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Supplemental Information

Damaged Myofiber-Derived Metabolic Enzymes Act as Activators of Muscle Satellite Cells

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Figure S1 (Related to Figure 2)

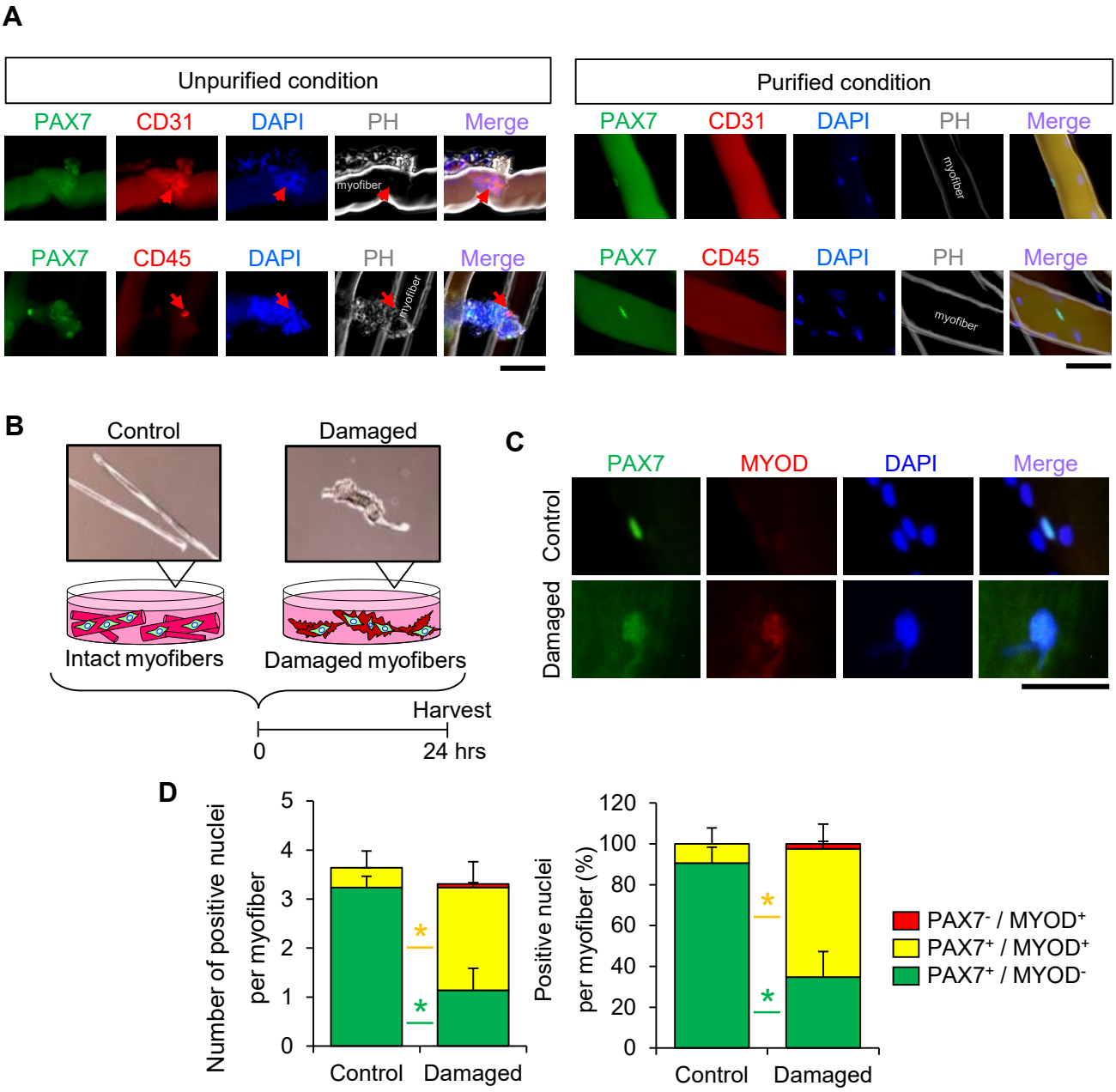

Figure S1. Satellite cell culture conditions

(A) Myofibers associated with interstitial cells were visible under the light microscope and avoidable in our culture model. In the unpurified condition, interstitial cells attached myofibers were visualized by immunostaining for CD31 and CD45, whereas these cells were not observed in the purified condition. Scale bars, 50 μm.

(B-D) To test whether intact myofibers themselves were responded to DMDFs and then indirectly stimulated their associated satellite cells, satellite cells associated with damaged (shrunken) myofibers were cultured in DMEM for 72 hours **(B)** and then immunostained for PAX7 and MYOD **(C)** (quantified in **(D)**). Isolated myofibers were mechanically damaged with a Pasteur pipette. Scale bars, 25 μm. *P < 0.05. Values are means ± SE (n=4 in each condition).

Figure S2 (Related to Figure 5)

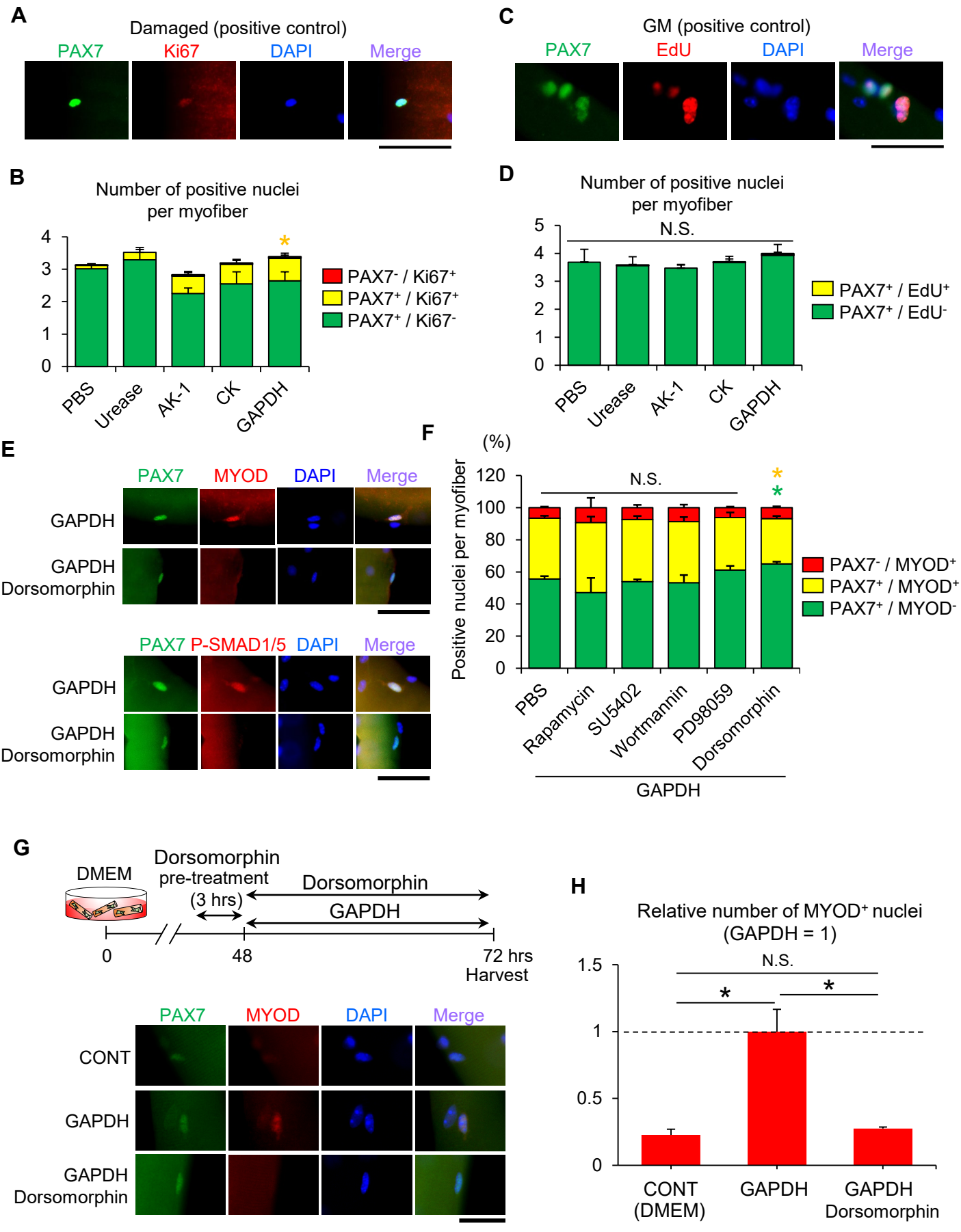

Figure S2. Effects of DMDFs on satellite cells

To examine whether DMDFs stimulate induction of entry into the cell-cycle, satellite cells associated with individual myofibers were freshly isolated from EDL muscles and treated with recombinant metabolic enzyme proteins in DMEM for 72 hours. PBS and urease were used as negative control and a non-muscle enzymatic control, respectively.

(A-B) Immunostaining of satellite cells associated with myofibers for PAX7 and Ki67 **(A)**(quantified in **B**). Damaged myofibers (Damaged) were used as a positive control. Asterisk (*) indicates differences compared with urease control. Values are means ± SE (n=4 mice in each condition). *P < 0.05. Scale bar, 50 μm.

(C-D) Immunostaining of satellite cells associated with individual myofibers for PAX7 and EdU **(C)**(quantified in **D**). Myofibers cultured in GM were used as a positive control. Values are means ± SE (n=4 mice in each condition). Scale bars, 50 μm.

(E-F) Satellite cells associated with myofibers were treated with chemical compounds in the presence of GAPDH in DMEM for 72 hours. **(E)** Immunostaining for PAX7 and MYOD (quantified in **F**) or Pax7 and p-SMAD1/5 (BMP signaling downstream targets) was performed. Values are means ± SE (n=3-5 mice, >15 individual myofibers per mouse were counted). Asterisk (*) indicate differences compared with PBS control. *P < 0.05. Scale bars, 50 μm.

(G-H) Satellite cells associated with myofibers were treated with Dorsomorphin in the presence of GAPDH in DMEM for 24 hours followed by pre-treatment with Dorsomorphin for 3 hours. **(G)** Immunostaining for PAX7 and MYOD (quantified in **H**). Values are means ± SE (n=3 mice, 10 individual myofibers per mouse were counted). *P < 0.05. Scale bars, 25 μm.

Supplemental Information

Supplemental Experimental Procedures

Muscle damage model

To induce muscle injury, 30 μL of 1.2 % $BaCl_2$ was injected intramuscularly into the TA muscle in both the right and left legs of anesthetized mice using a 29G 1/2 insulin syringe at 24 hours following the administration of recombinant proteins into the TA muscle. Regenerating muscles were collected further 24 or 48 hours after $BaCl_2$ injection. The cryosections were frozen by 2-methylbutane chilled in liquid nitrogen immediately after dissection and stored at −80°C until cryosectioning. Transverse muscle sections were obtained using a cryostat. The dose of GAPDH recombinant protein for *in vivo* experiments was 3.6 U per TA muscle (approximately 50 mg). EdU was intraperitoneally injected to mice 24 hours before sacrifice.

Proteome analysis

Proteins were fractionated in 4-20% gradient SDS polyacrylamide gel (Cosmo-Bio, Japan), and protein bands were detected using GelCode Blue Stain Reagent (Thermo Fischer Scientific, Waltham, MA). The protein bands ranging from 10 to 50 kDa were recovered from SDS-PAGE gel. The protein in gel was treated by trypsin and eluted from the gel following to the manufacture's manual (Bruker Daltonics, Germany). In brief, the sliced gel was destained with destaining solution (25 mM ammonium bicarbonate (ABC), 50% acetonitrile (ACN)), completely dehydrated with 100% ACN, and then treated with the reduction buffer (10 mM dithiothreitol (DTT), 25 mM ABC) at 56°C for 45 min. After removed the reduction buffer and washed with 25 mM ABC, the gel was treated with alkylating solution (55 mM iodoacetamide, 25 mM ABC) at room temperature for 30 min. After removed the alkylating buffer and washed with 25 mM ABC, the gel was completely dehydrated with acetonitrile again. The gel was

soaked with 20 ng/μl Trypsin Gold (Promega, Fitchburg, WI), and incubated at 37°C over night. The gel was mixed with the elution buffer (50% acetonitrile, 5% trifluoroacetate (TFA)) and sonicated for 3 min, vortexed for 30 min. The elution containing trypsin-digested peptides was recovered, and then reduced volume to less than 10 μl by evaporator. The peptide sample was fractioned with L-column 2 (CERI, Japan) by using the combination with EASY-nLCII (Thermo Fischer Scientific, Waltham, MA) and PROTEINNER fcII (Bruker Daltonics, Germany) following to the manufacture's manual (Bruker Daltonics, Germany). The fractioned sample was mixed with same volume of 0.7 mg/ml α-cyano-4-hydroxycinnamic acid (HCCA) (Bruker Daltonics, Germany) as a matrix. HCCA-mixed sample was analyzed to obtain m/z peak data by UltraflexIII matrix assisted laser desorption / ionization–time of flight mass spectrometry (MALDI-TOF/MS) with the equipped software (flexcontrol (ver. 3.3) and flexanalysis (ver. 3.3)) (Bruker Daltonics, Germany). The information of m/z signal data were analyzed by biotools (ver. 3.2) (Bruker Daltonics, Germany) and matrix server (Matrix Science, Boston, MA) by using public database (Swiss-Prot).

Recombinant proteins

Recombinant proteins were purchased as follows: adenylate kinase-1 (AK-1, Novus Biologicals, CO), creatine kinase-MM (CK, Oriental Yeast, Tokyo, Japan), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Abcam, Cambridge, UK), phosphoglycerate mutase-2 (PGAM-2, Oriental Yeast, Tokyo, Japan) and triose phosphate isomerase (TPI, Abcam, Cambridge, UK). These recombinant proteins were added in DMEM in a floating culture condition.

Immunofluorescence

For immunocytochemistry, satellite cells associated with myofibers were fixed with 2% paraformaldehyde, blocked/permeabilized with PBS containing 0.3% Triton X100 and 5% goat serum for 20 minutes at room temperature, and incubated with primary antibodies at 4°C overnight as described previously. For immunohistochemistry, frozen transverse sections of TA muscle were fixed with 4% paraformaldehyde, blocked/permeabilized containing 0.3% Triton X100 in PBS for 20-30 minutes at room temperature, and incubated with primary antibodies at 4°C overnight. All immunostaining samples were visualized using appropriate species-specific Alexa Fluor 488 and/or 546 fluorescence-conjugated secondary antibodies (Thermo Fisher Scientific). Stained samples were viewed on an Olympus IX83 (Olympus, Tokyo, Japan) microscope. Digital images were acquired with a DP80 camera and quantified using cellSens software (Olympus). Images were optimized globally and assembled into figures using Fiji.

Antibodies were obtained from the following sources: Mouse anti-PAX7 antibody (PAX7, sc-81648) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit anti-MYOD antibody (sc-760) (Santa Cruz Biotechnology); rat anti-Ki67 antibody (TEC-3, M7249); Rat anti-GFP antibody (NACALAI TESQUE, Kyoto, Japan) ; Rabbit anti-P-SMAD1/5 antibody (Cell Signaling Technology); Rat anti-CD31 (Biolegend, CA); Rat anti-CD45 (Biolegend, CA); Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific).

Quantitative revere transcription-PCR (Q-PCR)

Total RNA was extracted from TA muscle with ISOGEN II (NipponGene, Tokyo, Japan). cDNAs were prepared with a ReverTraAce kit (Toyobo, Tokyo, Japan). Q-PCR was performed with a THUNDERBIRD Probe qPCR Mix and a CFX96 real time PCR detection system (Bio Rad, Tokyo, Japan) according to the manufacturer's instructions. Primer

sequences were listed as follows: Pax7 (F5′-GTGCCCTCAGTGAGTTCGATTAGC-3′ and R5′-CCACATCTGAGCCCTCATCCA-3′); Myf5 (F5′- TGAGGGAACAGGTGGAGAAC-3′ and R5′- AGCTGGACACGGAGCTTTTA-3′); MyoD (F5′- AGCACTACAGTGGCGACTCA-3′ and R5′- GCTCCACTATGCTGGACAGG-3′); Myogenin (F5′- CTACAGGCCTTGCTCAGCTC-3′ and R5′- AGATTGTGGGCGTCTGTAGG-3′); TATA box binding protein as a normalizer (F5′-CAGATGTGCGTCAGGCGTTC-3′ and R5′- TAGTGATGCTGGGCACTGCG-3′).