Load-mediated growth stimulus

Satellite cell extracellular vesicles (EVs) deliver tdTomato protein and/or transcript

Prior to Satellite Cell Fusion

Satellite cell EVs deliver miRNAs, mRNAs, and/or proteins

ECM-related gene expression

Muscle Fiber Membrane

Significant long-term growth then ensues with satellite cell EV communication and delayed myonuclear addition
Title: Fusion-Independent Satellite Cell Communication to Muscle Fibers During Load-Induced Hypertrophy

Authors: Kevin A. Murach\textsuperscript{1,2}, Ivan J. Vechetti Jr.\textsuperscript{1,3}, Douglas W. Van Pelt\textsuperscript{1,2}, Samuel E. Crow\textsuperscript{1,2}, Cory M. Dungan\textsuperscript{1,2}, Vandre C. Figueiredo\textsuperscript{1,2}, Kate Kosmac\textsuperscript{1,2}, Xu Fu\textsuperscript{4}, Christopher I. Richards\textsuperscript{4}, Christopher S. Fry\textsuperscript{1,5}, John J. McCarthy\textsuperscript{1,3}, Charlotte A. Peterson\textsuperscript{1,2,3*}

Affiliations: \textsuperscript{1}The Center for Muscle Biology, University of Kentucky, Lexington, Kentucky 40536
\textsuperscript{2}Department of Physical Therapy, College of Health Sciences, University of Kentucky, Lexington, KY 40536
\textsuperscript{3}Department of Physiology, College of Medicine, University of Kentucky, Lexington, KY 40536
\textsuperscript{4}Department of Chemistry, College of Arts and Sciences, University of Kentucky, Lexington, KY 40536
\textsuperscript{5}Department of Athletic Training and Clinical Nutrition, College of Health Sciences, University of Kentucky, Lexington, KY 40536

Running head: Satellite cell extracellular vesicle communication to muscle fibers

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*Correspondence: Charlotte A. Peterson
900 S. Limestone, CTW 439
University of Kentucky
Lexington, KY 40536
859-218-0476 (phone)
cpete4@uky.edu

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Abstract

The “canonical” function of Pax7+ muscle stem cells (satellite cells) during hypertrophic growth of adult muscle fibers is myonuclear donation via fusion to support increased transcriptional output. In recent years, however, emerging evidence suggests that satellite cells play an important secretory role in promoting load-mediated growth. Utilizing genetically modified mouse models of delayed satellite cell fusion and in vivo extracellular vesicle tracking, we provide evidence for satellite cell communication to muscle fibers during hypertrophy. Myogenic progenitor cell extracellular vesicle-mediated communication to myotubes in vitro influences extracellular matrix (ECM)-related gene expression, which is congruent with in vivo overload experiments involving satellite cell depletion, as well as in silico analyses. Satellite cell-derived extracellular vesicles can transfer a Cre-induced, cytoplasmic-localized fluorescent reporter to muscle cells as well as miRNAs that regulate ECM genes such as matrix metalloproteinase 9 (Mmp9), which may facilitate growth. Delayed satellite cell fusion did not limit long-term load-induced muscle hypertrophy indicating that early fusion-independent communication from satellite cells to muscle fibers is an under-appreciated aspect of satellite cell biology. We cannot exclude the possibility that satellite cell-mediated myonuclear accretion is necessary to maintain prolonged growth, specifically in the later phases of adaptation, but these data collectively highlight how extracellular vesicle delivery from satellite cells can directly contribute to mechanical load-induced muscle fiber hypertrophy, independent of cell fusion to the fiber.
Introduction

The primary muscle stem cell population is Pax7+ satellite cells (108). Throughout post-natal development, these cells fuse to growing muscle fibers and contribute new myonuclei (131). When satellite cells are depleted using a genetic approach in pre-pubertal murine muscle, muscle fibers are smaller by adulthood (6), and when depleted in young adulthood, cannot mount an effective hypertrophic response to mechanical loading (31, 75, 84). Conversely, adult (>4 months old) murine muscle can sustain short-term load-mediated hypertrophy (≤2 weeks) in the absence of satellite cells (39, 40, 61, 76, 84), although long-term hypertrophy (≥8 weeks) is attenuated (34, 39, 40). Some evidence suggests that the addition of new myonuclei to muscle fibers undergoing hypertrophy is necessary before growth ensues (12, 45) or is required beyond a certain threshold of growth to control the “myonuclear domain” (22, 95, 96), which is the maximal area that each myonucleus can transcriptionally govern, although this limit is equivocal and may be muscle and/or fiber type-specific (22, 42, 61, 82, 83).

Aside from myonuclear addition, another avenue whereby satellite cells could directly support muscle fiber hypertrophy is via secreted factors, which is plausible since satellite cells reside in very close proximity to muscle fibers, increase dramatically in abundance with mechanical overload, and are known to communicate with mononuclear cells in muscle to facilitate adaptation (39, 40, 85, 92, 122). Recently, our laboratory described how satellite cells deliver miRNA to fibrogenic cells via extracellular vesicles (EVs) early during mechanical loading to support long-term hypertrophic muscle growth (39). We hypothesized that satellite cells may also be communicating with muscle fibers, the largest cells by volume in muscle, via EVs early during loading to potentiate the long-term growth response. To test this hypothesis, we developed murine genetic models of delayed satellite cell fusion and in vivo EV tracking, complemented with in vivo satellite cell depletion experiments, in vitro primary myogenic progenitor cell (MPC) culture, unbiased transcriptome profiling, and in silico analyses. Our experiments revealed that satellite cells communicate with muscle fibers via EVs and contribute to load-mediated muscle hypertrophy in adult animals, and that this involves the repression of matrix metalloproteinase 9 (Mmp9). Furthermore, delayed satellite cell fusion did not inhibit long-term muscle hypertrophy, which may in part be due to satellite cell EV communication to muscle fibers. Our findings, for the first time, reveal EV-mediated intercellular communication between satellite cells and muscle fibers during hypertrophy, and point to new therapeutic frontiers for targeting these cells to preserve or enhance muscle mass.
Results

Primary myogenic progenitor cell extracellular vesicles can package tdTomato protein and transcript

Numerous investigations provide evidence for non-fusion-mediated transfer of genetically-driven cytoplasm-localized fluorescent reporters in vivo (24, 89, 94, 104, 126). Most recently, using a Cre/Lox genetic inducible model, cytoplasmic tdTomato (tdT) fluorescent protein was shown to be packaged in EVs from adipocytes (37). In context with our previous work showing EV communication from satellite cells to fibrogenic cells during hypertrophy (39), we hypothesized that tdT protein and/or transcript could be packaged in satellite cell EVs. We generated satellite cell-specific tamoxifen-inducible tdT mice (Pax7\textsuperscript{CreERT}-tdT\textsuperscript{fl/+}, or Pax7-tdT), as previously described (59, 73, 93, 114), then isolated satellite cells from vehicle and tamoxifen-treated mice via fluorescent activated cell sorting (FACS) (68). In vitro, EVs (characterized via nanoparticle tracking analysis) from proliferating tdT\textsuperscript{+} primary MPCs contained tdT protein (determined via single vesicle fluorescent correlational spectroscopy, high magnification fluorescent imaging, and western blot) and mRNA (determined via qPCR) (Supplemental Figure 1). We isolated EVs via a polymer-based method (ExoQuick) as described by our laboratory (39), a polymer-based method combined with density gradient ultracentrifugation (DGUC), as well as ultracentrifugation (UC), and consistently found tdT transcript (Supplemental Figure 1). Collectively, these data agree with evidence for cytoplasmic protein and/or mRNA being packaged in EVs (24, 37, 38, 56) and show that MPC EVs contain tdT protein and transcript.

Fusion-independent satellite cell communication to muscle fibers during mechanical overload

Neural Wiskott-Aldrich Syndrome protein (N-WASp) regulates actin polymerization and is reportedly essential for myoblast fusion throughout development and in vitro (46). To prevent satellite cell fusion during synergist ablation-induced mechanical overload (MOV) of the plantaris muscle, we developed Pax7\textsuperscript{CreERT}-N-WASp\textsuperscript{f/f}-tdT\textsuperscript{f/+} mice (termed N-WASp/tdT onward) that, upon tamoxifen-induced recombination, demonstrate a loss of N-WASp gene expression simultaneous with tdT expression specifically in satellite cells (Figure 1A and B). We hypothesized that fusion-incompetent N-WASp-depleted satellite cells would communicate with muscle fibers via EVs during MOV, which could be detected by the appearance of tdT in muscle fibers. Following vehicle and tamoxifen administration in vivo and a minimum two-week washout, we isolated N-WASp/tdT MPCs via FACS and confirmed tdT expression and N-WASp knockout (p<0.05, Figure 1C and D) with tamoxifen. The loss of N-WASp did not affect EV biogenesis or release as N-WASp-/tdT+ MPCs generated a similar amount of EVs as MPCs.
isolated from vehicle-treated N-WASp+/tdT- mice (Figure 1E). Furthermore, N-WASp-/tdT+ EVs contained tdT mRNA (Figure 1F). N-WASp depletion did not affect MPC proliferation (Figure 1G), but reduced myosin heavy chain area and myotube formation in vitro (p<0.05, Figure 1H-J). We then subjected N-WASp+/tdT- and N-WASp-/tdT+ plantaris muscles to MOV for 7 days; sham-operated mice served as controls (Figure 1K). Satellite cell proliferation after 7 days of MOV was similar between N-WASp+/tdT- and N-WASp-/tdT+ mice (Figure 1L), but myonuclear accretion was abolished in the N-WASp-/tdT+ condition (p<0.05, Figure 1M and 1N). In the absence of satellite cell fusion and myonuclear accretion, muscle fibers in N-WASp-/tdT+ mice displayed tdT fluorescence, pointing to fusion-independent delivery of tdT (Figure 1O). To validate this finding, we modeled tdT transfer by EVs in vitro by incubating myotubes generated from wild-type C57BL/6J MPCs with EVs collected from proliferating N-WASp-/tdT+ MPCs (Figure 1P). We observed tdT puncta in myotubes that appeared similar to cytoplasmic tdT transferred via EVs reported in other cell culture models (56) (Figure 1Q); tdT puncta in myotubes were not apparent when cultured with EVs from vehicle-treated N-WASp+/tdT- MPCs (not shown). These findings align with previous work showing mRNA and protein transfer via EVs between myogenic cells in vitro (62), as well as cytoplasmic reporter transfer between myogenic cells (38). Collectively, these data indicate that satellite cells communicate to muscle fibers before fusing during MOV, which is in part mediated by EVs.

**Myogenic progenitor cell extracellular vesicles alter muscle cell transcriptional profiles in vitro in a fashion consistent with satellite cell-dependency in vivo**

Effectively blocking EV release in a cell type-specific fashion in vivo is complicated and presents a variety of technical challenges (120). In order to search for candidate muscle fiber genes whose expression is potentially affected by satellite cell EVs during hypertrophy in vivo, we turned to our previously published murine model of inducible satellite cell depletion, the Pax7-DTA model (40, 76, 84). Specifically, using an unbiased approach, we aimed to identify genes that were similarly expressed between satellite cell replete and deplete muscle at rest, but were lower in the presence of satellite cells following MOV, which could indicate EV-mediated delivery of repressive miRNAs to target cells by activated satellite cells (118). Plantaris muscles of adult mice with and without satellite cells underwent sham surgery or 7 days of MOV, and then muscles were profiled via microarray (Figure 2A). Mmp9 was the most differentially expressed gene in the microarray dataset that was lower in the presence of satellite cells after MOV (+41% in satellite cell replete and +80% in depleted muscle), fitting our above criteria (Figure 2B, Supplemental Table 1). Mmp9 is synthesized and secreted by myotubes (28), regulates ECM quality and turnover (15), and is robustly up-regulated in bioengineered myofibers after mechanical loading (99), indicating it is mechanosensitive and expressed by...
muscle fibers during hypertrophy; Mmp9 is also highly-induced in myotubes by the presence of inflammatory cues (66). Worth noting is that Mmp9 is enriched in activated satellite cells (19, 137, 142), so lower transcript levels in the presence of satellite cells during MOV points to the presence of a repressive satellite cell-mediated mechanism (e.g. EV delivery of miRNA to target cells). We then profiled MPC EVs via miRNA microarray to identify miRNAs that may inhibit Mmp9. We recently showed that miR-206 is transferred by satellite cell EVs to fibrogenic cells during MOV to regulate collagen deposition (39), and confirm here that miR-206 is the most abundant miRNA in MPC EVs (Figure 2C). Expanding on our earlier work, we found numerous miRNAs enriched in MPC EVs (Figure 2C) that are experimentally shown to lower Mmp9 expression, such as miR-24 (14, 69, 139), miR-149 (36, 49, 67, 90, 135, 138), and miR-486 (52, 140), as well as miRNAs that are validated to target the 3' UTR of Mmp9 mRNA and reduce transcript levels, including Let-7e (121), miR-133a and -133b (136, 141), and miR-320 (11) (Figure 2D). 

In silico predicted miRNA-mRNA target analysis (123) further revealed that ECM remodeling is the most regulated process by MPC EV miRNAs (Figure 2E, Supplemental Table 2 and 3). Although the in vivo results from satellite cell-depleted mice could be explained by dysregulated communication from other cell types throughout muscle, we speculate that satellite cell-derived EVs contribute to Mmp9 repression specifically in muscle fibers during MOV.

We sought to experimentally validate the effects of satellite cell communication to muscle fibers via EVs using an in vitro approach coupled with unbiased RNA sequencing. We incubated myotubes with MPC EVs for 12 and 24 hours and found that Mmp9 was robustly down-regulated at both time points (adj. p<0.05, Figure 2F). A variety of transcripts associated with the Reactome term “ECM Organization” (50) were also repressed at 12 or 24 hours (Bmp2, Col8a1, Col12a1, Ltpb2, P4ha3, Scube3, Smoc2, Timp3, adj. p<0.05, Supplemental Table 4). We further identified mRNAs whose expression was higher in myotubes incubated with MPC EVs. Nr4a1, which is strongly associated with exercise adaptation (102), was elevated at both 12 and 24 hours (adj. p<0.05). The complete list of up- and down-regulated genes in myotubes incubated with MPC EVs is found in Supplemental Table 4. It is possible that EVs from other cell types in muscle could mediate the effects observed here, but collectively, these data show that MPC EVs elicit marked transcriptional changes in myotubes that simulate the effect of satellite cell EVs on muscle fibers during hypertrophy.

**Delayed satellite cell fusion does not inhibit long-term muscle hypertrophy**

Satellite cell fusion was prevented during the first week of MOV in N-WASp-/tdT+ muscle (see Figure 1N), which aligned with our in vitro data showing impaired MPC fusion (see Figure 1H-J). Recent evidence from our laboratory suggests that the presence of satellite cells and modest
myonuclear accretion during the first week of MOV is sufficient to support robust long-term growth (up to 8 wks) without continued myonuclear addition (39), but whether there is an early "critical window" for satellite cell fusion that is required to sustain prolonged hypertrophy is not clear (12, 39, 45, 71). To determine the necessity of satellite cell fusion for maximal long-term hypertrophy, we subjected N-WASp+/tdT- and N-WASp-/tdT+ mice to 8 weeks of MOV; we also overloaded vehicle and tamoxifen-treated Pax7CreERT-N-WASpfl/fl mice (N-WASp+) for 2 weeks, and sham-operated mice served as controls (Figure 3A). Myonuclear accretion was attenuated at 2 weeks in the absence of N-WASp (p<0.05), consistent with our 7-day experiments; however, myonuclear number was similar between conditions by 8 weeks (Figure 3B). Satellite cell abundance (Figure 3C), muscle hypertrophy (Figure 3D-G), and ECM content (3H and I) were not different between groups after 8 weeks. In our hands, N-WASp-/tdT+ MPCs eventually fuse to form large myotubes with enough time in culture (not shown), congruent with the in vivo data. Although we were not able to test the necessity of myonuclear accretion for maximal growth using this model, our results collectively suggest that delayed satellite cell fusion during MOV does not inhibit long-term skeletal muscle hypertrophy or result in excess ECM accumulation, and that the delivery of satellite cell secretory products to muscle fibers, specifically early during loading via EVs, may help promote prolonged muscle growth.
Discussion

Our laboratory and others report that adult muscle fibers can grow in the absence of satellite cell fusion and myonuclear addition, indicating flexibility within the myonuclear domain (82, 83). Hypertrophy without increased myonuclear number has been demonstrated with mechanical (34, 39, 40, 60, 76, 84), pharmacological (33, 45, 63, 127), and genetic approaches (5, 9, 63, 88, 100, 128), although this finding is not universal (12, 31, 43-45, 51, 80, 101), which could be explained by a variety of factors (75, 83). In recent years, a deeper appreciation for the secretory function of muscle stem cells in general (35, 78, 86, 110, 132), and satellite cells specifically (39, 40, 92, 122) has emerged in the context of skeletal muscle remodeling, with evidence for EV communication between mononuclear cells (39). It is becoming clear that the importance of satellite cells for promoting muscle hypertrophy extends beyond fusion and myonuclear addition, and the findings from this investigation provide the first evidence of EV-mediated communication to muscle fibers during mechanical loading, independent from satellite cell fusion. EV communication to muscle fibers early during hypertrophy may influence ECM-related signaling within muscle fibers, specifically targeting Mmp9. Furthermore, we show that early satellite cell fusion during loading is not necessary to produce maximal long-term growth.

We attribute the appearance of tdT fluorescence in muscle fibers of N-WASp-tdT+ mice following 7 days of MOV at least in part to EV-mediated transfer of tdT protein and/or transcript from satellite cells, which is supported by our cell culture work. Other investigations in different experimental systems document the transfer of genetic cytoplasm-localized reporter fluorescence between cells (24, 38, 56, 89, 94, 104, 126), as well as to distant tissues specifically via EVs (24). Mesenchymal stem cells have also been shown to deliver transgene mRNAs to recipient cells via EVs that then elicit a biological effect (3). Recently, Flaherty et al. used an adipocyte-specific Cre-inducible cytoplasmic tdT model to show the packaging of tdT protein into EVs (37), consistent with what we find in our satellite cell-specific tdT model. The appearance of tdT in muscle fibers is likely not due to recombination in resident myonuclei since the Pax7-tdT cytoplasmic reporter is highly-specific to satellite cells (59, 73, 93, 114), and Cre-inducible reporters using other lineage-specific promoters have consistently shown reporter gene expression is restricted to the cell that is expressing Cre, even when Cre-mediated “leakiness” does occur (4, 18, 112, 119). Another intriguing explanation for tdT transfer from satellite cells to myofibers is tunneling nanotubes (113), that are capable of material transfer between myogenic progenitors and muscle fibers in culture (115), deserving further investigation. We ultimately conclude that reporter fluorescence in satellite cells can be
transmitted to muscle fibers in the absence of fusion, specifically during times of adaptive remodeling.

MPC EVs lowered gene expression related to ECM regulation in myotubes, which we used to model the influence of satellite cell-derived EVs on muscle fibers in vivo. Myogenic cell-derived EVs promote effective regeneration in vivo (20), which could in part be mediated by the effect of EVs on growing de novo muscle fibers. In our data, Mmp9 was repressed at 12 and 24 hours in EV-treated myotubes determined via RNA-seq, as well as in the presence of satellite cells during MOV determined via microarray. Mmp9 regulates ECM turnover and its inhibition improves muscle regeneration, controls fibrosis (143), and is protective of muscle mass in the face of pathological inflammation and muscular dystrophy (65, 66), specifically in the early phase of dystrophic disease progression (109). Somewhat paradoxically, lifelong genetic depletion of Mmp9 is deleterious to muscle mass (77) while constitutive muscle-specific over-expression promotes growth (25). Mmp9 is pleiotropic (27, 109, 130) and its regulation by muscle fibers during loading is not entirely clear (99). Mmp9 may affect myogenic and hypertrophic signaling in muscle (25, 109), but it is also known to cleave collagen IV and laminin, and high levels of Mmp9 induce muscle membrane damage (19, 25, 65). Satellite cells could therefore mediate a “goldilocks” effect by fine-tuning muscle fiber Mmp9 levels through EV delivery of miRNAs early during MOV to manage ECM integrity and facilitate the long-term hypertrophic response (39, 40). Fibroblasts and fibro-adipogenic progenitors (FAPs) contribute heavily to ECM deposition and fibrotic protein production, but numerous ECM-related genes are epigenetically regulated specifically in myonuclei during MOV, suggesting muscle fiber participation in these processes (125). Recent work using muscle-specific genetic mice reinforce the important role muscle fibers play in ECM regulation during adaptation (1, 97). MPC EVs also increased gene expression of some genes in myotubes, although the precise explanation for this deserves further study. Interestingly, Nr4a1 was elevated by EVs, and nuclear receptors were recently identified via large-scale meta-analyses as the most highly responsive genes to any type of exercise in skeletal muscle, including resistance exercise (98, 102). Classically associated with substrate utilization in skeletal muscle (17, 57, 74), Nr4a1 is epigenetically modified with resistance retraining after detraining in muscle (107), controls adult muscle mass (23, 117), and is a potent inhibitor of fibrosis (91). Satellite cell control of ECM dynamics via intercellular communication is consistent with the broader influence of satellite cell secretory products reported by us and others (2, 32, 39, 40, 47, 85, 116, 122), and the effects of satellite cell EVs directly on muscle fibers should not be overlooked.
The rigidity of the myonuclear domain has recently been disputed (30, 75), but numerous investigations across species show at least modest flexibility in the myonuclear domain of adult skeletal muscle during hypertrophy (82). Satellite cell fusion early during hypertrophy was posited to ameliorate muscle fiber membrane damage to support late-stage adaptation (45); however, muscle fiber membrane repair is efficiently carried out by resident intracellular machinery (13, 26, 48, 64, 105), and MOV-induced muscle fiber hypertrophy in our hands proceeds even when satellite cell fusion is delayed during loading. In context with our present findings, we speculate that when satellite cell fusion is effectively prevented by genetic means (44, 45, 101), that other secretory functions of the cell may also be affected which could negatively affect hypertrophy (8). Along these lines, mTOR activation in skeletal muscle is preserved during MOV in the complete absence of satellite cells (76) but is abolished in the presence of Myomaker-null fusion-incompetent satellite cells (44), which could point to altered satellite cell secretory function. In our hands, N-WASp deficient MPCs eventually fuse to form large myotubes, which is congruent with our 8 week N-WASp MOV data. N-WASp depletion in our model involves excision of exons six to nine and results in a truncated protein generated by exons one through five (70), which may explain why we did not observe the complete prevention of fusion that was reported using a floxed exon two construct (46), and may also explain intact secretory function. We speculate that impaired fusion with N-WASp disruption could be due to compromised MPC migration (58) since we observed that differences between conditions were less apparent at higher seeding densities in vitro. It is also conceivable that compensatory mechanisms were activated that circumvented the loss of N-WASp during prolonged MOV, or that the path of satellite cell myogenic progression and fusion differs during hypertrophy versus developmental growth and regeneration (43). Nevertheless, delayed satellite cell fusion during MOV did not inhibit long-term skeletal muscle hypertrophy or result in excess ECM accumulation.

Numerous investigations have linked the level of satellite cell proliferation and subsequent myonuclear accretion to the magnitude of skeletal muscle hypertrophy (7, 22, 54, 71, 95). Our data suggest that robust satellite cell proliferation in response to loading may promote long-term muscle fiber growth through fusion-dependent as well as -independent mechanisms. Our results also indicate that Cre-induced cytoplasmic-localized fluorescent reporters should be used with caution for accurately determining the contribution of satellite cell fusion to muscle fibers (21, 59, 73, 93, 114). Future experiments involving satellite cell-specific inhibition of EV release, as well as genetic manipulation of specific miRNAs in satellite cells in vivo will help provide more detailed insight into satellite cell regulation of muscle fiber gene expression by fusion-independent mechanisms. Satellite cells coordinate adaptation to support maximal long-term...
muscle hypertrophy (83), which may in part be due to EV delivery of miRNA to muscle fibers that affect ECM-related gene expression.
Materials and Methods

Animals

Pax7<sup>CreERT/+</sup> tdTomato<sup>fl/+</sup> mice were generated by crossing male Pax7<sup>CreERT/CreERT</sup> mice to female ai9 Rosa26 tdTomato<sup>fl/fl</sup> mice (72), purchased from the Jackson Laboratory; these were called Pax7-tdT. Pax7<sup>CreERT/+</sup> N-WASp<sup>fl/fl</sup> tdTomato<sup>fl/+</sup> mice were generated by crossing male Pax7<sup>CreERT/CreERT</sup> mice with female N-WASp<sup>fl/fl</sup> mice (70), that through several rounds of breeding, produced male Pax7<sup>CreERT/+</sup> N-WASP<sup>fl/fl</sup> mice (N-WASp). Male double-mutant mice were crossed to female N-WASp<sup>fl/fl</sup> tdTomato<sup>fl/fl</sup> mice that were bred to homozygosity by crossing tdTomato<sup>fl/fl</sup> mice with N-WASp<sup>fl/fl</sup> mice; the triple-mutant mouse was termed N-WASp/tdT. Pax7<sup>CreERT/+</sup> Rosa26<sup>DTA/+</sup> were generated by crossing male Pax7<sup>CreERT/CreERT</sup> mice with female Rosa26<sup>DTA/DTA</sup> mice (76, 87, 133), both purchased from the Jackson Laboratory (Bar Harbor, ME, USA); this mouse was termed Pax7-DTA. All Pax7-tdT, N-WASp/tdT, and N-WASp experiments involved a balanced mix of males and females, and Pax7-DTA experiments involved male mice. In all mice, tamoxifen treatment induces Cre activity, which recombines out stop cassettes in front of DTA or tdT constructs that were knocked into the Rosa26 locus, and recombines out exons six through nine flanked by loxP sites in the N-WASp mice. Administration of tamoxifen to Pax7-tdT and N-WASp/tdT mice results in robust expression of tdT [shown here and elsewhere (59, 73, 93, 114)] and depletion of N-WASp (in N-WASp/tdT), and to Pax7-DTA mice results in specific and significant depletion of satellite cells across various skeletal muscles regardless of age (41, 76, 81). Mice were genotyped via tail snip and NaOH digestion, then polymerase chain reaction for 40 cycles using GoTaq G2 chemistry (Promega, Madison, WI, USA), followed by electroporation on 1.5-2.0% agarose gel to visualize DNA bands (see Supplemental 5 for genotyping primer sequences). C57BL/6J mice (obtained from the Jackson Laboratory) were used to obtain primary MPCs for in vitro experiments. Experiments and animal care were performed in accordance with the University of Kentucky Institutional Animal Care and Use Committee. All mice were housed in a temperature- and humidity-controlled room and maintained on a 14:10 light:dark cycle, with standard chow and water ad libitum. All mice were >4 months old at the time of experimentation, consistent with our previous work (84).

Mouse treatment, synergist ablation mechanical overload surgery (MOV), and tissue collection

Mice were injected intraperitoneally with vehicle (15% ethanol in sunflower seed oil) or tamoxifen (2 mg/day, suspended in ethanol and sunflower seed oil) for five days, then given a two-week washout period before any experimentation ensued (76). Mice underwent bilateral synergist ablation surgery to induce hypertrophy of the plantaris muscle, as previously
described by our laboratory (39, 60). Briefly, mice were anesthetized using 95% oxygen and 5% isoflurane gas, then ~1/3 of the gastrocnemius/soleus complex was removed, careful not to disturb neural or vascular supply. Sham surgery involved all procedures except for excision of muscle. Following recovery from surgery, mice were euthanized via lethal dosage of sodium pentobarbital and cervical dislocation at the appropriate time point. Hind limb muscles for immunohistochemistry analysis were weighed, covered in Tissue Tek OTC compound (Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen-cooled isopentane, and stored at -80°C until the time of sectioning. For single muscle fiber myonuclear count analysis, whole hind limbs were excised and the ankle was fixed at 90° (to control for sarcomere spacing) in 4% paraformaldehyde (PFA) for 48 hours. For N-WASP/tdT mice, after 48 hours in PFA, the top ¼ of the plantaris was removed and incubated in 30% sucrose overnight at 4°C, then frozen for visualization of tdT, as described elsewhere (93). The remainder of the muscle was stored in phosphate buffered saline (PBS) until the time of single fiber analysis. The entire plantaris muscle was flash-frozen and used for molecular analysis in Pax7-DTA experiments.

Muscle histology and quantification

All histological analyses are described in detail in previous publications from our laboratory (39, 40, 76, 84). In brief, frozen tissue was sectioned (7 µm) and air-dried for at least one hour. For Pax7, sections were fixed in 4% PFA then subjected to epitope retrieval using sodium citrate (10 mM, pH 6.5) at 92°C for 20 minutes. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in PBS for 7 minutes, followed by a mouse-on-mouse block (Vector Laboratories, Burlingame, VT, USA). Sections were then incubated overnight with a Pax7 antibody and a laminin antibody, followed by incubation with a biotin-conjugated secondary antibody for Pax7, and a fluorophore-conjugated antibody for laminin; sections were then incubated with 4',6-diamidino-2-phenylindole (DAPI, 1:10,000) for 10 minutes. The Pax7 signal was amplified using streptavidin horseradish peroxidase (SA-HRP) followed by a tyramide signal amplification fluorophore (TSA). Pax7 staining was conducted only in conjunction with laminin, accepting the limitation that a very small percentage of myonuclei may co-stain with Pax7 protein for some unknown reason (106). For fiber cross-sectional area, sections were incubated with an antibody against dystrophin, followed by a fluorescent secondary antibody. To analyze ECM, sections were incubated with directly-conjugated α-wheat germ agglutinin (WGA, 1 mg/mL, Invitrogen) for two hours. All sections with fluorescence were mounted using VectaShield (Vector) and stored in the dark until the time of imaging. To visualize endogenous tdT expression, PFA-fixed sections were cut then dried for 1 hour in the dark, fixed again in 4% PFA for 10 minutes, washed in PBS, then mounted with VectaShield (93). See Supplemental 5
for antibody information. Muscle sections were imaged using a Zeiss upright microscope (AxioImager M1, Oberkochen, Germany), and analyzed with Zen software or MyoVision software (129). A size correction factor previously published by our laboratory was used to normalize fiber cross sectional area for experiments involving both male and female mice (61). For Pax7, entire muscle cross-sections were imaged at 20x magnification using the mosaic image stitching function in Zen. Pax7+ satellite cells were identified as Pax7+/DAPI+ and residing underneath the basement membrane, and were quantified manually by a blinded using tools in the Zen software (normalized to muscle fiber number). Similarly, entire muscle cross-sections were captured at 20x magnification, and muscle fiber cross sectional area was determined using semi-automated analysis software (129). WGA was analyzed by capturing 2-3 artifact-free 20x images that were then analyzed using a threshold tool in Zen.

Single muscle fiber isolation and quantification

Single fiber myonuclear counts were obtained according to previously published methods (10, 76, 84). In brief, 48-hour PFA-fixed muscles were manually dissected into bundles then incubated in 40% NaOH for two hours at room temperature while rotating. Fibers were then washed liberally with PBS atop a 40 µm strainer, gently moved into tubes using tweezers, triturated lightly until completely separated using low retention pipette tips, then dispersed on glass slides. Fibers were mounted with a glass cover slip using VectaShield with DAPI (Vector). For analysis, linear sections (~300-500 µm) from 30 randomly selected individual muscle fibers per muscle were imaged at 20x magnification using the z-stack function in Zen. Myonuclei were manually counted by a blinded trained technician, and fiber length was determined using a tool in Zen. A aggregate mean value from the 30 fibers analyzed from each mouse was used for statistics. To visualize tdT-expressing satellite cells on longitudinal muscle fibers (see Figure 1A), PFA-fixed muscle fibers were manually dissected in PBS using a dissecting microscope. Isolated muscle fibers were plated on glass slides and mounted in a mixture of phalloidin in PBS (1:100) and VectaShield with DAPI. Satellite cells were visualized at 40x magnification using a Zeiss LSM 880 with Airyscan confocal microscope.

Primary myogenic progenitor cell (MPC) isolation and maintenance

To obtain MPCs for in vitro experiments, the detailed FACS protocol from the Rando laboratory was employed (68). In brief, minced muscle was sequentially digested with collagenase, then collagenase and dispase at 37°C while rocking. Suspensions were aspirated with a 20 gauge needle, strained through 40 µm filters, pelleted at 500 x g for five minutes, then re-suspended. The single cell suspensions were incubated with antibodies against Vcam, Cd31, Cd45, and...
Sca1 at 4°C; a biotinylated secondary antibody was used for Vcam. Cells were pelleted at 500 x g, re-suspended, and sorted using an iCyt FACS machine (Sony Biotechnology, Champaign, IL, USA). Appropriate isotype-specific controls were used to validate antibody specificity, and dead cells were gated out using forward/side scatter profiles and/or propidium iodide (non-tdT experiments). Satellite cells were identified as Vcam+/Cd31-/Cd45-/Sca1-. MPCs were maintained in 5% O₂ and expanded in growth media made of Ham’s F10+15% normal horse serum+1% penicillin/streptomycin supplemented with basic fibroblast growth factor (5 ng/ml, Millipore) on plates coated with ECM gel diluted 1:100 (Sigma-Aldrich). Cells were differentiated on ECM-coated plates (35 mm or 8-well plastic chamber slides) in differentiation media (DM) made of high-glucose DMEM+10% normal horse serum+1% penicillin/streptomycin. Cells from in vivo vehicle or tamoxifen treated mice had a minimum two-week washout before being isolated. See Supplemental 5 for antibody information.

**Extracellular vesicle (EV) isolation and characterization**

To obtain EVs from MPCs, 10 cm plates were expanded to high confluence. MPC conditioned growth media was collected after 24-48 hours and stored at -80°C (note: EVs from fresh media were used for imaging analysis, described below). At the time of experimentation, EVs were isolated using ExoQuick TC according to System Bioscience’s protocol and as previously described by our laboratory (39). For further validation, EVs were isolated via ultracentrifugation (UC) and density gradient ultracentrifugation (DGUC, see Supplemental Figure 1). Briefly, for ExoQuick TC isolation of EVs, MPC-conditioned media was cleared of cellular debris via centrifugation for 15 minutes at 3,000 x g, moved to a new sterile vessel, incubated with Exoquick TC for a minimum of 12 hours at 4°C, then centrifuged for 30 minutes at 1,500 x g. The EV pellet was then re-suspended in 300 µl of PBS or DM (depending on downstream application). Particle size and concentration was carried out on a ZetaView nanoparticle tracking analyzer (NTA, Particle Metrix, Meerbusch, Germany) after filtration through a 0.22 µm filter (1:3000 in PBS, sensitivity 76, shutter 350). For UC isolation of EVs, conditioned media was cleared of debris as described above, diluted in PBS, then spun at 118,000 x g for 6 hours. The resulting EV pellet was reconstituted in 100 µl of PBS and used for NTA, RNA, protein, and high-resolution imaging analysis. For additional DGUC EV analyses, we used an OptiPrep gradient as previously described by Jimenez et al. (53). Briefly, Exoquick TC-collected EVs were layered on top of a discontinuous density gradient of 5%, 10%, 20%, and 40% iodixanol. The iodixanol dilutions were prepared by diluting OptiPrep (60% aqueous iodixanol) with 0.25 M sucrose/10 mM Tris, pH 7.5. After an 18 h centrifugation at 100,000 x g, 12 density gradient fractions were collected, diluted in PBS, and centrifuged at 100,000 x g for 6 hours. NTA
analysis and protein quantification validated the fraction enriched with EVs, and this fraction was then used for protein and RNA analysis.

**Fluorescent correlational spectroscopy (FCS) and high-magnification fluorescent microscopy of individual vesicles**

To fluorescently label vesicles, 5 µM Dil was added to the vesicle suspension and incubated at 37°C for 30 minutes. Vesicles were then purified using a 0.45 µm sterile syringe filter. For FCS measurements, 40 µl of freshly prepared fluorescently labeled sample was placed onto a coverslip mounted on an Olympus IX83 microscope (Olympus, Shinjuku, Tokyo, Japan) equipped with a PicoQuant PicoHarp 300 Time-Correlated Single Photon Counting system (Picoquant, Berlin, Germany). A 60x water immersion objective was used to focus a 532 nm CW laser into the sample solution. Two avalanche photodiodes were used for photon detection. All FCS results are an average of at least 3 measurements. To diminish the background signal from immobilized molecules, we performed all measurements 30 µm above the glass surface in the sample solution. Data analysis was performed using SymPhoTime 64 software (Picoquant). An autocorrelation of the fluorescence time trace was then calculated and fit to extract the diffusion time (79, 134). Measurements of vesicles labeled with Dil and of free recombinant tdTomato protein were used to establish the expected diffusion time of tdTomato in vesicles as compared to freely diffusing protein. To visualize tdTomato-containing vesicles in solution, a 40 µL sample of the vesicle suspension was placed on a glass coverslip. The fluorescence was imaged on an inverted microscope (Olympus IX81) using a 60X 1.49 NA oil immersion objective, an electron multiplying charge couple device, and 561 nm laser excitation. Sequential frames (10 ms) were collected for a duration of 1 minute. Images were processed and analyzed using imageJ.

**Western blot analysis of EVs**

Protein was isolated from EVs using RIPA buffer supplemented with protease and phosphatase inhibitors (ThermoFisher). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Bio-Rad, Hercules, CA, USA). For quantification of protein abundance, 27 µg of protein was loaded, separated on a discontinuous acrylamide gel (4-15%, Bio-Rad), and transferred to a nitrocellulose membrane. Total protein transfer was visualized using Revert 700 total protein stain (Licor, Lincoln, NE, USA). The membrane was blocked using 5% non-fat dry milk in TBS+0.1% Tween (TBS-T) for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies (1:1000 dilution in 5% non-fat dry milk). After primary antibody incubation, membranes were washed with TBS-T and incubated with appropriate goat
anti-rabbit secondary antibodies at 1:10000 dilution for 1 hour at room temperature in 5% non-fat dry milk. Blots were developed using enhanced chemiluminescence (Clarity Western ECL substrate, Bio-Rad), and imaged on a Chemidoc imaging system (Bio-Rad).

Immunocytochemistry

To determine N-WASp+/tdT- and N-WASp-/tdT+ MPC proliferation rate, 1x10^4 cells per well were plated on 8-well chamber slides. MPC growth media was spiked with five µM of 5-Ethynyl-2’-Deoxyuridine (EdU) for 24 hours. Cells were fixed in warmed 4% PFA for 15 minutes, then EdU was visualized using a detection cocktail described by Kirby et al. (61). Briefly, fixed cells were permeabilized in 0.5% Triton X-100 (Sigma-Aldrich), washed with 3% bovine serum albumin (BSA) in PBS, then incubated in the EdU detection cocktail containing tris base (100 mM), biotin azide (100 µM), copper sulfate (4 mM), ascorbic acid (100 mM), and deionized H2O. Following PBS washes, cells were incubated in SA-HRP followed by fluorescent TSA. Slides were mounted using VectaShield with DAPI, images were captured at 20x magnification, and EdU+/- cells were quantified relative to DAPI. To determine fusion dynamics, myosin heavy chain (MyHC) area and fusion index was quantified. On 8-well chamber slides, 5x10^4 cells were differentiated for 30 hours, then fixed in warm 4% PFA. To visualize MyHC, myotubes were washed with 0.2% Triton X-100, blocked with 1% BSA for an hour, and incubated with a pan-myosin primary antibody in PBS (1:100). Myotubes were then incubated with a biotinylated secondary antibody, followed by a fluorophore-conjugated streptavidin. After being mounted with DAPI, 2-3 images were captured at 20x magnification, and MyHC area normalized to DAPI content was quantified using a quantification tool in Zen. Fusion index (myotubes containing ≥5 myonuclei) was quantified by a trained technician. To determine whether tdT message could be transferred to myotubes, 2x10^4 C57BL/6J MPCs were differentiated in 8-well chamber slides until large multi-nucleated myotubes were observed (30 hours), then myotubes were incubated with EVs from N-WASp+/tdT- or N-WASp-/tdT+ MPCs for 20 hours. Myotubes were then fixed with warm 4% PFA, incubated with phalloidin in PBS (1:100) for 2 hours, then mounted with DAPI to visualize tdT fluorescence. Cell culture experiments were conducted at or before passage six. Antibody information is found in Supplemental 5.

Gene expression experiments involving MPCs, myotubes, EVs, and muscle tissue

For cellular and tissue RNA extraction, cells were lysed in TRIzol reagent, and RNA was extracted using the Zymo MiniPrep kit (Zymo Research, Irvine, CA, USA) with slight modification; the aqueous phase from TRIzol combined with 3-bromo-chloropropane following phase-separation was added directly to the spin column, and not the TRIzol itself (as
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recommended by the manufacturer). RNA concentration and quality were assessed via NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and Bioanalyzer using Nano chips (Agilent, Santa Clara, CA, USA). For qPCR on cells, 2 nanograms of cDNA was used per reaction, and reactions were performed using Sybr chemistry on a QuantStudio 3 (Thermofisher Scientific) in duplicate with Gapdh as the housekeeping gene. Reaction efficiency was evaluated using LinReg software (103), and data are presented as relative gene expression. To model in vitro what satellite cells may communicate to muscle fibers, 3x10^5 C57BL/6J MPCs were differentiated on 35 mm plates until large multi-nucleated myotubes were observed (32 hours), and Exoquick-isolated EVs from C57BL/6J MPCs were then incubated with myotubes in DM for 12 and 24 hours; ExoQuick-isolated particles from non-MPC conditioned GM served as the control treatment. For RNA sequencing, myotube RNA was prepared as a 250-300 bp non strand-specific library using the NEBNext Ultra RNA Library Prep Kit (NEB) followed by pair-end sequencing using an Illumina NovaSeq 6000 s4 platform. Mapping to the mouse mm10 transcriptome was performed using HISAT2, data were normalized according to Novogene’s standard practices (29), quality was assessed and reads were filtered by removing reads that contained adapters, reads that contained N>0.1% (N represents base that could not be determined), and low-quality reads (quality value of over 50% of bases of the read ≤20), and differential analysis was performed using DESeq2 v2 with a model based on the negative binomial distribution. To evaluate gene expression in EVs via qPCR, total RNA was extracted using the ExoRNeasy Serum/Plasma kit (Qiagen). RNA concentration was evaluated using a bioanalyzer with small or pico chips (Agilent), cDNA was generated using the Vilo IV reverse transcription kit (Thermo Fisher Scientific), and qPCR was used to detect tdT message (normalized to 18s, which was present in EVs according to Agilent analysis as well as a previous investigation of myogenic cell EVs (62)). Reactions were performed using Sybr chemistry on a QuantStudio 3 (Thermofisher Scientific) in duplicate with 0.2 ng of cDNA, and presented as relative gene expression (2^(-ΔΔCt)). Primer sequences for qPCR are found in Supplemental 5. From homogenized Pax7-DTA muscle tissue, microarray analysis was carried out as previously described by our laboratory (16); the microarray results from the Pax7-DTA experiments are part of a larger cohort of mice with additional time-points that has not been published yet, but we present the sham and 7 day time points here. Briefly, RNA quality, concentration, and integrity was determined by spectrophotometer and bioanalyzer, and 250 ng of RNA per sample was submitted to the University of Kentucky Genomics core for analysis using an Affymetrix GCS 3000 7G scanner and mouse gene 2.0 ST array chips (Thermo Fisher Scientific). Similarly, for microarray analysis of MPC EV miRNA (isolated from multiple 10 cm cell culture plates of proliferating C57BL/6J primary MPCs), 70 nanograms of pooled EV RNA
was submitted to the University of Kentucky microarray core. Using the Flashtag Biotin HSR kit (Thermo Fisher Scientific) and following a prolonged ~40 hour hybridization step, miRNA was analyzed using an Affymetrix GeneChip miRNA 4.0 array. Microarray data were obtained using GeneChip Command Console software, analyzed with Affymetrix Expression Console software (Thermo Fisher Scientific), and normalized using the Robust Multichip Analysis (RMA) method.

**Pathway and in silico analyses**

Gene expression pathway analysis was carried out using ConcensusPathDB with the overrepresentation feature for mouse genes in KEGG, Reactome, and Wikipathway databases, and a minimum input list overlap of “2” and p-value cutoff of $p=0.01$ (55). DIANA miRpath was used to determine which pathways were targeted by MPC EV miRNAs (124), and specific in silico miRNA-mRNA 3’UTR interactions were predicted using miRWalk (111).

**Statistical Analyses**

For statistical comparison of two groups, unpaired two-tailed Student’s t-tests were employed. For comparisons of two groups with two conditions, two-way ANOVAs were employed and, when an interaction effect was identified, a Tukey’s HSD post-hoc test was employed. All data were normal according to the Kolmogorov-Smirnov test. Differences among groups were considered significant when the probability value, $p$, was less than 0.05. Outliers greater than two standard deviations from the mean were excluded from analysis. For RNA sequencing, false discovery rate (FDR) was controlled for using the Benjamini and Hochberg’s approach, with adjusted $p<0.05$ and a log2 fold change >1.
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Author Contributions
K.A.M, C.A.P, and J.J.M. designed experiments. K.A.M, I.J.V, D.W.VP, S.E.C., C.M.D., V.C-F., X.F., K.K., C.I.R. and C.S.F. performed experiments. K.A.M, I.J.V., D.W.VP, S.E.C., X.F., C.S.F. and C.I.R. analyzed data. K.A.M wrote the manuscript and prepared the figures. C.A.P. and J.J.M provided funding support and supervised the study. C.A.P. and J.J.M. assisted with data interpretation and manuscript writing. All authors edited and approved the final manuscript.

Declaration of Interests
The authors have no conflicts to declare.
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**Figure Legends**

**Figure 1** Delayed satellite cell fusion and extracellular vesicle (EV)-dependent communication to muscle fibers during 7 days of mechanical overload (MOV). **A** Confocal image of a tdTomato (tdT)-expressing satellite cell (red) overlayed with DAPI (blue) on a muscle fiber (stained for F-actin, gray), illustrating Pax7Cre-mediated recombination of the floxed tdT transgene in the presence of tamoxifen. **B** Histochemical image depicting Pax7 immunostaining (green) that coincided with tdT fluorescence (red) in the correct anatomical location (beneath laminin, gray, and overlaid with DAPI, blue) after tamoxifen treatment. **C** Flow cytometry plot illustrating tdT+ cells co-expressing Vcam, which was used to identify and purify satellite cells via fluorescent activated cell sorting (FACS) in tamoxifen-treated mice; Vcam+/Cd31-/Cd45-/Sca1- cells were purified in vehicle-treated mice, as described previously (68), and tdT recombination was minimal (not shown). **D** Gene expression to confirm N-WASp recombination from *in vivo*-treated vehicle- (N-WASp+/tdT-) versus tamoxifen-treated (N-WASp-/tdT+) primary myogenic progenitor cells (MPCs) purified via FACS, n=2 biological replicates per condition. **E** Nanoparticle tracking analysis showing EV particle abundance normalized to cell count from N-WASp+/tdT- and N-WASp-/tdT+ MPCs, n = 2 biological replicates per condition. **F** Relative tdT mRNA abundance in EVs isolated from N-WASp+/tdT- (n=2 biological replicates) and N-WASp-/tdT+ MPCs (n=3 biological replicates). **G** Proliferation in N-WASp-/tdT+ and N-WASp+/tdT- MPCs (n=3 biological replicates per condition). **H** Myosin heavy chain (MyHC) area after 30 hours of differentiation (n=3 biological replicates per condition). **I** Fusion index (n=3 biological replicates per condition). **J** Representative images of MPC fusion in N-WASp+/tdT- versus N-WASp-/tdT+ conditions; green is MyHC and blue is DAPI. **K** Study design schematic illustrating vehicle (Veh) and tamoxifen (Tam) treatment followed by sham or 7 days of MOV in N-WASp/tdT mice. **L** Satellite cells normalized to muscle fiber count in N-WASp+/tdT- (n=5 sham and 5 MOV) versus N-WASp-/tdT+ (n=6 sham and 7 MOV) mice. **M** Representative images of myonuclei in isolated single muscle fibers from MOV mice. **N** Myonuclear content analysis on isolated single muscle fibers. **O** Representative image of tdT fluorescence in MOV N-WASp-/tdT+ muscle fibers despite the prevention of satellite cell-mediated myonuclear accretion. **P** Study design schematic illustrating the treatment of wild-type C57BL/6J myotubes with EVs from N-WASp-/tdT+ MPCs. **Q** Representative image showing tdT puncta (red) in C57BL/6J myotubes (stained for F-actin, green, and DAPI, blue) incubated with N-WASp-/tdT+ MPC EVs. *p<0.05, #p<0.05 effect of MOV, scale bars=100 µm, all data presented as mean±SE.
Figure 2 Evidence for the impact of extracellular vesicle (EV)-mediated communication to muscle fibers in vitro and in vivo. A Study design schematic illustrating vehicle (Veh, SC+) and tamoxifen (Tam, SC-) treatment followed by sham or 7 days of mechanical overload (MOV) in Pax7-DTA mice. B Mmp9 mRNA levels in sham versus MOV in the presence and absence of satellite cells; n = 6 mice per group, 2 separate pools of 3 plantaris muscles for each group resulting in n=2 pools per group. C miRNAs enriched in MPC EVs that influence Mmp9 levels in different experimental models; miR-206 was the most abundant miRNA measured. D Summary of evidence for miRNAs that are enriched in MPC EVs that affect Mmp9 via direct 3’UTR targeting or indirectly via experimental manipulation using miRNA mimics and/or antagoniRs (see Results for specific studies). E DIANA miRpath analysis of miRNAs enriched in myogenic progenitor cell (MPC) EVs using the top 100 miRNAs. F Mmp9 mRNA levels in C57BL/6J myotubes incubated with MPC EVs for 12 or 24 hours; 1 primary cell line was used to generate myotubes, and were incubated with MPC EVs from 2 separate cell lines at each time point (n=2 per time point: 12 and 24 h control, 12 and 24 h EV treatment). *adjusted p<0.05, all data presented as mean±SE.

Figure 3 Delayed satellite cell fusion does not inhibit long-term muscle hypertrophy A Study design schematic illustrating vehicle (Veh) and tamoxifen (Tam) treatment followed by sham, 2 weeks, or 8 weeks of mechanical overload (MOV) in N-WASp (2 wk) and N-WASp/tdT (8 wk) mice. B Myonuclear content analysis on isolated single muscle fibers at 2 weeks (n=5 sham and 8 MOV N-WASp+, n=7 sham and 11 MOV N-WASp-) and 8 weeks (n=4 sham and 4 MOV N-WASp+/tdT-, n=4 sham and 5 MOV N-WASp-/tdT+). C Satellite cells normalized to muscle fiber count after 8 weeks of MOV. D Plantaris muscle wet weight normalized to body weight. E Representative plantaris muscle cross-sections labeled with dystrophin (pink), scale bar=500 μm. F Magnified representative images of dystrophin, scale bar=50 μm. G Average muscle fiber cross-sectional area (CSA). H Representative images of extracellular matrix glycosaminoglycan area evaluated by wheat germ agglutinin staining (WGA), scale bar=50 μm. I WGA area normalized to muscle fiber count. *p<0.05 effect of MOV, all data presented as mean±SE.
Supplemental Figure 1 Validation of tdTomato (tdT) protein and gene expression

A&B Nanoparticle tracking analysis of EVs from vehicle (Veh)- (A) and tamoxifen (Tam)-treated (B) Pax7-tdT myogenic progenitor cells. 

C Fluorescence correlation spectroscopy of recombinant tdT protein (control), Dil membrane-labeled extracellular vesicles (EVs) from myogenic progenitor cells (MPCs) isolated via ultracentrifugation (UC), and ultracentrifuge-derived particles from Tam-treated Pax7-tdT MPCs. The diffusion time of tdT fluorescent events closely resembles membrane-labeled EVs, indicating tdT protein packaged in EVs. Analysis of particles from Veh-treated Pax7-tdT MPCs (veh) revealed no fluorescent EVs (data not shown). 

D High resolution fluorescent microscopy showing tdT fluorescent EVs in solution (white arrows) from tamoxifen-treated Pax7-tdT MPCs. Image taken at 60X magnification. 

E Total protein from Veh and Tam treated Pax7-tdT MPCs and EVs. DGUC = density gradient ultracentrifugation. 

F Western blot illustrating a low amount of tdT protein in vehicle Pax7-tdT MPCs, a high amount in tamoxifen MPCs, and a detectable amount in EVs from tamoxifen-treated MPCs isolated via UC (see over-exposed inset). MPCs were cultured in growth media comprised of 15% horse serum in order to promote proliferation and EV release, so a portion of isolated EVs were from serum, which likely contributed to the mismatch between CD81 levels (a well-accepted exosome marker enriched in EV fractions, lower bands) and tdT levels. More EVs were obtained via UC versus the EV-enriched DGUC fraction, which may explain why tdT was more readily detectable in the UC preparation. 

G Efficiency and specificity of the tdT primer used for qPCR. Efficiency was determined using recombined MPCs that were constitutively expressing tdT. "X" is tdT expression in myotubes from C57BL/6J primaries that did not contain tdT in the genome. 

H Levels of tdT transcript in EVs isolated via ExoQuick (n=2 biological replicates per condition), UC, and DGUC (n=1 per condition).
Figure 1

A

B

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D

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K

L

M

N

O

P

Q

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Figure 2

A) 7 d MOV: Pax7-DTA

B) Pax7-DTA 7 d MOV: Mmp9

C) MPC EV miRNAs → Mmp9

D) MPC EV miRNAs → Mmp9

E) MPC EV miRNA In Silico Prediction Analysis

F) MPC EVs → Myotubes: Mmp9
Figure 3

A. 2 & 8 wk MOV: N-WASp and N-WASp/tdT

B. 2 wk MOV

C. 8 wk MOV

D. p=0.07

E. N-WASp/tdT-

F. N-WASp/tdT-

G. #

H. N-WASp/tdT-

I. WGA Area/Fiber

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