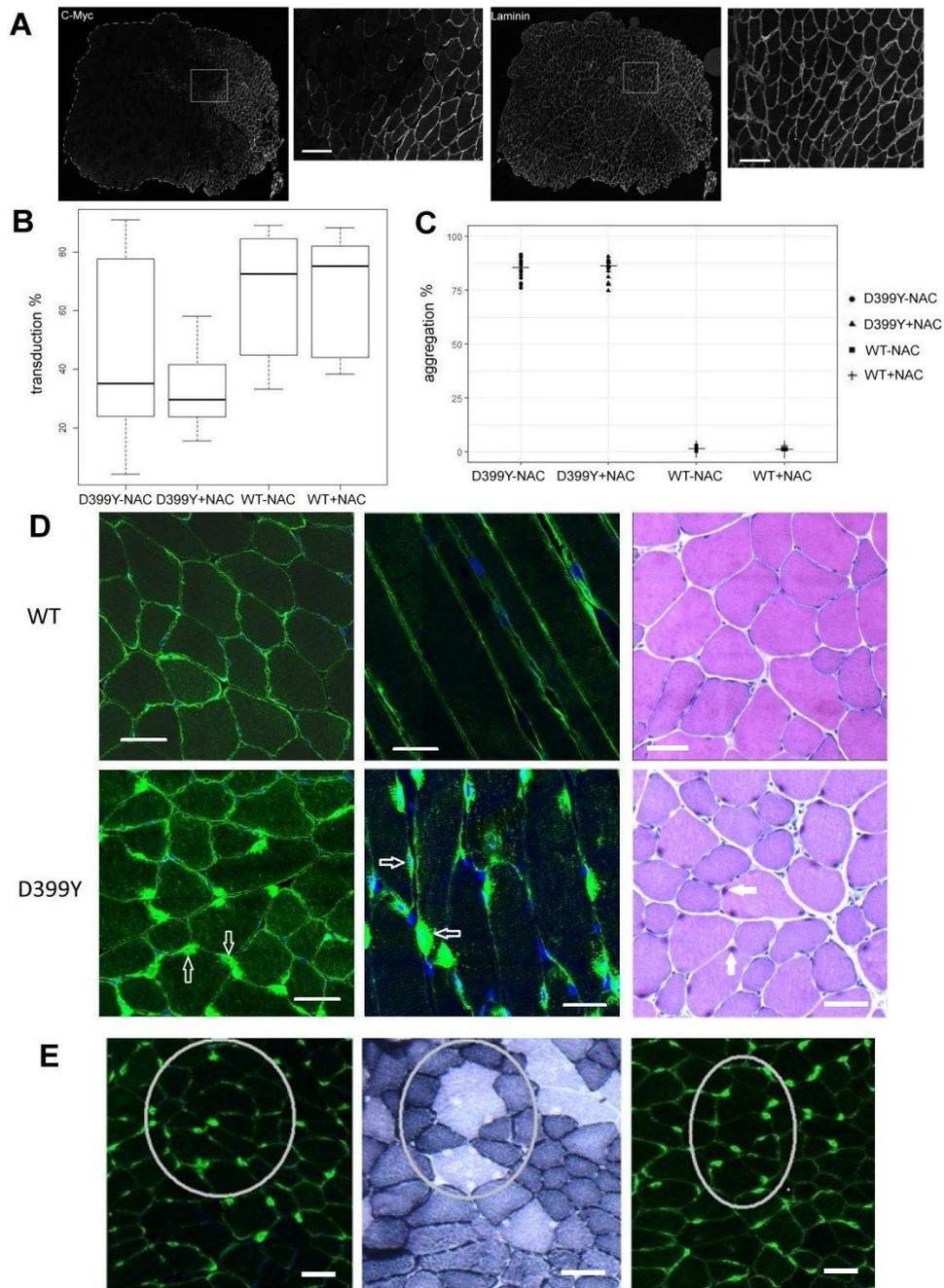
539 of NAC. NAC especially reduced the percentage of oxidized reporter in cells
540 expressing desD399Y (Figure 5G). Differences in the averages between untreated
541 and treated showed that this decrease was ~16%–25% for both.

542 Finally, we investigated the mitochondrial effect of H₂O₂ incubation using the
543 mitochondrial matrix-targeted HyPer reporter. Redox balance is distinct in this
544 organelle, as the reporter was more oxidized even without treatment and remained
545 unchanged in all experiment conditions (Figure 5H). This indicates that mitochondria
546 are not affected as cytoplasm in response to oxidation.

547 **Expression of desD399Y *in vivo* induces aggregation with slightly altered SDH**
548 **repartition in adult mouse muscle fibers.**

549 Heat-shock and oxidative stress are two types of environmental variations that can
550 be found in muscle physiology (47). DesD399Y induces aggregation following these
551 environmental stresses, as do other mutations, such as desQ389P or desR406W.
552 We used a specific desD399Y mouse model with AAV-injected TA muscle to confirm
553 the anti-aggregative effect of NAC *in vivo*. We initially injected AAV containing
554 desWT or desD399Y in TA muscle of C57BL/6J mice and then analyzed resulting
555 desmin expression and aggregation with or without NAC treatment after 1–2 months.

556 We calculated transduction efficiency by counting anti-Myc-stained fibers compared
557 to all fibers stained with laminin (Figure 6A and B). Surprisingly, transduction
558 efficiency was variable and higher for desWT (mean: 75%) than desD399Y (~50%),
559 regardless of the duration before analysis (1 or 2 months).



560

561 **Figure 6: AAV-injected muscles overexpressing desD399Y mimic some important features of**
 562 **human desminopathies: an *in vivo* model to test the effect of NAC as a putative anti-**
 563 **aggregative molecule.** (A) Representative images of immunohistochemical staining of muscle cross-
 564 sections with anti-Myc antibodies to visualize transduced fibers (left) and laminin to visualize all fibers
 565 (right). Results are quantified in plots of (B) transduction percentage and (C) aggregation percentage
 566 from 4 injected mouse TA muscles for each condition. (D) Confocal micrographs of transversal (left)
 567 and longitudinal (middle) sections of muscle expressing desWT or desD399Y stained with anti-Myc
 568 antibodies (green). Nuclei are counterstained with Hoechst dye (blue). Right panels show HE-stained
 569 transverse sections imaged with a stereomicroscope. White arrows indicate aggregates. Images taken
 570 with a 40x objective; white bar = 25 μ m. (E) Confocal micrographs of a series of desD399Y-injected
 571 muscle cross-sections stained with anti-Myc antibodies (green) and Hoechst dye (blue) (left and right
 572 panels) or SDH (middle panel). Circles highlight area with aggregation and the absence of SDH
 573 staining. Images taken with a 40x objective.

574 As expected, expression of ectopic desmin was detected in muscle protein extracts
575 after western blotting with anti-Myc or anti-desmin antibodies (Supp. Data 2).
576 Aggregation percentages were evaluated by counting fibers with aggregates,
577 regardless of the number of aggregates, compared to all fibers (Figure 6C). In AAV-
578 desWT-injected TA, <5% of fibers presented aggregates, while ~95% of AAV-
579 desD399Y-injected fibers were positive for aggregates. As observed in stable cell
580 lines, aggregates were always localized around nuclei (Figure 6D). Disorganization of
581 surrounding z-disks, as observed in patient biopsies with aggregates, was not noted.
582 In a few cases, fibers with aggregates had some areas without SDH staining (in
583 circles in Figure 6E). Those small spots indicated absence of enzyme activity and
584 were smaller than rubbed-out fibers observed in patient biopsies (i.e. where, in some
585 muscle fibers, SDH stain looks as though it has been rubbed away inside the fiber).
586 In addition, we quantified fiber size in injected muscles. Minimum Feret diameters
587 (min Feret) is commonly considered a better marker than area measurement for
588 muscle physiology comparisons, because it minimizes the effects of oblique
589 sectioning. AAV-desD399Y-injected muscles have slightly decreased min Feret
590 diameter of fibers compared to AAV-desWT-injected muscles (Supp. Data 3).
591 Altogether, data indicate that AAV-desD399Y injected muscle mimics some main
592 human desminopathy features and could serve as a model to test the antioxidative
593 effects of NAC.

594

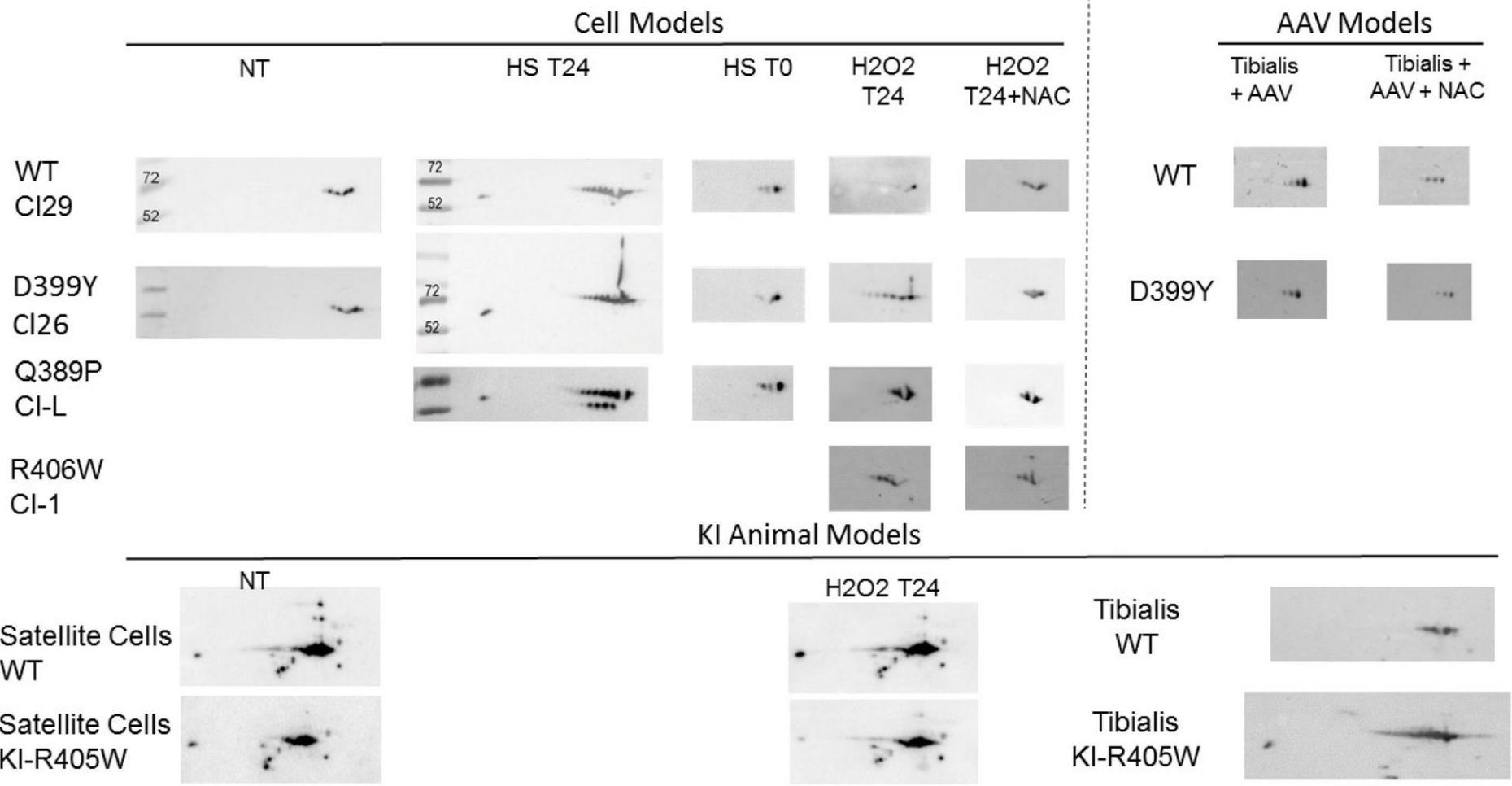
595 The day following injection, we split mice into two groups: one with untreated drinking
596 water, and one with 20 mM of NAC solution in the drinking water. Despite results with
597 cell lines expressing desD399Y, aggregation was similar with or without NAC
598 treatment (Figure 6C). However, unexpectedly and independently of the desD399Y

599 mutant, NAC treatment increased the total number of fibers (Supp. Data 3A) and
600 seemed to be associated with a slight decrease in min Feret diameter (Supp. Data
601 3B).

602 **2B desmin mutants exhibit different PTM patterns *in vitro* and *in vivo*.**

603 To understand some molecular alterations of desminopathies upon stress, we
604 investigated PTMs in our cell and mouse models of pathological mutations under
605 different environmental factors. We hypothesized that PTM differences can be linked
606 to stress-induced aggregation, as highlighted previously. We thus used bidirectional
607 gels to look for differences between exogenous desmin PTMs in stable clones
608 expressing desWT versus mutants 24 h after heat shock or oxidative stress, or in
609 injected muscles.

610 As expected, human Myc-tagged desmin presented a pI of ~5.2 (coherent with Table
611 1). However, we observed several patterns. Untreated desWT cells (clone 29) had
612 four spots, whereas heat-shocked cells had at least eight spots with the same
613 molecular weight (Figure 7, first line, two first columns). DesD399Y cells showed the
614 same pattern, although they presented several additional spots with higher molecular
615 weights (Figure 7, second line, two first columns). We found similar results after
616 oxidative stress by H₂O₂ (Figure 7, fourth column), although treated desWT-
617 expressing clones did not have as many spots as expected (Figure 7, WT/H₂O₂ T24).



619 **Figure 7: Stress induces various desmin PTMs, depending on the mutation.** For cell models
620 (left): 2D-PAGE analysis of total protein extracts from stable clones expressing desWT (WT-CI29) or
621 mutant desD399Y (D399Y-CI26), desQ389P (Q389P-CI-L), or desR406W (R406W CI-1) or satellite
622 cells from KI-R405W mice. Samples represent different time points (T0, just after stress; T24, 24 h
623 after stress) and stresses [thermal, HS, or oxidative (H₂O₂)] with or without NAC pretreatment. For
624 muscles (right): 2D-PAGE analysis of total protein extracts of TA muscles, either AAV-injected (with
625 desWT or desD399Y), treated with NAC for 2 months or untreated, or from KI-R405W mice.

626 After exposure to heat shock or oxidative stress, extracts from the stable clone
627 expressing desQ389P presented two forms of desmin with close molecular weights
628 (Figure 7, third line). One is the expected size of desmin (53 kDa) with several
629 additional spots that could represent desD399Y, and the second is close to 49 kDa.
630 The day after oxidative stress, desR406W also had a different migration profile from
631 desWT. We observed four spots, with some additional lower-molecular-weight spots
632 close to 49 kDa, similar to Q389P but with a higher pI (~5.4).

633 Because aggregates are already present 24 h after stress, we wanted to determine
634 whether the observed PTMs take place just after the stress, when no aggregation is
635 detectable. For all analyzed cell lines, desmin patterns at T0 were the same as for
636 untreated cells (four spots) (Figure 7, third column). We also examined the patterns
637 at T4h, when aggregation begins for the three previously studied cell lines (33).
638 Profiles were similar to those obtained at T0—four spots of different pIs but similar
639 molecular weights (data not shown). Thus, these results suggest that PTMs are
640 probably a post-aggregative process.

641 We also analyzed desmin patterns by 2D-PAGE after stressing cells that were
642 pretreated with NAC. Except for desR406W, all mutant patterns in the presence of
643 NAC were similar to desWT without stress (Figure 7, fifth column). For desR406W,
644 spots corresponding to potential proteolysis remained present, potentially with one
645 additional higher-molecular-weight spot.

646 To further analyze our *in vivo* results, we compared these patterns to protein extracts
647 from AAV-desWT-injected or AAV-des-D399Y-injected muscles. None of the
648 modifications observed in cells could be detected in muscle given the high presence
649 of aggregates and morphological alterations presented above (Figure 7, last two
650 columns). Indeed, in AAV-desWT-injected as well as AAV-D399Y-injected muscles in
651 the absence of NAC, four forms of desmin appeared at the expected molecular
652 weights. After pretreatment with NAC, the profile appeared to be globally identical,
653 although only three spots seemed to remain, with probable disappearance of the
654 highest-pI form.

655 Finally, patterns obtained with muscles or satellite cells from KI-R405W (Figure 7,
656 last lines) displayed many PTMs around the values in Tables 1 and 2, indicating both
657 molecular weight and potential known phosphorylation modifications.

658 **Discussion:**

659 Usually, no proline residue is present in the central domain in desmin alpha helices.
660 This residue is known to create a bend of $\sim 25^\circ$ in the helix (48). However, with Pymol
661 software, it is only possible to visualize that the alpha amine of this residue no longer
662 participates in alpha helix stabilization. Helix 2B length and rigidity may be sufficient
663 to attenuate this deformation. In the case of replacement of alanine with a proline
664 (A357P), this position supposedly stabilizes the dimer with ionic interactions.
665 Substitution of proline at least alters the alpha helix and should be enough to drive
666 the pathological phenotype. Size and bulkiness of the side chain has a moderate
667 effect, as shown in Figure 2B, and affects hydrophobicity. Pymol software indicates
668 that the helix deformation for Q389P comes with a moderate potential change
669 outside the coiled coil, which can affect interactions between several dimers and may

670 impact polymerization. Proline extends less beyond the helix than glutamine, which
671 may affect assembly of the dimers in tetramer and/or radial compaction. In fact,
672 Q389P could be compared to other mutations leading to replacement of a residue by
673 a proline, such as R350P (b in heptad as Q389P), A357P (e in heptad as D399Y),
674 and L370P (inside the heptad, leading to a lost hydrophobic interaction). However,
675 R350P is upstream of the stutter, A357P is in the stutter, and L370P creates a bend
676 in a conserved sequence, similar to Q389P, but at different coiled coil locations. It is
677 therefore possible that proline destabilization of the alpha helix is not equivalent for
678 these four positions, even if their pathogenic effect is established (37). On the other
679 hand, abolishing interactions between side chains [C–O of aspartate (399) or
680 glutamate (401) with NH of lysine (395) or arginine (406)] may destabilize the dimer.
681 In addition, tyrosine spreads much more than aspartate and changes the charge at
682 this position. For tryptophan, its orientation and size are also very different from those
683 of arginine. These two mutations could also interfere with dimer assembly in the
684 tetramer and/or with radial compaction. For R406W, modeling has already been used
685 to study the interactions of desmin and synemin (49), which strengthens the desmin
686 network. These anchors are not permitted by the mutation, even though the 406
687 residue is not in charge of the interaction. Again, this indicates that the effect of
688 substitution on the secondary structure might not be predominant, that side chain
689 size could disturb tetramer or later assembly, and that accumulation affects
690 construction of the network, which could have a disturbing influence. However, the
691 dimeric structure does not allow us to see this cumulative process.

692 Our inducible cell lines allow comparisons between desmin mutants in an isogenic
693 environment. The basic properties of the cell lines (proliferation and inducibility) were
694 similar to cells expressing desWT or desD399Y. In addition, expression levels of the

695 exogenous proteins are close to or below a ratio of 1:1 with respect to endogenous
696 murine desmin, minimizing the effect of overexpression by maintaining closer-to-
697 physiological conditions. These isogenic contexts enable comparisons of only
698 mutation effects and allow analysis of their behavior in parallel. Random insertion
699 matter could be bypassed with new gene technologies. However, we tested several
700 clones for each mutation to ensure that the observed effect is indeed dependent on
701 mutation and not insertion. Moreover, for R406W mutation, redox effects have been
702 validated in satellite cells from R405W-KI mice. Additionally, these clones present an
703 inducible expression that avoid deleterious effects of overexpression. Finally, it would
704 be interesting to have cells or muscles from patients to confirm these results;
705 unfortunately, they are extremely rare and no samples are currently available for the
706 mutations studied.

707 Currently to detect desmin point mutations, there is only one specific antibody for
708 R349P. To sense specifically exogenous human desmin, we therefore used a Myc-
709 tag. Indeed, it allows first to quantify (via a shift with desmin antibody) the exogenous
710 desmin vs endogenous and second to detect specifically the human desmin carrying
711 the 6 mutations studied. In addition, we have previously shown that the Myc-tag in N-
712 terminal position, does not cause any alteration in the formation of the WT desmin
713 network *in cellulo*, with or without environment changes (31). Surprisingly, stress did
714 not induce similar aggregation patterns for all 2B mutations as it did in cells
715 expressing the desD399Y mutation. These results suggest that, despite the presence
716 of proline in the rod domain, desA357P-expressing cells are not so sensitive to
717 external stress, unlike desQ389P. These results are similar to those for desS46Y and
718 desS460I, located respectively in the head and tail domains, which were previously
719 shown to have no stress-induced aggregation (33). Our results here show that

720 desQ389P behaves like desD399Y in terms of aggregate shape and formation upon
721 stress, while desR406W responds to stress to a lesser extent than desQ389P or
722 desD399Y, indicating desR406W may be more sensitive to environmental changes in
723 each experiment. Transient experiments show that all mutations have the same
724 dominant negative effect and lose the ability to form a network without IFs
725 (16,17,37,50). However, our stable clones reveal specific characteristics for each
726 mutation in response to stress. This sensitivity to stress cannot be related to the type
727 of mutation (e.g., proline substitution) or region in the protein (e.g., 2B domain),
728 confirming the variability of aggregate induction mechanisms.

729 To decipher the underlying molecular mechanisms, we constructed compartment-
730 specific genetically encoded redox reporters. The cyto-Grx1-roGFP2 reporter did not
731 reveal any difference of cytoplasmic GSH/GSSG redox state between C2C12 cells
732 expressing desWT and desD399Y. It is possible that desD399Y expression induces
733 only a slight change of GSH/GSSG ratio that is beyond detection by cyto-Grx1-
734 roGFP2 reporter. Consistently, we were unable to detect any GSSG in C2C12 cells
735 and in our clones by high performance liquid chromatography (data not shown). In
736 contrast, cyto-HyPer reacted differently in cells expressing desWT and in some
737 desmin mutants to oxidative stress-induced aggregation. A significant shift was
738 observed at 60 min, with a greater amount of the oxidized probe found in clones
739 expressing desD399Y and desR406W (Figure 5B). In addition, the delayed return to
740 a normal redox ratio took up to 120 min, especially for the desD399Y clone. Delayed
741 oxidation kinetics related to H₂O₂ metabolism could reflect an imbalanced redox
742 homeostasis in mutant cells, although no H₂O₂ increase was detectable at steady-
743 state. Furthermore, satellite cells extracted from KI-R405W mice exhibited similar
744 results, confirming the implication of redox balance pathways. Finally, observations

745 with mito-HyPer reporter imply that it is the cytoplasmic redox homeostasis that is
746 mostly affected in desmin mutants.

747 Cytoplasmic redox dynamics in response to oxidative stress varied in various
748 mutants. Cells expressing desWT and desQ389P could rapidly restore the redox
749 balance as indicated by HyPer reporter, while cells expressing desD399Y and
750 desR406W required more recovery time. On the other hand, desQ389P and
751 desD399Y displayed similar cellular aggregation. Therefore, these mutants may rely
752 on different altered enzymatic pathways and each mutation may affect specific redox
753 mechanism.

754 Finally, NAC pretreatment induced WT-like results in desD399Y and desR406W
755 cells. As in the case of SEPN1- Knock Out (KO) cells, NAC pretreatment reduces
756 abnormal sensitivity to oxidative stress induced by H₂O₂ (30). Cytoplasmic redox
757 balance related to H₂O₂ content is therefore weakened in the context of D399Y or
758 R406W mutation. We have previously shown that, in cells expressing desD399Y,
759 cadmium chloride (non-oxidative, heavy metal) disturbs the redox dynamic and
760 induces moderate aggregation (33). Therefore, redox homeostasis seems to be
761 fragile in these mutant desmin-expressing cells, so reducing treatment can cause
762 destabilization. This may have deleterious effects on desmin network stability in the
763 context of mutants and NAC would indirectly reduce this sensitivity through
764 glutathione synthesis.

765 Therefore, it may be important for trials of therapeutic molecules to test a wider range
766 of mutations expressed in murine models. Our laboratory has recently experimented
767 with a new type of mouse model that is faster to produce than the KI model. We
768 introduced two desmin mutations (R406W and E413K) into AAV vectors and injected

769 11-week-old mouse TA muscles (45). Analysis of these muscles showed
770 morphological changes and decreased contractile force, as observed in patients with
771 myofibrillar myopathies. The relatively fast timeline of these experiments suggests an
772 ability to study several mutations in parallel.

773 The AAVs used in this study were produced by a platform at the National Institute for
774 Health and Medical Research in France, which is different from the laboratory that
775 constructed the desR406W and desE413K viral vectors (45). Differences in
776 transduction may therefore be due to preparation and technical details as well as the
777 individual batch. However, since we injected a maximal volume (70 μ L) with respect
778 to muscle size, we could not increase transduction efficiency. So, the variability
779 associated with limited muscle transduction was quite acceptable for a phenotypic
780 study of the impact of desmin over-expression. Indeed as in cells, desmin level
781 remains moderate (Supp. Data 2). Nonetheless, muscle fibers had a very high level
782 of aggregation, up to 90% in fibers expressing exogenous desD399Y, while only a
783 few percent in those expressing desWT. Thus, the location of D399Y is disturbed,
784 similar to patients' muscles, despite expression in an already mature muscle.
785 Moreover, as for the cell lines desD399Y, aggregates are located close to the nuclei
786 like in R406W but not in E413K, shown in a previous study (45). This comparison
787 also applies to SDH staining. In addition, some fibers with aggregates had lighter
788 SDH-stained areas, which could be explained by the low number of mitochondria in
789 this fiber type. In R406W fibers, these non-stained areas are more extensive and
790 seem to affect all fiber types.

791 As mouse muscles presented pathophysiological characteristics that mimic some of
792 those found in human patients, we treated mice with 20 mM NAC, which is the

793 commonly used dose for such experiments (49), and higher concentrations seem to
794 have secondary effects on WT mice (unpublished data from our lab). After 1–2
795 months of NAC pretreatment fibers showed comparable aggregation with or without
796 treatment. Furthermore, NAC increased the number of muscle fibers and decreased
797 their size. Antioxidant supplementation can have a negative impact on acute
798 exercise, suggesting that adaptation to such stress is more dependent on redox
799 homeostasis (51). This is consistent with the role of ROS as intracellular signaling
800 molecules, so the observed hyperplasia may be due to lack of ROS in the presence
801 of NAC, which can have such an effect even without exercise.

802 Regarding defects in aggregation prevention, one question is whether the dose of
803 NAC was appropriate. We do not have internal markers to ensure NAC was delivered
804 to muscle. However, NAC-treated mice drank less than untreated mice, although no
805 dehydration was observed (Supp. Data 4). This decreased drinking volume could be
806 due to the very low pH of NAC solution or the taste of NAC solution. Nonetheless, the
807 daily NAC dose that mice absorbed remained constant over time. Furthermore, the
808 NAC quantities absorbed in our study were similar to those in other studies, where
809 NAC was effective (e.g., KO-SEPN1 mice) (30). Thus, accessibility of NAC to
810 muscles should have been appropriate in our study.

811 Another question then arises—why did NAC have no effect in muscle while it
812 prevents D399Y aggregation in cells? Among several hypotheses, one of the most
813 probable is that expression of mutant desmin in the muscle sarcoplasm happens too
814 quickly and/or reaches levels too high for the antioxidant effect to be preventive.
815 Thus, once aggregation has been established, using antioxidant molecules has no
816 effect. NAC treatment may therefore be more appropriate as a first step before

817 injection, identical to cellular models. However, this experimental course is more
818 difficult to extrapolate to therapy for patients who have the mutation at birth.
819 Alternatively, we could test other anti-aggregative molecules, such as PP242 or NCS
820 23766, or combine the effects of several molecules, as is done in cell lines (34). An
821 alternative hypothesis is that expression of desD399Y in mature muscles does not
822 initially affect redox balance but may do so in a secondary manner. In that case,
823 studies would need to test the effect of NAC treatment for a longer period of time, for
824 which the KI mouse model could be useful.

825 Desmin PTMs in both myoblast and muscle contexts were consistent with the
826 absence of network alterations immediately or a few hours after heat shock or
827 oxidative stress. These behaviors indicate that stress is not directly related to
828 aggregation, but that the cellular stress response may be connected to the presence
829 of mutations on soluble-desmin protein or associated in filaments. These results also
830 show singular reactions of desmin to adapt to intrinsic alteration with environmental
831 changes. These changes thus seem to be a post-aggregative process. In fact,
832 aligned spots of the same molecular weight seem to indicate potential known desmin
833 phosphorylations, with some additional for stresses exposition. Nowadays, no other
834 small modifications are described for desmin and it seems here not to participate in
835 early pathophysiological mechanisms. So hyper-phosphorylation 24 h after stress
836 could be one common point. Stress may not depolymerize the desmin network or
837 alter repolymerization in the mutant context (52). Rather, phosphorylation seems to
838 drive the depolymerization/repolymerization process preferentially for aggregated
839 proteins. PTMs with superior weights, seen for desD399Y, could correspond to PTMs
840 known for desmin or other IFs due to short peptides (i.e., ubiquitination or
841 sumoylation). This signal addresses peptides to various degradation processes that

842 are more complex than proteolysis (ubiquitin proteasome system or autophagy).
843 Alternatively, Olivé et al. (53) found an aberrant form of ubiquitin, UBB+1, that co-
844 localizes with aggregates in skeletal muscles biopsies of patients with myotilinopathy
845 or desminopathy. Other IFs, lamins, and keratins are sumoylated or even hyper-
846 sumoylated under pathological conditions (54). Additional studies could provide a link
847 between these processes in terms of aggregate elimination mechanisms in regards
848 to mutation location.

849 In the same way, proteolysis related to calpain and caspases could explain the lower-
850 weight forms of desQ389P and desR406W as it has been shown in meat (55).
851 Compared with native desmin isolated from bovine muscle, degradation of oxidized
852 desmin is enhanced by caspases but suppressed by μ -calpain (55). This study also
853 used circular dichroism spectra of desmin to show that oxidation modifies desmin's
854 secondary structure— α -helix content decreased from 76.2% to 52.0%, while random
855 coil content increased from 8.0% to 22.4%.

856 We did not detect any extra-PTMs in cells or injected muscles, similar to results
857 described for patient muscles (31,32,56). One hypothesis is that no additional PTMs
858 occur, even in desmin aggregates. Indeed, desmin overexpression might overload
859 enzymes and prevent further PTMs. Another hypothesis is that muscle response is
860 delayed and thus may occur at a later timepoint than our observations. However,
861 supplementary PTMs are present in KI-R405W mouse muscles and even untreated
862 satellite cells, suggesting an early effect on desmin PTMs patterns, similar to
863 phosphorylations. Nonetheless, this could be due to an early alteration of redox
864 balance in the homozygous KI context, as has been suggested for linked ROS and
865 PTMs in muscle exercise (57).

866

867 **Conclusion**

868 Our study demonstrates that redox balance can be altered by expression of at least
869 some desmin mutants. Our results highlight that each mutation has unique
870 pathological molecular mechanisms such as maintaining redox balance and/or
871 protein regulation and aggregation. Therefore, it will be important to integrate these
872 specific behaviors when considering future therapeutics.

873 **ACKNOWLEDGMENTS**

874 We thank Centre d'expérimentation Fonctionnelle (CEF), Pitié Salpêtrière Hospital,
875 Paris and the "plateforme d'Hébergement et d'expérimentation animale Buffon"
876 respectively for AAV-injected and KI-R405W mice. Confocal images were acquired at
877 the imaging facility of the BFA unit. Thanks to Françoise Balter and Philippe Moullier
878 for AAV obtained from Vector Production Center of French National Institute of
879 Health and Medical Research (UMR 1089). We thank Solène Boitard for teaching us
880 the injection method, Dorothée Baïlle and Laurence Vernis for their help in the
881 construction of cyto-HyPer and cyto-Grx1-roGFP2 expression plasmids. We are also
882 grateful to Ana Ferreiro for her constructive advice on muscle physiology and use of
883 NAC. We also thank Elitsa Ivanova for English language improvement. Finally, we
884 acknowledge Linh Chi Bui of the Bioprofiler platform at the BFA unit for her help
885 quantifying GSSG/GSH ratio by HPLC.

886 **FUNDING** : F.D., C.H., F.B.R., E.C., A.L., O.A., P.V., and S.B.P. were supported by
887 Paris Diderot University, the French National Center for Scientific Research (CNRS),
888 and the Agence Nationale de la Recherche (ANR) (3BSV5-0017-DESMECA). This

889 work was supported by the Association Française contre les Myopathies (AFM)
890 (grant numbers 15454, 18358, and 20802); and ANR (grant number ANR-13-BSV5-
891 0017). S.B.D. is a fellow of AFM.

892

893 **AUTHOR CONTRIBUTIONS**

894 F.D. and S.B.P. designed the research, performed experiments, analyzed data, and
895 wrote the paper. B.D.S., C.H., F.B.R., E.C., and G.C. performed experiments. M.E.H.
896 provided reporters and analyzed data. P.V. critically read the paper. A.L. extracted
897 muscles from KI-R405 mice, analyzed data, and critically read the paper. O.A.
898 designed AAV experiments.

899 **DECLARATION OF INTEREST** None

900 **Bibliography**

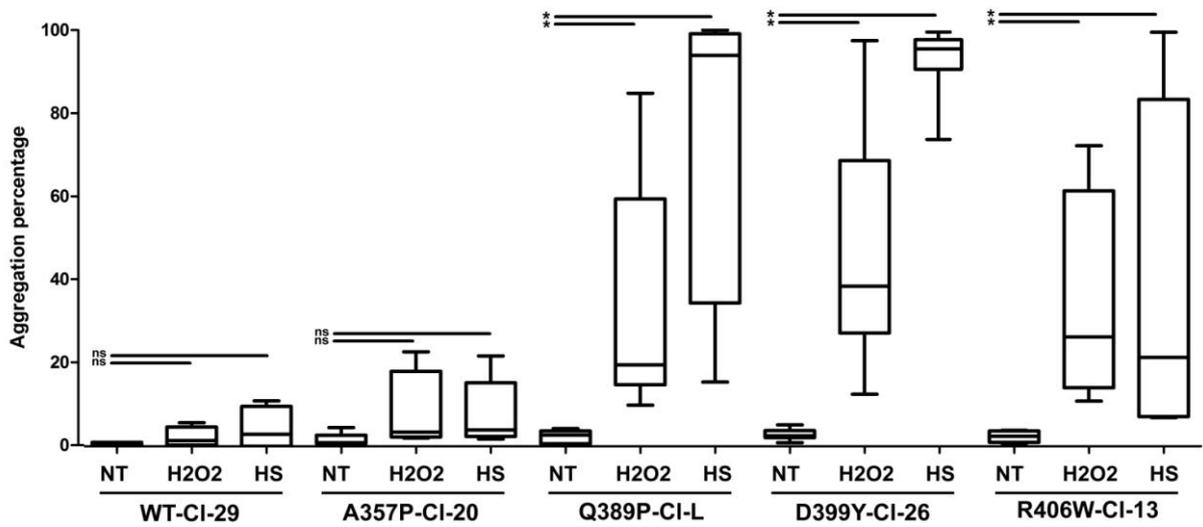
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1065

1066 **Supporting Information**



1067

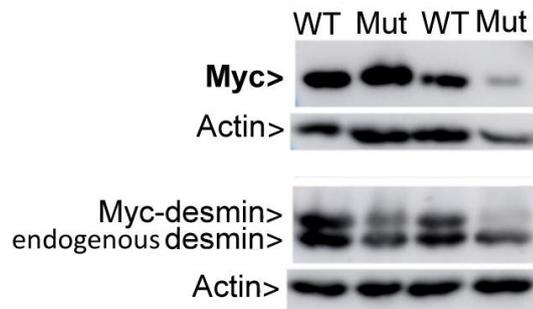
1068 **Supp. Data 1:** Box plot representing the aggregation percentages 24 h after stress (T24) compared to
 1069 untreated cells. Three different treatments are shown: heat shock (HS), oxidative stress (H₂O₂), and
 1070 no stress (NT). Cells were counted in at least 3 independent experiments (>100 cells each) (ns: not
 1071 significant; **p* < 0.05, compared to unstressed).

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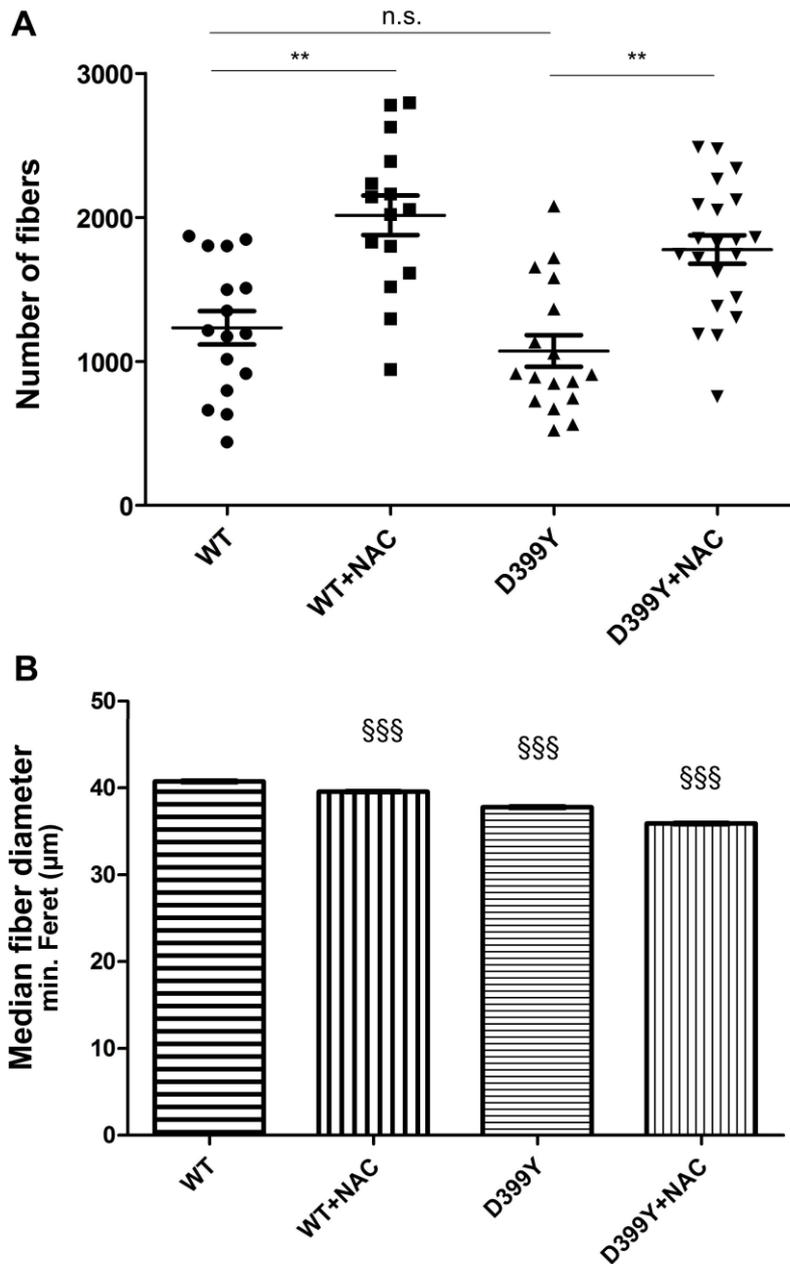
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Supp. Data 2: Desmin expression levels in AAV-infected muscles. Representative Western blot analysis of endogenous and human Myc-desmin in muscles injected with AAV expressing WT or D399Y (Mut) desmin. Actin serves as a loading control.



1080

1081 **Supp. Data 3: Effect of NAC on fiber number and median fiber diameter in TA muscles**
 1082 **expressing ectopic human desmin.** (A) Quantification of number of fibers in TA muscles expressing
 1083 WT or D399Y mutant desmin, untreated or treated with NAC. Plot shows the average of the total
 1084 number of fibers in 4 sections from 4 injected muscles for each condition (n.s.: $p > 0.05$; **: $p < 0.01$).
 1085 (B) Quantification of median fiber diameters (min. Feret values) in TA muscles expressing WT or
 1086 D399Y mutant desmin, untreated or treated with NAC. Diameters were measured on whole muscle
 1087 transversal sections stained with laminin. Medians were calculated with 5 sections from 3 injected
 1088 muscles for each condition (§§§: $p < 0.001$, each construct compared to others).

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Treatment	Mean volume drank (mL/day)	Ratio volume drank (without NAC/with NAC)	Mean absorbed NAC (mg/mouse/day)	Mean absorbed NAC (g/kg/day)
Without NAC 1 month	4.53	1.3	–	–
With NAC 1 month	3.45	–	11.5	0.38
Without NAC 2 months	5.68	1.9	–	–
With NAC 2 months	2.97	–	10	0.33

1092 **Supp. Data 4: Measures of amount drank by mice.** Amount of liquid (water or NAC solution) drank
1093 by mice after 1 or 2 months. Each group contains an equivalent number of animals injected with AAV
1094 expressing WT or D399Y mutant desmin.