Supplemental Data

Figure S1. Depletion of satellite cells in Pax7-DTA Mice, related to Figure 1

A) Immunohistochemical identification of satellite cells using an antibody against Pax7 (red) co-stained with laminin (green) in both SC-WT and SC-Dep muscle cross-sections at Sham, SA1 and SA2 time points (quantified in C). Arrows identify Pax7+ cells. Scale bar=50 µM. B) Plantaris muscle mass normalized to body weight. † denotes main effect for surgery; p < 0.05 (N = 6-9 mice/group/time point). C) Quantification of satellite cell content in SC-WT and SC-Dep skeletal muscle. Related to Figure 1. * denotes significant difference within the respective time point; † denotes significant increase relative to Sham condition within that respective group; p < 0.05 (N = 6-9 animals/group/time point).
Figure S2. No effect of satellite cell depletion on pSMAD3 and exosomal identification from surface marker expression, related to Figures 1 and 2

A) Immunohistochemical identification of pSMAD3 + nuclei (red) co-stained with DAPI (blue) in both SC-WT and SC-Dep muscle cross-sections at Sham and SA1 time points (quantified in B). Scale bar=20 µM. B) Quantification of percentage of nuclei that are p-SMAD3+ in SC-WT and SC-Dep skeletal muscle at sham and SA1. Related to Figure 1. * denotes significant increase relative to Sham condition; p < 0.05 (N = 6 animals/group/time point). C) MPC exosomes were identified following their isolation from MPC conditioned media (CM) by Western blot analysis of exosomal markers CD63, CD81 and HSP70. Actin was used as a control. Related to Figure 2.
Figure S3. miR-206 regulates fibrogenic cell collagen expression through ribosome binding protein 1 (Rrbp1), related to Figure 2

A) Luciferase activity. * denotes significant effect of miR-206; p < 0.05 (4 independent samples (n = 4) each performed in triplicate). B) Sequence alignment of miR-206 and the Rrbp1 3'-untranslated region (UTR) containing the predicted miR-206 binding site. The seed sequence within miR-206/Rrbp1 binding site is underlined, and the mutated seed nucleotides of the mutant Rrbp1 (Rrbp1m) 3'-UTR are boldfaced. C) qRT-PCR quantification of primary fibrogenic cell collagen expression levels. * denotes significant effect of miR-206; p < 0.05 (N = 3 isolates studied in duplicate). D) qRT-PCR analysis demonstrated efficient knockdown of Rrbp1 mRNA following transfection of Rrbp1 siRNA in fibrogenic cells compared to a scrambled control siRNA. * denotes significant effect of Rrbp1 siRNA; p < 0.05 (N = 3 isolates studied in duplicate). E) Western blot analysis demonstrated efficient knockdown of Rrbp1 protein content following transfection of Rrbp1 siRNA compared to a scrambled control siRNA. * denotes significant effect of Rrbp1 siRNA, P < 0.05. (N = 3 isolates). F) qRT-PCR quantification of fibrogenic cells collagen expression levels. * denotes significant effect of Rrbp1 siRNA; p < 0.05 (N = 3 isolates studied in duplicate).

Data represented as Mean ± SEM. Related to Figure 2.
Figure S4. Quantification of time course microarray analysis of various collagen and Rrnbp1 mRNAs following synergist ablation, related to Figure 4

Note the peak for the most abundant collagen transcripts (Col1a1, Col1a2, Col12a1, and Col3a1) at 7 days post-surgery (SA1). Inset - Change in expression of Rrnbp1 during mechanical overload (see Chailou et al., for complete dataset). Related to Figure 4.
Figure S5. No effect of satellite cell depletion on collagen expression in sham-operated animals, related to Figure 4

Quantification of Col1a2, Col12a1 and Col3a1 mRNA levels. Related to Figure 4.
Figure S6. Muscle fibrogenic cells largely express Tcf4 and Myogenic progenitor cells (MPCs) are almost exclusively MyoD+, related to Figures 2 and 3
A) Immunocytochemical identification of Tcf4 in muscle fibrogenic cells in vitro. (B) Co-stained image depicting nuclei stained with DAPI. C) Merged image showing muscle fibrogenic cells were uniformly Tcf4+. D) Quantification of percentage of muscle fibrogenic cells positive for Tcf4. E) Immunocytochemical identification of MyoD in MPCs. F) Co-stained image depicting nuclei stained with DAPI. G) Merged image showing MPCs were nearly 100% MyoD+. H) Quantification of percentage of MPCs positive for MyoD. For both experiments, N = 3 isolates and scale bar=50 μM. Related to Figure 2 and 3.
Figure S7. Presence of activated satellite cells/MPCs during the acute phase of ECM remodeling prevents expansion of fibrogenic cells.
Table S1. Complete dataset of miRNA expression profiling of MPC's, MPC exosomes and fibroblasts using qRT-PCR based miRNA arrays, related to Figure 2 and 3.

See separate Excel sheet for Table S1.
Table S2. List of predicted miR-206 targets using TargetScan, related to Figure 2 and 3.

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Table S3. List of predicted miR-206 targets that are elevated in satellite cell-depleted muscle following one week of overload, related to Figures 2 and 3.

See separate Excel sheet for Table S3.
Supplemental Materials and Methods

**Generation of mouse strains**

The Pax7\(^{\text{CreER}/+}\); Dicer\(^{\text{fl/fl}}\) strain, designated Pax7-Dicer, was generated by crossing male Pax7\(^{\text{CreER}/\text{CreER}}\) and female Dicer\(^{\text{fl/\text{fl}}}\) strains. Male heterozygotes for Pax7\(^{\text{CreER}/+}\) and Dicer\(^{\text{fl/+}}\) were then backcrossed to female Dicer\(^{\text{fl/\text{fl}}}\) to generate Pax7\(^{\text{CreER}/+}\); Dicer\(^{\text{fl/\text{fl}}}\) offspring. Pax7-Dicer mouse allows satellite cell-specific expression of the Cre recombinase, driven by the Pax7 promoter. Tamoxifen treatment induces Cre activity, which recombines out DNA flanked by loxP sites encoding most of the second RNaseIII domain of the Dicer enzyme (Barberi et al., 2013) in Pax7-expressing satellite cells. Limb muscles from adult (4 months of age), male Pax7-Dicer mice were isolated and digested for the isolation of primary cells. The Pax7\(^{\text{CreER}/+}\); Rosa26\(^{\text{DTA}+/+}\) strain, designated Pax7-DTA, was generated by crossing male Pax7\(^{\text{CreER}/\text{CreER}}\) and female Rosa26\(^{\text{DTA}/\text{DTA}}\) strains. Administration of tamoxifen results in the specific depletion of satellite cells (Fry et al., 2014; Fry et al., 2015; Lee et al., 2015; McCarthy et al., 2011).

**Synergist ablation surgery and tissue collection**

Mice were subjected to bilateral synergist ablation surgery to induce hypertrophy of the plantaris muscle as previously described in detail (McCarthy and Esser, 2007). Briefly, following anesthetization with a mixture of 95% oxygen and 5% isoflurane gas, 50% of the soleus and gastrocnemius muscles were surgically removed via an incision of the hindlimb. Particular attention was made to ensure neural and vascular supply to the plantaris remained intact and undamaged. Sham surgery controls for day zero involved similar procedures without gastrocnemius and soleus muscle excision. Following recovery from surgery, mice were euthanized at the designated time point (1, 2 or 8 weeks following surgery) and plantaris muscles were excised and weighed. Tissue used for RNA was flash frozen in liquid nitrogen and stored at -80°C until further use. Tissue used for microscopy was pinned to a cork block at resting length, covered with a thin layer of Tissue Tek
optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA), and then quickly frozen in liquid nitrogen-cooled isopentane and stored at -80°C until sectioning.

**Immunohistochemistry and immunocytochemistry**

Frozen tissue was sectioned (7 µm) and air dried for approximately 1 hr. Pax7 and fiber type cross-sectional area methods have been published previously (Fry et al., 2014). Briefly for Pax7, sections were fixed in 4% paraformaldehyde, followed by epitope retrieval using sodium citrate (10 mM, pH 6.5) at 92°C for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 7 min, followed by blocking step with Mouse-on-Mouse Blocking Reagent (Vector Laboratories). Incubation with Pax7 antibody (1:100) (Developmental Studies Hybridoma Bank, Iowa City, IA) was followed by incubation with the biotin-conjugated secondary antibody (1:1000) (Jackson ImmunoResearch, West Grove, PA) and the signal was amplified using streptavidin-horseradish peroxidase (HRP) included within a tyramide signal amplification kit (Life Technologies, Carlsbad, CA # T20935). For Tcf4/αSMA, sections were fixed in 4% paraformaldehyde, followed by epitope retrieval using sodium citrate (10 mM, pH 6.5) at 92°C for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 7 min. Incubation with Tcf4 antibody (1:100) (Cell Signaling, Danvers, MA) and αSMA (1:100) (Santa Cruz, Santa Cruz, CA) was followed by incubation with goat anti-mouse IgG2a AF555 (ThermoFisher, Waltham, MA) and biotin-conjugated secondary antibody (1:1000) (Jackson ImmunoResearch) and the signal was amplified using streptavidin-horseradish peroxidase (HRP) included within a tyramide signal amplification kit (Life Technologies, Carlsbad, CA # T20935). For fiber type-specific cross-sectional area, unfixed sections were incubated in antibodies against myosin heavy chain (MyHC) types 1, 2a, and 2b (type 1: BA.D5; 2a: SC.71; and 2b: BF.F3, Developmental Studies Hybridoma Bank) in addition to laminin (1:100, Sigma). MyHC type 2x expression was assumed from unstained fibers. Fluorescent-conjugated secondary antibodies against various mouse immunoglobulin subtypes were applied to visualize MyHC expression and laminin. Sections were postfixed in 4% PFA prior to mounting. For pSMAD3, sections were fixed in 4% paraformaldehyde,
followed by epitope retrieval using sodium citrate (10 mM, pH 6.5) at 92°C for 20 min prior to incubation with pSMAD3 antibody (1:75) (Cell Signaling) overnight. On the second day, slides were incubated with goat-anti rabbit AF555 (Life Technologies).

For MyoD (MPCs) and Tcf4 (fibrogenic cells) immunocytochemistry, cells grown on cover slips were fixed in 4% PFA for 20 min, washed with PBS and permeabilized in PBS + 0.1% Triton-X100. Cells were blocked in 1% BSA, and incubated in primary antibody (MyoD 1:100, BD Biosciences # 554130 or Tcf4 1:100, Cell Signaling #2569) for 2 hr at room temperature. Cells were then washed in PBS, then incubated for 1 hr in secondary antibodies (Gt anti-Ms biotin, Jackson ImmunoResearch #115-065-205; Gt anti-Rb biotin, Jackson ImmunoResearch # 111-065-045), followed by 1 hr with streptavidin-HRP included with the TSA kit (Life Technologies). TSA-Alexa Fluor 488 or 594 was used to visualize antibody-binding and cells were co-stained with 4’,6-diamidino-2-phenylindole (DAPI, #D35471; Life Technologies).

**Image acquisition and analysis**

Images were captured at 20X objective magnification at room temperature with a Zeiss upright microscope (AxioImager M1, Oberkochen, Germany) and analysis carried out using the AxioVision Rel software (v4.8). Analysis of fiber type-specific cross-sectional area (CSA) of the plantaris was performed manually with identification of fiber type by expression of MyHC isoforms and then manual analysis of fiber-type specific CSA using AxioVision software. Satellite cell abundance was assessed manually by counting cells that were scored as Pax7+ and DAPI+ residing within the laminin border and were expressed as Pax7+ cells/fiber. Tcf4 and Tcf4/αSMA abundance were assessed manually by counting cells that were scored as Tcf4+ / DAPI+, and Tcf4+ / αSMA+ / DAPI+. pSMAD3 was assessed with the use of the automated counting program within AxioVision, and scored as pSMAD3+ and DAPI+.

**Single fiber isolation and myonuclear quantification**

After careful removal of the other plantar flexor muscles, plantaris muscle was left on the tibia and fixed *in situ* at resting length in 4% paraformaldehyde for 48 hr. Single fibers were isolated using a
previously described method (Brack et al., 2005; Fry et al., 2015). Briefly, following digestion for 2 hr in 40% NaOH, single fibers were mechanically teased apart, strained, and washed with PBS before being stained with DAPI for nuclear visualization. Suspended fibers were dispersed on a slide and mounted with Vectashield fluorescent mounting media (Vector Laboratories). A minimum of 15 fibers from each animal were used to determine myonuclear number and domain size. Nuclei from each fiber were counted by z-stack analysis to determine the number of myonuclei within a defined fiber segment (nuclei/100 μM). Myonuclear domain is defined as the amount of cytoplasm per myonucleus and was calculated by multiplying \( \pi \times \text{one-half the fiber width (radius)}^2 \times \text{length of the measured fiber segment} \), to give a fiber segment volume (μm\(^3\)), which was then divided by the total number of myonuclei within the segment to generate the myonuclear domain.

**Histochemistry**

For quantification of extracellular collagen content, muscle sections were fixed for 15 min in Bouin's solution at 37°C. After a brief wash, tissue was incubated in Sirius Red solution (0.1% in Saturated Picric Acid) for 2 hr at room temperature. Tissue was washed, dehydrated and mounted in Xylene-based mounting media. Images were captured using a BX61VS inverted microscope (Olympus) and analyzed using Adobe Photoshop. For quantification of collagen area, red pixels (Sirius Red+ collagen) were selected in Adobe Photoshop, and all non-red pixels were deleted, converting the resulting image to a binary image and counting red+ pixels using the ImageJ Analyze Particles function. For extracellular matrix (ECM) accumulation, muscle sections were pre-fixed in 4% PFA, and then incubated with Texas-Red-conjugated α-wheat germ agglutinin (1 mg/mL; Invitrogen W21405). Wheat germ agglutinin (WGA) staining was quantified to measure the area that the muscle ECM occupied using the thresholding feature of the AxioVision software. The area occupied by WGA was expressed relative to total area of the muscle cross-section.

**Isolation and culture of primary myogenic progenitor cells (MPCs) and muscle fibrogenic cells**

Detailed procedures have been published previously (Fry et al., 2014; Hidestrand et al., 2008). Briefly, MPCs were purified from the gastrocnemius, soleus, plantaris and tibialis anterior of 12 male
adult (4-month-old) C57BL/6 mice and 4 male adult (4-month-old) Pax7-Dicer mice according to the preplate protocol (Rando and Blau, 1994). Muscles were thoroughly minced in PBS containing dispase II (2.4 U/mL, Roche Applied Science, Indianapolis, IN, USA) and collagenase D (1 mg/mL, Sigma, St. Louis, MO, USA), and incubated at 37 °C for 60 min, then passed through a 40-µm filter (BD Biosciences, San Jose, CA, USA). The filtrates were spun at 1300g for 10 min to pellet the cells. To enrich for MPCs or fibrogenic cells, cells were plated on uncoated 60-mm tissue culture plastic and incubated for 30 min at 37 °C in growth media (GM, DMEM + 20% fetal bovine serum) to allow adherent cells (fibrogenic cells) to attach. Adherent cells are collectively referred to as fibrogenic cells with a likely heterogeneous composition enriched for FAPS and fibroblasts. We chose not to purify adherent cells of a specific phenotype to better represent the milieu of cells surrounding muscle fibers and satellite cells within the muscle niche. Immunocytochemistry was performed on fibrogenic cells with a known muscle connective tissue fibroblast marker (Tcf4) (Mathew et al., 2011; Merrell et al., 2015; Miller et al., 2005; Murphy et al., 2011) and isolates used in experiments were >95% Tcf4+ (Figure S6A-D), demonstrating a population of adherent cells significantly enriched for muscle fibroblasts. The non-adherent cells (MPCs) were collected and transferred to a 60-mm collagen-coated dish (BD Biosciences, San Jose, CA) (passage 0). GM+ (GM + 5 ng/ml basic fibroblast growth factor) was changed every other day between preplating. MPCs were almost exclusively MyoD+ as determined by immunocytochemistry (>97% MyoD+, Figure S6E-H). Fibrogenic cells were maintained on uncoated 60-mm tissue culture plastic plates in GM, changed every other day. MPCs from Pax7-Dicer mice were also treated with either 4-OH tamoxifen (conditional knockout [cKO]; Sigma, St. Louis, MO) or DMSO (control) at a final concentration of 1µM in GM+ and passaged 6 times following recombination of Dicer to deplete the MPCs and MPC exosomes of miRNA.

**Conditioned medium**

Medium from fibrogenic cells and MPCs from both C57BL/6 and Pax7-Dicer mice was collected following a 24 hr conditioning period and cells/large debris pelleted at 1300 x g for 10 min. Fibrogenic cells (8 × 10⁵/plate) were seeded on uncoated plates with MPC conditioned medium (CM) or
fibrogenic cell CM (control) for 24 hr (n = 3-5 isolates studied in duplicate). Following the 24 hr culture with CM medium, the fibrogenic cells were collected for RNA/protein analysis.

**Exosome isolation and labeling**

MPCs were cultured at 70-80% confluency for collection of CM for exosome isolation. 10 ml of MPC CM was spun at 1500g for 10 min in 15 ml conical tubes using an Eppendorf 5810R centrifuge with an A-4-81 rotor to pellet large debris and cells, and the remaining supernatant combined with ExoQuick-TC reagent overnight at 4°C (10 ml CM supernatant with 2 ml Exo-Quick-TC). Sequential centrifugation (1500g for 30 min and 5 min) yielded a pellet of exosomes. Following isolation, exosomes were immediately harvested for protein and RNA content, or resuspended in fibroblast CM. The RNA of a subset of MPC exosomes was labeled to detect the docking and delivery of MPC exosomes with fibroblasts with acridine orange according to manufacturer instructions (Systems Biosciences, Mountain View, CA), and then incubated with fibrogenic cells. MPC exosome docking and delivery was imaged on a Zeiss upright AxioImager microscopes (200X magnification) and analyzed with AxioVision (version 4.9). MPC exosomes were incubated with fibrogenic cells for a period of 24-48 hr prior to the collection of RNA and protein from fibrogenic cells.

**Total RNA isolation and quantitative reverse transcription (qRT)-PCR**

Total RNA from fibrogenic cells, MPCs, MPC exosomes and whole tissue was isolated using TRIzol (Invitrogen) according to manufacturer’s instructions. RNA content and purity were assessed using the NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA). For *in vitro* studies, first-strand cDNA synthesis from total RNA (500 ng) was performed using qScript cDNA Supermix (Quanta Biosciences) according to manufacturer’s instructions. A subset of RNA from Pax7-Dicer MPC exosomes was reverse transcribed using the Universal miRNA cDNA Synthesis Kit from Exiqon (Woburn, MA) following manufacturer’s instructions to generate cDNA from miRNAs. For *in vivo* studies, cDNA was generated using miScript® II RT Kit (Qiagen, Hilden, Germany). The resultant cDNA was amplified with a 1 × SYBR Green PCR Master Mix (Applied Biosystems) or 1 x Taqman Master Mix plus 0.3 µmol/l of gene-specific upstream and downstream primers during 40 cycles on an
Applied Biosystems 7900 Fast Realtime Cycler. A standard curve was generated using pooled cDNA from the experimental samples included in each set of experiments, and Ct values were within the linear portion of the amplification range. The expression of the specific genes of interest was reported as relative arbitrary units, as the value obtained for the specific gene was normalized to the value obtained for housekeeping gene. For in vitro studies, genes were normalized to Gapdh, for in vivo studies mRNAs were normalized to the geometric mean of Gapdh, Tmx4 and Capbz, with miRNAs being normalized to the small nuclear RNA, Rn7sk. In every case, the Ct values were taken when the efficiency of the reaction was between 90 and 110%. In every run, product purity was confirmed by examining the melt curve that showed a single peak. All the real-time experimental runs were repeated twice. Primers for Col1a2, Col3a1, Col6a2 and fibronectin expression in vitro were predesigned Taqman primers; primers for miR-206, -133a and -709 were predesigned Exiqon or Qiagen primers. All primers used are designed to span intron/exon borders.

### Primers used for SYBR Green qPCR reactions

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### qRT-PCR-based miRNA microarray

Total RNA, isolated as described above, was treated with TURBO DNase (Ambion, Austin, TX) to remove genomic DNA contamination. The total RNA concentration and purity were assessed by measuring the optical density (230, 260 and 280 nm) with the Nanodrop 2000 Spectrophotometer (ThermoFisher). miRNA cDNA synthesis from total RNA (100 ng) was performed using the Taqman miRNA RT kit and megaplex RT and preamp rodent pools set version 3.0 (Life Technologies). 750
unique miRNAs were profiled using rodent miRNA A and B version 3.0 Taqman low density array cards. Expression levels of all profiled miRNAs are presented in Supplemental Table 1.

**Western blot analysis**

Fibrogenic cell, MPC, MPC exosome and whole tissue protein was harvested using ice-cold RIPA buffer with 10 μl/ml protease inhibitor cocktail for mammalian tissues (P8340, Sigma-Aldrich, St. Louis, MO) and protein concentration determined by the BCA (ThermoFisher) method. Thirty μg for cells and 20 μg for whole tissue of protein were electrophoresed in 4–15% SDS polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred onto PVDF membranes at 0.08 A for 2 hr at 4°C. Membranes were blocked for 30 min with Odyssey Blocking Blocker (LI-COR, Lincoln, NE) prior to immunoblotting with the following antibodies: actin (Santa Cruz #sc-1616, Santa Cruz, CA) and Rrbp1 (Bethyl #A303, Montgomery, TX) overnight at 4°C with gentle rocking. After washing, the blots were incubated for 20 min at room temperature with goat anti-rabbit IgG (Alexa 680) or donkey anti-goat (Alexa 800) (LI-COR). Bands were visualized and quantified using the Odyssey Infrared Imaging System Application Software Version 3.0.21 and normalized to actin. Results are expressed as arbitrary densitometric units.

**Transfection experiments**

Mouse primary muscle fibrogenic cells were plated on 6-well plates at a confluency of 40-50%. The following day, fibrogenic cells were washed once with PBS before incubation in serum-free medium (DMEM). Fibrogenic cells were transfected with either a miR-206 mimic, antago-miR-206, siRNA against Rrbp1 or scrambled controls (Scr) (Life Technology) at a final concentration of 50nM using Lipofectamine 2000 (Life Technology) according to manufacturer instructions for a period of 6 hr. Following transfection, cells were washed and placed in GM. Fibrogenic cells were harvested for RNA and protein 24 hr following transfection.

**Validation of Rrbp1 mRNA as a miR-206 target**

To construct a Rrbp1 3′-UTR luciferase reporter plasmid, the mouse Rrbp1 3′-UTR was amplified from total mouse DNA and cloned into the Xbal site downstream of the luciferase gene in the pGL3-
promoter vector (Promega, Madison, WI). The predicted miR-206 binding site in the Rrbp1 3'-UTR is underlined in Figure S3B. The single miR-206 site within the cloned rat Rrbp1 3'-UTR was mutated by altering the middle three nucleotides of the miR-206 seed sequence using Gene Editor System (Promega) (mutated nucleotides are bolded, Figure S3B). Orientation and sequence of the wild-type and mutated Rrbp1 3'-UTR were confirmed by sequencing. NIH/3T3 cells (5 x 10⁴) were plated in a 12-well plate and transfected 24 hr later with Rrbp1 3'-UTR reporter gene (80 ng/well), with or without miR-206 mimic (20 nM final concentration, Life Technology) and pRL-CMV vector (20 ng) for normalization using Lipofectamine 2000 as described above. Following transfection, cells were washed and placed in GM. Twenty-four hours post-transfection, cells were lysed and luciferase activities measured using the Dual-Luciferase Assay according the manufacturer’s directions (Promega). Results are normalized to luciferase activity with Scr from 4 independent samples (n = 4), each performed in triplicate.
Supplemental References


