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Roles of the synaptic molecules Hevin and SPARC in mouse neuromuscular junction development and repair

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Abstract

Hevin and secreted protein acidic and rich in cysteine (SPARC) are highly homologous matricellular proteins that function in concert to guide the formation of brain synapses. Here, we investigated the role of these glycoproteins in neuromuscular junction (NMJ) maturation, stability, and repair following injury. Hevin and SPARC mRNA levels in developing (postnatal day 9), adult (postnatal days 90 and 120), and injured (fibular nerve crush) skeletal muscles were assessed with qPCR. Muscle fiber size was analyzed in developing (P9) mice lacking SPARC, Hevin, and both SPARC and Hevin. NMJ morphology was assessed in developing (P9), adult (P90) and injured (fibular nerve crush) mice lacking SPARC, Hevin, and both SPARC and Hevin skeletal muscle. Hevin and SPARC are expressed in skeletal muscles and are upregulated following nerve injury. Hevin$^{-/-}$ mice exhibited delayed NMJ and muscle fiber development but displayed normal NMJ morphology in adulthood and accelerated NMJ reinnervation following nerve injury. Mice lacking SPARC displayed normal NMJ and muscle fiber development but exhibited smaller NMJs with fewer acetylcholine receptor islands in adulthood. Further, SPARC deletion did not result in overt changes in NMJ reformation following nerve injury. The combined deletion of Hevin and SPARC had little effect on NMJ phenotypes observed in single knockouts, however deletion of SPARC

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Disclosure of conflicts of interest

None of the authors has any conflict of interest to disclose.

in combination with Hevin reversed deficiencies in muscle fiber maturation observed in Hevin$^{-/-}$ muscle. These results identify SPARC and Hevin as extracellular matrix proteins with roles in NMJ development and repair.

Keywords

synapse; neuromuscular junction; Development; injury; skeletal muscle; Extracellular matrix

Introduction

The formation and maintenance of skeletal muscle fibers and their synapses with motor axons, known as neuromuscular junctions (NMJs), are dependent on the complex molecular composition of the muscle extracellular matrix (ECM) [1,2]. By selectively tethering receptors, ion channels, and growth factors at both synaptic and non-synaptic regions of skeletal muscle, the molecules that comprise the ECM are critical for NMJ structure and muscle function [3–5]. Therefore, a better understanding of the regulatory molecules that ensure proper assembly, distribution, and turnover of the ECM may lead to therapeutic targets that improve skeletal muscle fiber and NMJ formation and maintenance [6,7]. While much has been learned about the composition of the ECM that surrounds skeletal muscle fibers, particularly in the synaptic basal lamina, there is a pressing need to identify novel molecules that support the maintenance and repair of muscle fibers and NMJs.

Among novel ECM molecules with a potential role in muscle fiber and NMJ formation and repair are the proteins secreted protein acidic and rich in cysteine (SPARC, also known as osteonectin) [8] and Hevin (also known as SPARC-like 1 and synaptic cleft 1) [9]. These highly homologous matricellular proteins share a follistatin region that enables them to modulate growth factors such as fibroblast growth factor 2 (FGF2), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor beta (TGF-β) in the ECM [10–12]. Their role in synapse formation has been well characterized in the CNS where they are expressed by astrocytes during development and continue to be expressed at high levels throughout adulthood [13–18]. In the developing CNS, SPARC and Hevin play antagonistic roles to ensure timely development of excitatory synapses which is mediated by competitive binding of their shared follistatin region [18]. Outside of the CNS, SPARC and Hevin are expressed in skeletal muscle and are present at NMJs [16,19–27]. SPARC has been shown to regulate aspects of Schwann cell growth [28] and muscle regeneration [23–27], however, the function of these matricellular proteins in NMJ maturation and repair are not known.

To better understand the roles of SPARC and Hevin in NMJ and muscle fiber development and repair, we utilized transgenic SPARC$^{-/-}$ and Hevin$^{-/-}$ mice. We show that loss of Hevin, but not SPARC, slows the maturation of NMJs and muscle fibers during development. We also demonstrate that expression of both SPARC and Hevin is upregulated during NMJ reformation following motor neuron injury and that these genes regulate aspects of synapse repair. Additionally, we show that the combined deletion of SPARC and Hevin impacts NMJ maturation and reformation following nerve injury but has little effect on muscle fiber

development. Together, these results identify roles for the matricellular proteins Hevin and SPARC in skeletal muscle maturation and NMJ development and repair.

Materials and methods

Animals

Global Hevin ((SPARCl1$^{-/-}$, referred hereafter as Hevin$^{-/-}$) and SPARC (SPARC $^{-/-}$) knockout mice were the kind gift of Dr. Cagla Eroglu [18]. These lines were crossed with each other to generate SPARC$^{-/-}$;Hevin$^{-/-}$ double knockout mice. For NMJ analysis, each line was crossed with the Thy1YFP16 transgenic line, in which yellow fluorescent protein (YFP) is expressed under the Thy1 promoter in peripheral neurons [29], to generate SPARC$^{-/-}$;Thy1YFP, Hevin$^{-/-}$;Thy1YFP and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP mice. All mice were bred on a mixed background composed primarily of C57B1/6J and SV129. Animals that died before the conclusion of experiments were not included in subsequent analyses. Animals were housed with a 12-hour light/dark cycle with access to food and water ad libitum. Breeding, housing, and experimental use of animals were performed in a pathogen free environment in accordance with the National Institutes of Health and Virginia Tech Institutional Animal Care and Use Committee (Protocol# 18-148) guidelines.

Tissue fixation

Adult mice were sacrificed with isoflurane and perfused transcardially using 4% paraformaldehyde (PFA) diluted in phosphate buffer saline (PBS). Internal organs and skin were removed, and mice were post-fixed in 4% PFA overnight at 4°C. Mice aged P9 were anaesthetized and decapitated, organs were removed, and carcasses were fixed in 4% PFA overnight at 4°C.

Fibular nerve crush

Surgeries were performed as described previously [30]. Briefly, mice were anesthetized with ketamine/xylazine, shaved, and administered an analgesic, buprenorphine, prior to surgery. To access the fibular nerve, a small incision in the skin was made near the crush site in the right hindleg. The common fibular nerve was crushed for five seconds at the point where the nerve crosses posterior to the gastrocnemius tendon using small forceps, and stitches were applied to close the wound. Mice were monitored after surgery and buprenorphine was administered as needed.

mRNA quantification

Mice were sacrificed with isoflurane and muscles were dissected and flash frozen in liquid nitrogen before homogenization in Trizol reagent (Life Technologies, Carlsbad, CA). C2C12 cells were washed three times in PBS before addition of Trizol (500 μL/well). Cells were scraped from wells and flash frozen in Trizol. Total RNA was isolated and treated with DNase using the Aurum Total RNA mini kit (Bio-Rad, Hercules, CA). RNA concentration was quantified using a NanoDrop Lite Spectrophotometer (Fisher Scientific, Waltham, MA). RNA was reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). Quantitative PCR (qPCR) was performed with iTAQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using a CFX Connect Real Time PCR System (Bio-Rad, Hercules,

CA). Expression values were normalized to 18S rRNA using the $2^{-\Delta\Delta CT}$ method. No reverse transcriptase and no template controls were used. All primers used for this study are listed in Table 1. Agarose gel electrophoresis was performed on qPCR products using a 2% agarose gel.

C2C12 Culture

C2C12 cells were seeded to 8-well chamber slides coated with poly-ornithine (3 μg/mL) and laminin (10 μg/mL). Proliferating C2C12 myoblasts were maintained in high glucose Dulbecco's modified eagle medium (DMEM, Life Technologies, Carlsbad, CA) containing 20% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), 0.1% penicillin/streptomycin (Pen/Strep, Life Technologies, Carlsbad, CA), 2 mm glutamine (Life Technologies, Carlsbad, CA), and 0.1% Fungizone (Life Technologies, Carlsbad, CA) at 37°C and 5% CO2. Differentiation of C2C12 myoblasts into myotubes was achieved by seeding cells at a high density (5 × 108 cells/well) in DMEM containing 2% horse serum (Life Technologies, Carlsbad, CA), 0.1% Pen/Strep, 2 mm glutamine, and 0.1% Fungizone.

Immunohistochemistry: muscle cross-sections

The tibialis anterior (TA) was dissected from PFA-fixed mice and dehydrated in 30% sucrose overnight at 4oC and embedded in tissue freezing medium (General Data Healthcare, Cincinnati, OH). Muscles were cross-sectioned using a cryostat at a thickness of 16 μm and mounted onto gelatin-coated slides. Sections were blocked at room temperature for 30 min in blocking buffer (5% lamb serum, 3% BSA, 0.1% Triton-X 100 in PBS). Rabbist anti- laminin (Sigma-Aldrich #L9393; St. Louis, MO, 1:250) was applied to sections in blocking buffer overnight at 4oC. After washing 3 times in PBS, Alexafluor 568 conjugated polyclonal goat anti-rabbit antibody (#A11036, Invitrogen, Carlsbad, CA) was applied to muscle sections at 1:1000 in blocking buffer for 1 hour at room temperature. Sections were washed twice and 4,6-Diamidino-2-phenylindole (DAPI, #D1306, Fisher Scientific, Waltham, MA) diluted 1:1000 in PBS was applied for 10 min. Sections were washed twice in PBS and Vectashield mounting medium (Vector Laboratories, Burlingame, CA) was applied prior to addition of a coverslip.

Immunohistochemistry: whole-mounted skeletal muscle

The extensor digitorum longus (EDL) was dissected from PFA-fixed mice and incubated in Alexafluor 555 conjugated alpha-bungarotoxin (#B35451, Invitrogen, Carlsbad, CA) diluted 1:1000 in PBS for 2 hours at room temperature to label post-synaptic acetylcholine receptors. Muscles were washed 3 times with PBS and whole-mounted to gelatin coated slides in Vectashield.

Imaging

Imaging was performed with a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss Microscopy, Berlin, Germany) using a 20× (0.8 numerical aperture) objective. Zeiss Zen software was used to generate maximum intensity projections and stitching of tile scans.

Muscle fiber cross sectional analysis

Cross sectional area (CSA) analysis was performed on tile scan images of 16 μm TA cross sections following laminin immunohistochemistry (IHC). Laminin positive outlines of individual muscle fibers were used for CSA measurement using ImageJ. The fractionator method [31] was used to randomly sample skeletal muscle fibers by superimposing a grid at a random orientation over the images using ImageJ.

Statistical analysis

Data are represented as mean ± SEM. Comparisons were performed with an unpaired student's t test with Welch's correction for unequal variance as necessary. Differences were considered significant if $p < 0.05$. Replicates for comparisons are listed in the figure legends. Statistical analyses were performed with the Microsoft Excel Analysis Tool pack.

Results

Hevin and SPARC are expressed in developing and adult skeletal muscle

SPARC and Hevin have been detected in myoblasts *in vitro* and skeletal muscle tissue *in vivo* [18,20,23,24,32]. Skeletal muscle tissue is comprised of multiple cell types, including myoblasts and mature muscle fibers [33], and it is unknown if mature muscle fibers, which constitute the postsynaptic region of NMJs [1], specifically express SPARC and Hevin. To better understand how SPARC and Hevin expression changes as myoblasts differentiate into mature muscle fibers, we evaluated mRNA levels in cultured C2C12 myoblasts and myotubes using reverse transcription quantitative polymerase chain reaction (qPCR). Specificity of qPCR primers against SPARC and Hevin transcripts were confirmed following qPCR using RNA isolated from Hevin$^{-/-}$ (Fig. 1 A) and SPARC$^{-/-}$ (Fig. 1 B) skeletal muscles. SPARC and Hevin transcripts were detected in both C2C12 myoblasts and differentiated myotubes, providing evidence that muscle fiber progenitors as well as mature muscle fibers express both SPARC and Hevin (Fig. 1 B,C). Levels of both genes were significantly higher in myotubes versus myoblasts (Fig. 1 B,C), suggesting that they are upregulated during myogenic differentiation.

To further explore this relationship *in vivo*, we assessed SPARC and Hevin transcripts in developing (P1 and P21) and mature (P70 and P120) Tibialis Anterior (TA) muscle. Both Hevin and SPARC were detected in the TA at all ages (Fig. 1 E–F). Levels of Hevin transcripts remained constant in skeletal muscle throughout development and early adulthood (Fig. 1E). In contrast, SPARC mRNA levels were highest in skeletal muscles during early development, at P1, and progressively declined as skeletal muscles matured (Fig. 1F). To confirm that SPARC and Hevin protein is expressed by skeletal muscles and present at the NMJ, we performed SPARC and Hevin IHC in developing skeletal muscle (P9-P21). Specificity of the SPARC and Hevin antibodies was confirmed in Hevin$^{-/-}$ (Fig. 1G) and SPARC$^{-/-}$ (Fig. 1I) EDL muscles. We observed distribution of Hevin (Fig. 1H) and SPARC (Fig. 1J) in both the non-synaptic and synaptic regions of EDL muscle cross sections. This observation supports the presence of Hevin and SPARC at developing NMJs and suggests that their roles in developing skeletal muscles may not be restricted to synaptic maturation. Together, these results demonstrate that both Hevin and SPARC are expressed in

developing and adult skeletal muscles. These results also support previous studies showing that SPARC and Hevin are present at rodent NMJs [16,22].

Loss of Hevin, but not SPARC, delays NMJ maturation and muscle fiber formation

In light of the known roles of Hevin and SPARC in excitatory synaptogenesis in the CNS [13–18] and their expression in developing and mature skeletal muscle [23–27], we reasoned that these matricellular proteins may act in concert to regulate the formation of the NMJ. We analyzed NMJ maturation in the extensor digitorum longus (EDL) muscle at P9, an age at which growth and alignment of the pre- and postsynapse is accompanied by the removal of supernumerary axons and axonal sprouts that extend beyond the presynapse [1]. To assess the function of SPARC and Hevin in this process we analyzed transgenic mice with global deletions of SPARC (SPARC$^{-/-}$), Hevin (Hevin$^{-/-}$), or both SPARC and Hevin (SPARC$^{-/-}$;Hevin$^{-/-}$). To facilitate visualization of motor axons, we crossed these lines with Thy1YFP transgenic mice, in which motor axons are labeled with yellow fluorescent protein (YFP), to generate SPARC$^{-/-}$;Thy1YFP, Hevin$^{-/-}$;Thy1YFP and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP transgenic mice. We observed deficiencies in NMJ maturation and refinement in mice lacking Hevin as compared to Thy1YFP controls, characterized by an overall smaller NMJ area (Fig. 2 A, B, E), increased incidence of axonal sprouting (Fig. 2 A, B, F), and an increase in the numbers of NMJs innervated by two or more axons, however the latter difference was not statistically significant (Fig. 2 A, B, G). By contrast, SPARC deletion had no outward effect on NMJ maturation, where similar NMJ area (Fig. 2 A, C, E), numbers of axonal sprouts (Fig. 2 A, C, F) and multiply innervated NMJs (Fig. 2 A, C, G) were observed in P9 SPARC$^{-/-}$;Thy1YFP versus Thy1YFP control EDL. Similar to muscles lacking Hevin, the combined deletion of Hevin and SPARC resulted in evidence of delayed NMJ maturation, where decreased NMJ size (Fig. 2 A, D, E) and increased numbers of axonal sprouts (Fig. 2 A, D, F) and multiply innervated NMJs (Fig. 2 A, D, G) were observed in P9 SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP as compared to Thy1YFP controls. These results suggest that, similar to excitatory synapses in the CNS [13–18], Hevin but not SPARC promotes the proper development of the NMJ.

Our observations of elevated Hevin and SPARC mRNA levels following the differentiation of C2C12 myoblasts into myotubes and the presence of both transcripts in developing skeletal muscles led us to test whether either gene plays a role in skeletal muscle fiber maturation. We evaluated the cross-sectional area (CSA) of skeletal muscle fibers of the TA in developing (P9) SPARC$^{-/-}$, Hevin$^{-/-}$, and SPARC$^{-/-}$;Hevin$^{-/-}$ mice. Our analysis revealed that Hevin deletion resulted in a modest, but statistically significant, decrease in average skeletal muscle fiber CSA at P9 (Fig. 3 A). Further analysis of muscle fiber CSA distribution revealed increased numbers of small muscle fibers (125-150 μm2) and decreased numbers of larger muscle fibers (275 and 325 μm2) as compared to controls, suggesting a delay in muscle fiber maturation at this age (Fig. 3 B). SPARC deletion resulted in a modest increase in average skeletal muscle fiber CSA that was not statistically significant (Fig. 3 A) and differences in muscle fiber CSA were not observed (Fig. 3 C). Interestingly, deletion of SPARC mitigated the effects of Hevin deletion, where no changes in average skeletal muscle fiber CSA (Fig. 3 A) or CSA distribution (Fig. 3 D) were observed in SPARC$^{-/-}$;Hevin$^{-/-}$ as compared to control muscle.

Hevin and Sparc are not required for NMJ maintenance in healthy skeletal muscle

We next determined whether SPARC and Hevin are necessary for maintenance of NMJs in healthy adult skeletal muscle. We evaluated multiple indices of synaptic health, including fragmentation of acetylcholine receptors (AChRs) on the postsynapse, NMJ area, and completeness of overlap between the pre- and postsynapse [34,35], in adult (P90) EDL muscles of SPARC$^{-/-}$;Thy1YFP, Hevin$^{-/-}$;Thy1YFP and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP transgenic mice. We did not observe a discernable effect of Hevin deletion on the integrity of NMJs in young adult mice (Fig. 4 A, B). The degree of AChR fragmentation (Fig. 4 E), NMJ area (Fig. 4 F), and synaptic overlap (Fig. 4 G) were similar between Hevin$^{-/-}$;Thy1YFP and control muscle. In both SPARC$^{-/-}$;Thy1YFP (Fig. 4 C) and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP (Fig. 4 D) muscle, we observed a reduction in both postsynaptic AChR cluster fragmentation (Fig. 4 E) and NMJ area (Fig. 4 F) as compared to control muscle. Similar to Hevin$^{-/-}$;Thy1YFP muscle, no differences were observed in synaptic overlap (Fig. 4 G) in SPARC$^{-/-}$;Thy1YFP and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP muscle. Additionally, we did not observe differences in axonal sprouting or NMJs with multiple motor axons in any of the three mouse lines when compared to Thy1YFP controls (Fig. 4 A–D). The increased uniformity of AChR clusters and decreased size of the NMJ associated with SPARC deletion, whether alone or in combination with Hevin deletion, suggest that SPARC may function to promote NMJ maturation during the latter stages of development (i.e., after P9). However, the overall absence of degenerative features in NMJs of adult Hevin$^{-/-}$ or SPARC$^{-/-}$ mice suggest that these genes have minimal function in maintaining NMJ integrity in healthy young adult muscles.

Hevin and SPARC deletion accelerates NMJ reinnervation but hinders synaptic refinement following nerve injury

Previous studies showing that Hevin and SPARC affect growth factor signaling in the ECM to promote excitatory synapse formation in the CNS [18] and Schwann cell recruitment in the PNS [28], along with our findings that deletion of Hevin delays NMJ maturation (Fig. 2), suggest that SPARC and Hevin may function to repair injured NMJs. To assess this possibility, we measured SPARC and Hevin mRNA levels during the process of NMJ reinnervation of the TA muscle following injury to the fibular nerve, via crush, in 3-month-old wild type mice. In the fibular nerve crush model, motor axons completely retract from the TA and EDL muscles by 1d post-injury, return and make initial contact with postsynaptic sites at 7d post-injury, and fully reoccupy NMJs by 12d post-injury [30]. Transcriptional analysis using qPCR revealed that both SPARC and Hevin transcripts are highly upregulated during the period of NMJ reinnervation, at 7d and 9d post-fibular nerve crush (Fig. 5 A, B). By 12d post-injury, when NMJ reformation is nearly complete, Hevin mRNA levels return to baseline while increases in SPARC mRNA are somewhat attenuated (Fig. 5 A, B), suggesting that these genes are upregulated to promote NMJ repair.

To determine the roles of Hevin and SPARC in repair of adult NMJs, we examined NMJs in the EDL muscle of 3-month-old SPARC$^{-/-}$;Thy1YFP, Hevin$^{-/-}$;Thy1YFP and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP at 9d following crushing of the fibular nerve. Surprisingly, deletion of SPARC, Hevin, or SPARC and Hevin resulted in modest increases in the numbers of fully innervated NMJs at 9d post-injury, although this difference was only

statistically significant in Hevin$^{-/-}$;Thy1YFP mice (Fig. 5 C–F, G). These results suggest that deletion of Hevin accelerates the rate of NMJ reinnervation following nerve injury. We also observed a statistically significant increase in the numbers of axonal sprouts in SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP EDL (Fig. 5 H). Modest increases in the numbers of multiply innervated NMJs were also observed in SPARC$^{-/-}$;Thy1YFP, Hevin$^{-/-}$;Thy1YFP and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP muscle, however these differences were not statistically significant (Fig. 5 I). The acceleration of NMJ reinnervation along with modest deficits in synaptic refinement suggest that Hevin and SPARC may be upregulated by muscles during reinnervation to orchestrate the fidelity of NMJ repair, at the expense of its rate of reinnervation. Recently, our group showed that both Hevin and SPARC are highly expressed by perisynaptic Schwann cells (PSCs), which are integral to synapse formation [22]. It is possible, therefore, that PSCs in addition to muscle fibers utilize Hevin and SPARC to mediate aspects of NMJ reformation following nerve injury.

Discussion

Previous studies have shown that SPARC and Hevin regulate excitatory synapse formation [13–18] and are expressed in muscle tissue [19–28], suggesting that these molecules could have important functions in skeletal muscle and NMJ development. Here we show that SPARC and Hevin are expressed in skeletal muscle fibers and influence skeletal muscle maturation, NMJ development, and NMJ repair. Hevin expression in skeletal muscle remains constant during development and adulthood and its deletion impacts maturation of skeletal muscle fibers and of NMJs. SPARC, on the other hand, is highly upregulated in developing skeletal muscle but its deletion has little effect on either skeletal muscle fiber or NMJ maturation. In adult SPARC$^{-/-}$, and SPARC$^{-/-}$; Hevin$^{-/-}$ muscles, NMJs are smaller in size and display a more contiguous AChR clustering pattern, suggesting that SPARC, and possibly Hevin, play a role in the latter stages of NMJ development. We show that both SPARC and Hevin are upregulated by skeletal muscle in response to nerve injury. Deletion of Hevin expedites NMJ reformation following nerve injury, although with a decreased degree of fidelity in the absence of SPARC as evidenced by increased numbers of axonal sprouts.

Our results showing delayed NMJ maturation in the absence of Hevin are in line with published findings showing that Hevin promotes synapse formation in the CNS [13,15,18]. In particular, Kucukdereli and colleagues demonstrated that astrocyte-secreted Hevin and SPARC work in an antagonistic fashion to orchestrate the formation of glutamatergic synapses, where Hevin deletion delays synapse formation and SPARC deletion accelerates it [18]. In contrast to these findings, we did not observe obvious changes in NMJ formation in the absence of SPARC. Further, the effects of Hevin deletion on NMJ development observed in our study are less robust than those observed by Kucukdereli and colleagues in glutamatergic synapse development [18]. These differences could be due to compensatory developmental effects that exist in skeletal muscles but not the CNS, including those related to Schwann cells [28]. They may also be attributable to fundamental differences between NMJs and glutamatergic synapses, including ECM composition, neurotransmitter type (i.e., cholinergic versus glutamatergic), or synaptic glia. With regard to synaptic glia, PSCs exclusively associate with synapses in the peripheral nervous system and serve similar

functions as astrocytes by promoting synapse formation during development, injury, and disease [36]. We have previously shown that PSCs in developing muscle express both SPARC and Hevin at very high levels [22]. PSCs are thus a source of SPARC and Hevin at the NMJ and it is possible that PSCs may utilize these two molecules to promote the development and repair of NMJs in addition to muscle fibers.

Our findings suggest that Hevin is among a growing family of ECM proteins, such as z-agrin [1] and FGFBP1 [34], that are known to influence NMJ development. Like z-agrin [37]. Hevin [9] has a follistatin region that is central to its ability to bind and mediate the function of a host of ECM molecules. It is possible, therefore, that Hevin influences z-agrin function, via its follistatin domain, by competing for ECM proteins that mediate z-agrin distribution and function at the NMJ. SPARC has been demonstrated to regulate a number of molecules that are present in skeletal muscles and known to influence NMJ repair, including TGF-β, collagen IV and, FGF2 [3,10–12,34,38]. To our knowledge, however, interactions between SPARC and Hevin and their known binding partners have not been demonstrated in skeletal muscles. One possible avenue of future studies is to determine the specific ECM molecules with which Hevin and SPARC interact during NMJ development and repair. Also unexplored are the roles of SPARC and Hevin in motor function. While we did not observe obvious motor function abnormalities in developing or adult mice lacking SPARC or Hevin, it is possible that their deletion causes subtle motor deficits that could be detected by behavioral tests, such as rotorod and hanging tests, or electromyography.

Previous studies have shown that SPARC is expressed by developing skeletal muscles [23,32], and its overexpression influences C2C12 myoblast differentiation into myotubes *in vitro* [26]. In our analysis, we found no obvious effects of SPARC deletion on skeletal muscle fiber maturation in P9 mice. The differences between our results and those described previously [26] could be attributed to the increased complexity of the ECM *in vivo*, versus *in vitro*, where the availability of additional ECM molecules with redundant functions *in vivo* may compensate for the loss of SPARC. It is also possible that, while SPARC is capable of promoting muscle fiber maturation, it is not necessary for this process.

Conclusions

Together, our findings demonstrate that both Hevin and SPARC are expressed by developing and mature skeletal muscles and suggest that Hevin may have a limited role in NMJ and muscle fiber maturation, while Hevin and SPARC may work in concert to influence NMJ repair. While much is to be learned about the ECM molecules and receptors that work with these proteins in muscles and specifically at the NMJ, this study provides initial evidence that Hevin and SPARC modulate aspects of NMJ and muscle development and repair following injury. It also raises the possibility that these two molecules may similarly influence repair of aged and disease-afflicted muscles and their NMJs.

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Highlights

- The matricellular proteins Hevin and SPARC are expressed by mouse skeletal muscles and present at the neuromuscular junction (NMJ) during its formation.
- Deletion of Hevin, but not SPARC, slows NMJ development.
- Hevin and SPARC are highly upregulated in adult skeletal muscles during NMJ repair.
- Deletion of Hevin and SPARC accelerates reinnervation of the NMJ but impairs synaptic refinement following nerve injury.

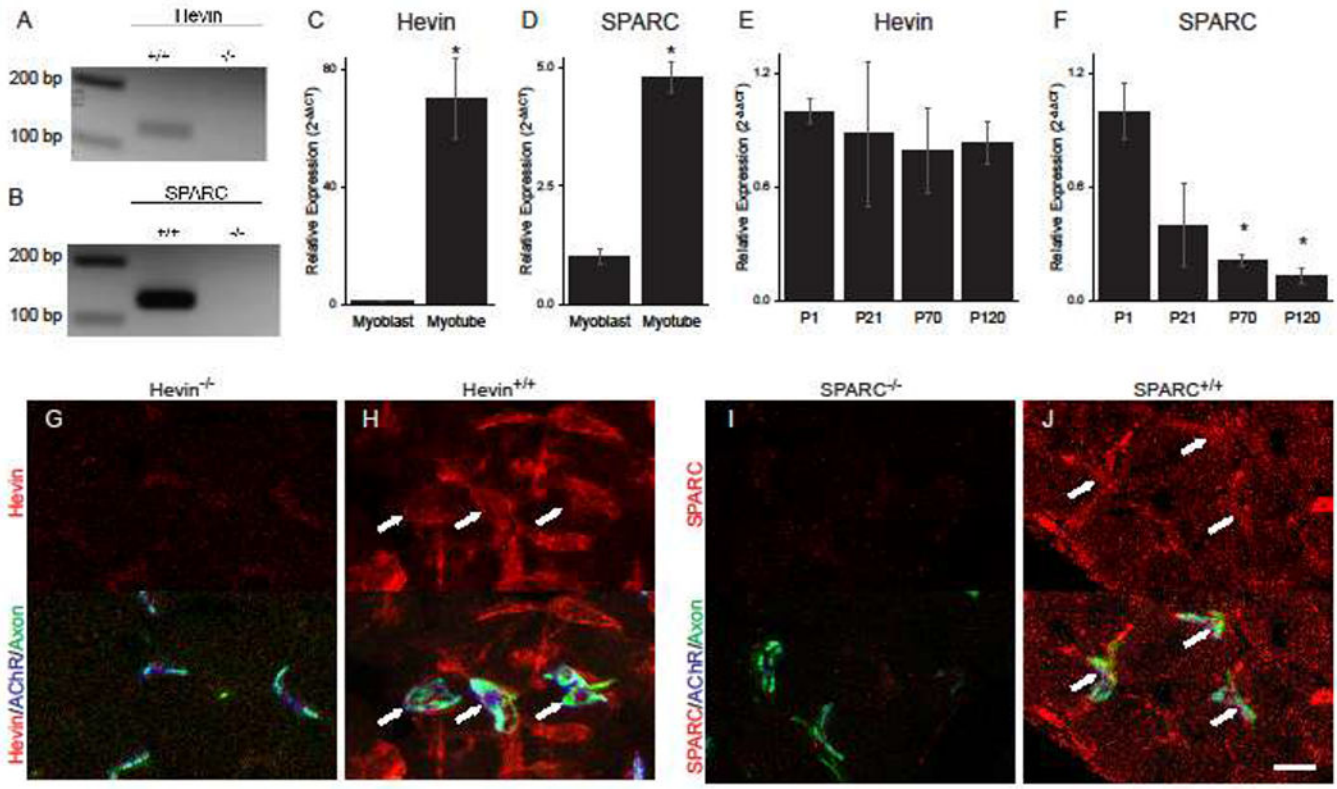

Figure 1.

Hevin and SPARC are expressed in skeletal muscle. (A-B) Hevin and SPARC qPCR primer specificity was confirmed by RT-PCR using RNA isolated from the TA of Hevin$^{-/-}$ (A) and SPARC$^{-/-}$ (B) mice and their wild type littermates. A representative image of agarose gel electrophoresis of PCR products shows bands at the predicted sizes (SPARC, 110 bp; Hevin, 125 bp) in wild type but not knockout samples. (C-D) RT-qPCR analysis of RNA collected from C2C12 myoblasts and myotubes, at 3 days post-differentiation, shows that both Hevin (C) and SPARC (D) transcripts are present in cultured myoblasts and are highly upregulated following differentiation into myotubes. An asterisk indicates $p < 0.05$ versus myoblasts, N = 3. (E-F) Analysis of developing (P1 and P21) and adult (P70 and P120) TA muscle by RT-qPCR shows that Hevin transcript levels remain constant throughout development and young adulthood (D). SPARC transcript levels are significantly lower in adult (P70 and P120) versus developing (P1) muscle (E). (G-H) Hevin IHC in P9 Hevin$^{-/-}$;Thy1YFP and Hevin$^{+/+}$;Thy1YFP TA muscle cross-sections. Fluorescently conjugated α bungarotoxin (fBTX)-labeled AChRs (blue) and yellow fluorescent protein (YFP)-labeled motor axons (green) mark the synaptic region (arrows) of the muscle fiber. (I-J) SPARC IHC in P21 SPARC$^{-/-}$;Thy1YFP and SPARC$^{+/+}$;Thy1YFP TA muscle cross-sections in which the synaptic region is marked by fBTX-labeled AChRs and YFP-labeled motor axons (arrows). An asterisk indicates $p < 0.05$ versus P1, N = 3 for all groups except P21 where N = 2. Expression values normalized to 18S rRNA using the $2^{-\Delta\Delta CT}$ method. All values reported as mean expression ± SEM. Scale bar = 10 μm.

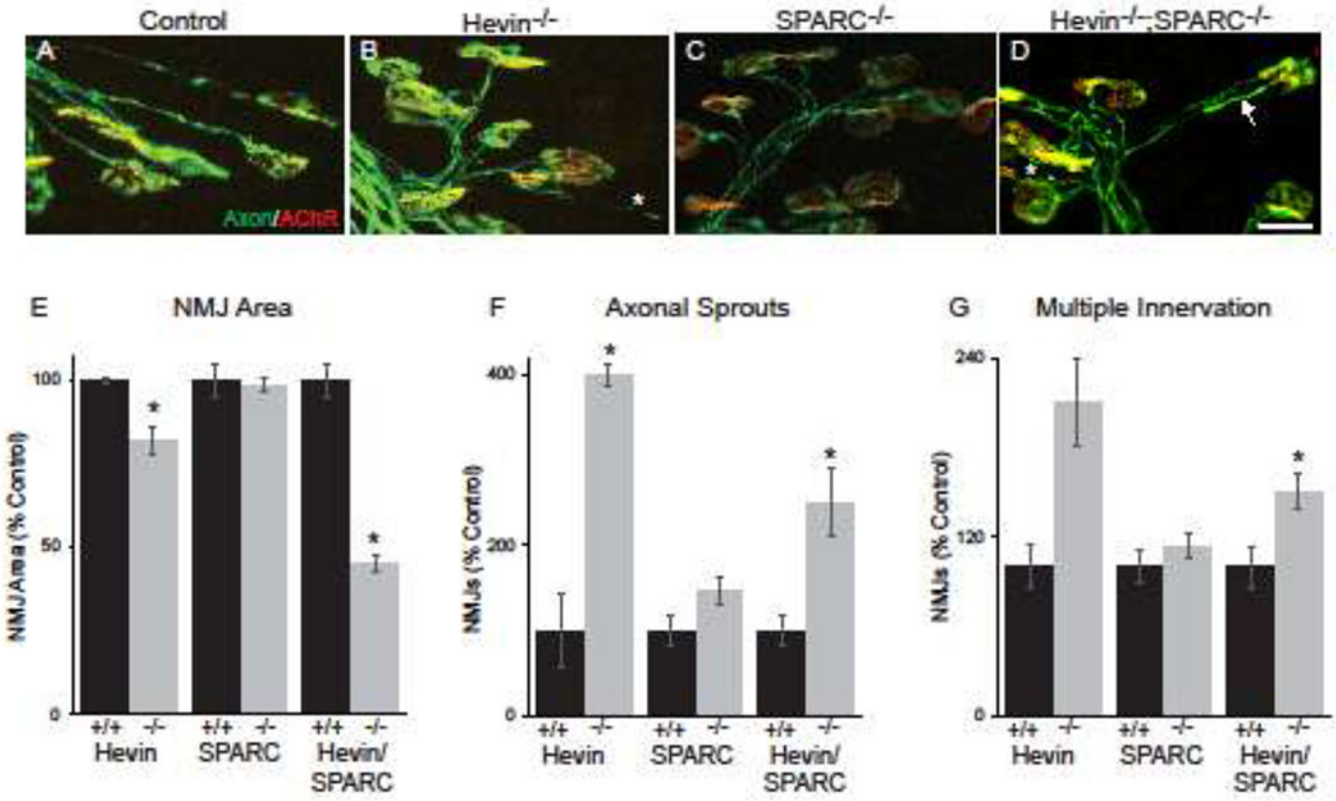

Figure 2.

Loss of Hevin but not SPARC delays NMJ maturation in developing (P9) muscle. (A-D) Representative images of NMJs of the EDL muscle of P9 Thy1YFP control (A), Hevin$^{-/-}$;Thy1YFP (B), SPARC$^{-/-}$;Thy1YFP(C), and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP (D), mice in which motor axons are labeled with YFP (green) and AChRs are labeled with fluorescently conjugated α bungarotoxin (fBTX, red). A white asterisk indicates axon sprouting. A white arrow indicates multiple innervation of an NMJ. (E) Measurements AChR+ area reveal a significant reduction in average NMJ area in EDL muscles from mice lacking Hevin or both Hevin and SPARC. (F) Analysis of the frequency of axon sprouts that extend beyond the postsynapse shows increased sprouting in EDL muscles from mice lacking Hevin or both Hevin and SPARC. (G) Quantification of the presence of NMJs that are innervated by two or more axons shows elevated numbers of multiply innervated NMJs in EDL muscles from mice lacking Hevin or both Hevin and SPARC, with a statistically significant difference in comparisons between SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP and Thy1YFP control EDL. All values reported as mean ± SEM. An asterisk indicates $p < 0.05$ versus wild type control, N= 3-4. Scale bar = 20 μm.

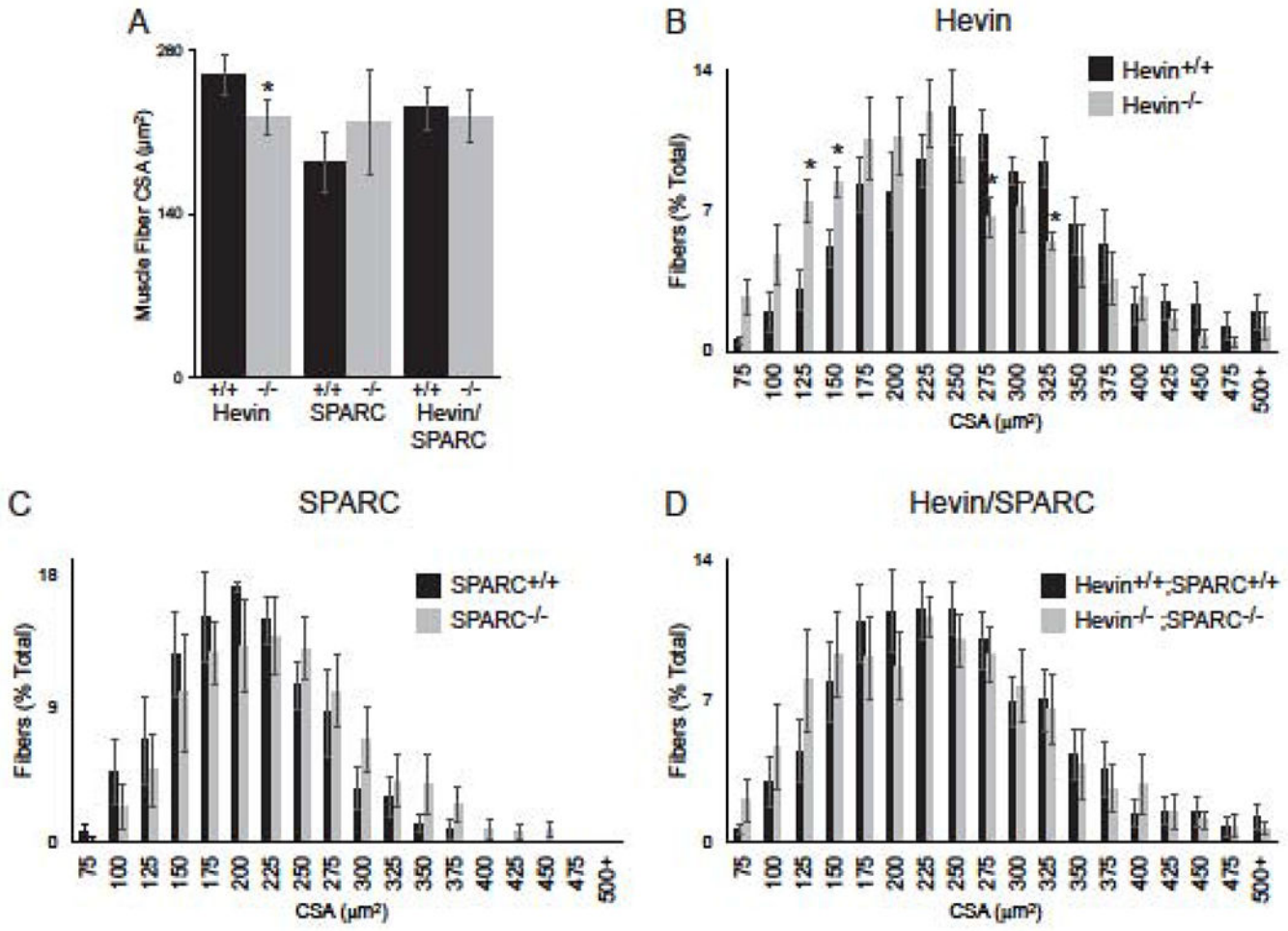

Figure 3.

Loss of Hevin but not SPARC delays skeletal muscle fiber maturation in developing (P9) muscle. (A) Measurements of average muscle fiber cross-sectional area (CSA) in the TA muscle of P9 control, Hevin$^{-/-}$, SPARC$^{-/-}$, and SPARC$^{-/-}$;Hevin$^{-/-}$ mice shows that deletion of Hevin, but not SPARC or SPARC and Hevin combined, reduce the size of developing skeletal muscle fibers. (B-D) Analysis of the distribution of skeletal muscle fiber CSA in P9 TA muscle reveals significantly higher numbers of muscle fibers with smaller CSAs and significantly lower numbers of muscle fibers with larger CSAs in Hevin$^{-/-}$ as compared to control muscle (B). These differences were not observed in SPARC$^{-/-}$ (C) or SPARC$^{-/-}$;Hevin$^{-/-}$ (D) muscle. All values reported as mean ± SEM. An asterisk indicates $p < 0.05$ versus wild type control, N= 3-5. Scale bar = 25 μm.

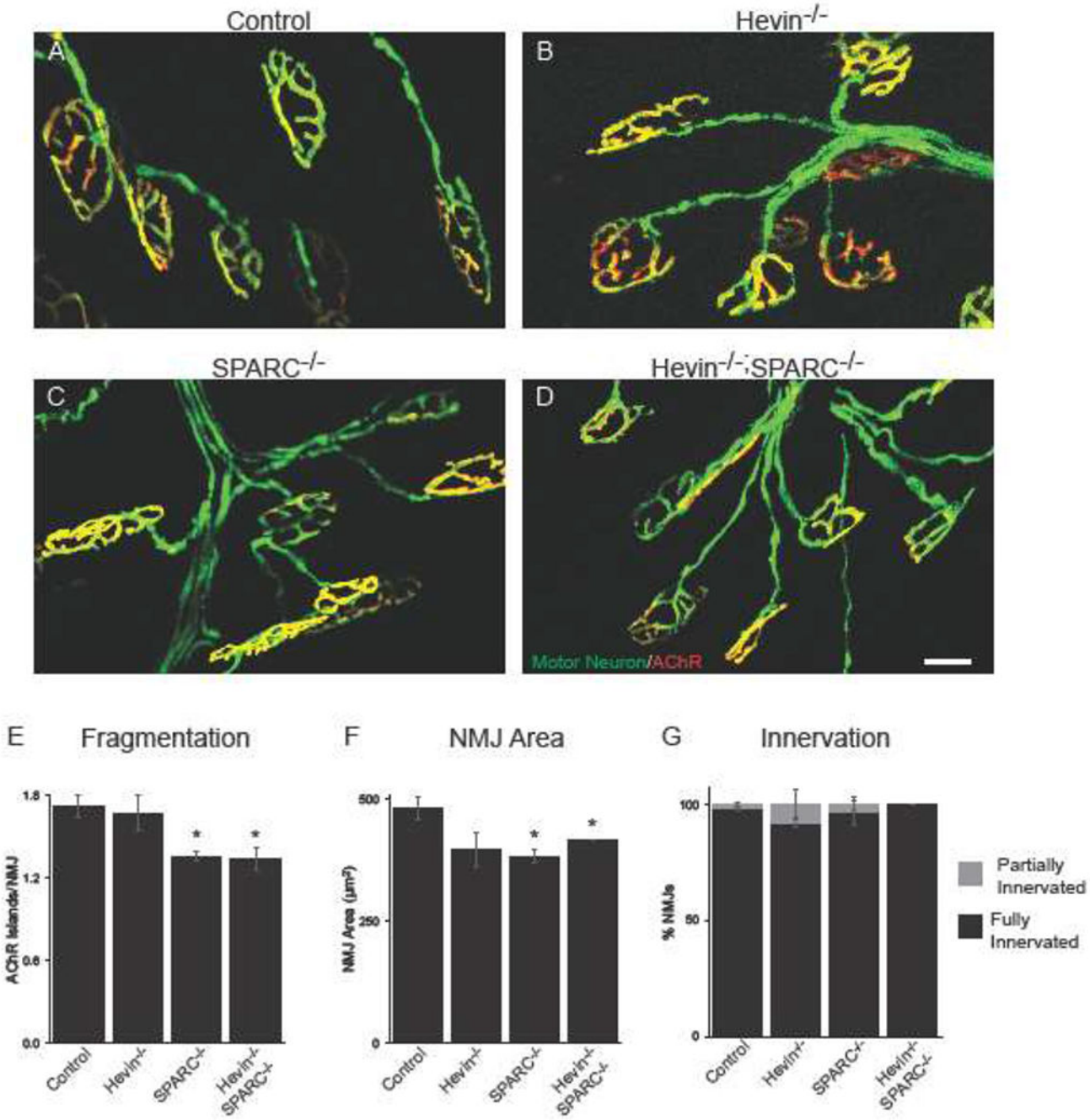

Figure 4.

Hevin and Sparc are not required for NMJ maintenance. (A-D) Representative images of NMJs of the EDL muscle of P90 Thy1YFP control (A), Hevin$^{-/-}$;Thy1YFP (B), SPARC$^{-/-}$;Thy1YFP(C), and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP (D), mice in which motor axons are labeled with YFP (green) and AChRs are labeled with fBTX, (red). (E) AChR cluster fragmentation of NMJs was evaluated by counting the number of AChR islands per NMJ in EDL muscles of P90 Thy1 YFP control, Hevin$^{-/-}$;Thy1YFP, SPARC$^{-/-}$;Thy1YFP, and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP mice. EDL muscles lacking SPARC or both SPARC and

Hevin had reduced numbers of AChR islands as compared to control. (F) Measurements AChR+ area reveal a significant reduction in average NMJ area in EDL muscles from mice lacking SPARC or both SPARC and Hevin, as compared to control. (G) Analysis of the degree of pre- and postsynaptic overlap at the NMJ showed that fully innervated NMJs constitute the majority of NMJs in each of the mouse lines analyzed. All values reported as mean ± SEM. An asterisk indicates $p < 0.05$ versus wild type control, N = 3. Scale bar = 25 μm.

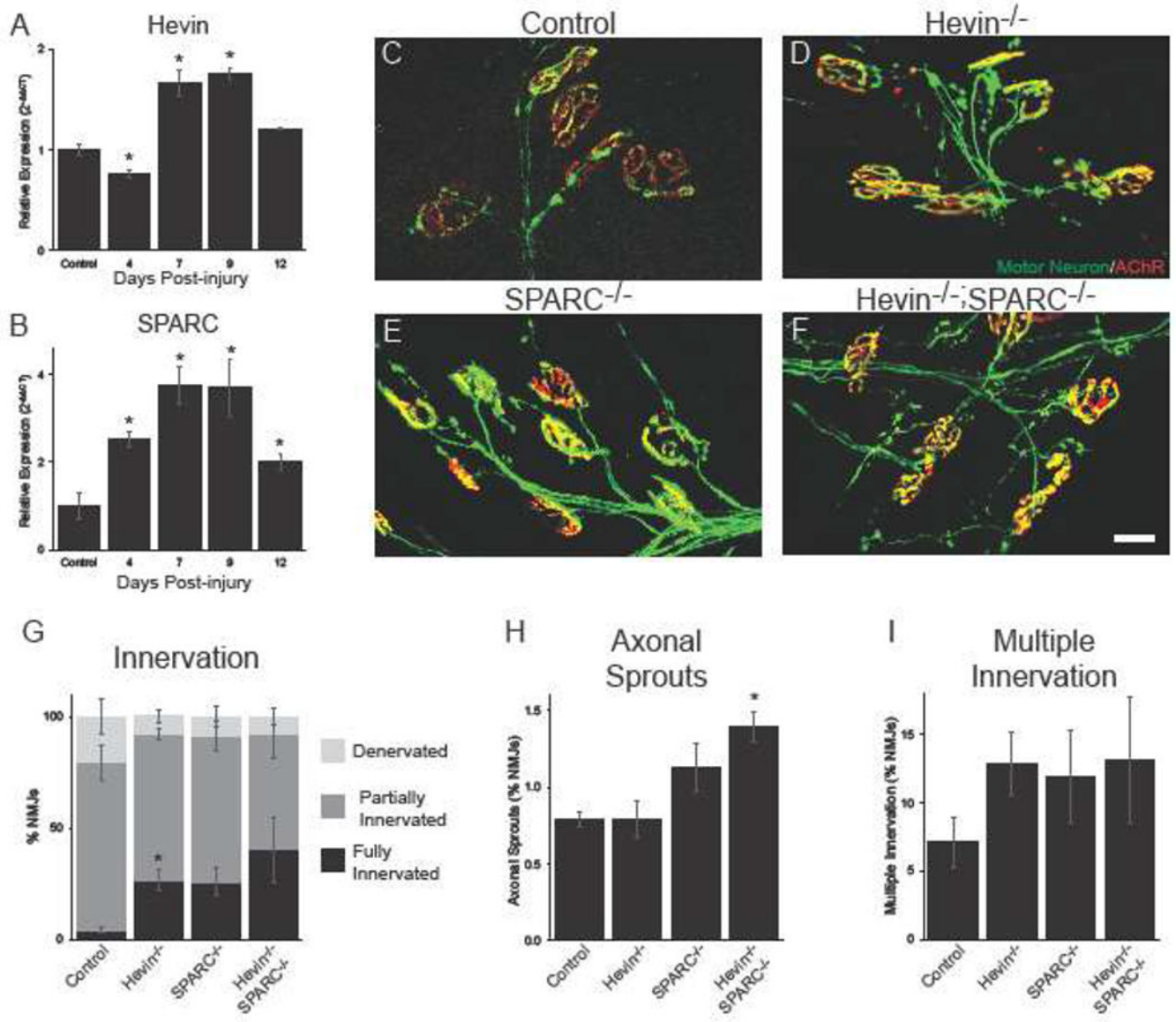

Figure 5.

Hevin and SPARC deletion promotes NMJ reinnervation following nerve injury. (A, B) Hevin and SPARC mRNA is elevated in adult (P90) control TA following fibular nerve crush. (C-F) Representative images of NMJs (motor neurons, green; AChRs, red) in the EDL muscle at 9 days post-fibular nerve crush in P90 Thy1YFP control (C), Hevin$^{-/-}$;Thy1YFP (D), SPARC$^{-/-}$;Thy1YFP (E), and SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP (F) mice. (G) Analysis of synaptic overlap shows a statistically significant increase in fully innervated NMJs in Hevin$^{-/-}$;Thy1YFP EDL as compared to Thy1YFP controls. (H) Analysis of the frequency of axon sprouts that extend beyond the postsynapse shows increased sprouting in SPARC$^{-/-}$;Hevin$^{-/-}$;Thy1YFP EDL muscle. (I) Analysis of multiply innervated NMJs. All values reported as mean ± SEM. Expression values normalized to 18S rRNA using the

$2^{-\Delta\Delta CT}$ method. An asterisk indicates $p < 0.05$ versus wild type control, N = 3. Scale bar = 25 μm.

Table 1.

qPCR primer sequences.

Gene	Forward Primer (5’-3’)	Reverse Primer (5’-3’)
18S rRNA	GCCAGTCGGCATCGTTTATG	GCGAGAAGAAAGCCAAACAC
Hevin	GCTAGCTCCTCTTGGGCATT	ATGCTGGCTAGATCTGCGG
SPARC	CATGCAAATACATCGCCCCC	TCAGTGAGGAGGTTGTTGCC