Peroxisome Proliferator-activated Receptor \(\beta/\delta\) Induces Myogenesis by Modulating Myostatin Activity*‡§

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Background: PPAR\(\beta/\delta\) has been implicated in muscle regeneration; however the signaling mechanism(s) is unclear.

Results: Activation of PPAR\(\beta/\delta\)-promoted Gasp-1 expression blocked myostatin activity and enhanced myogenesis.

Conclusion: Activation of PPAR\(\beta/\delta\) led to inhibition of myostatin activity and thus increased myogenesis.

Significance: PPAR\(\beta/\delta\) agonists are novel myostatin antagonists that have potential benefits toward improving postnatal muscle growth and repair.

WITHDRAWN
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This article has been withdrawn by the authors. In this article, we reported that PPAR\(\beta/\delta\) positively regulates myogenesis. After a thorough investigation by the Nanyang Technological University in Singapore, data falsifications have been found in some of the in vitro laboratory studies, which invalidate the results reported. Hence, the co-authors wish to withdraw this publication and offer our sincere apologies to all those investigators who may have been affected and misled by this.

Gasp-1 has been reported to bind to and inhibit the activity of myostatin; consistent with this, we found that enhanced secretion of Gasp-1, increased Gasp-1 myostatin interaction and significantly reduced myostatin activity upon L165041-mediated activation of PPAR\(\beta/\delta\). Moreover, we analyzed the ability of hGASP-1 to regulate myogenesis independently of PPAR\(\beta/\delta\) activation. The results revealed that hGASP-1 protein treatment enhances myoblast proliferation and differentiation, whereas silencing of hGASP-1 results in defective myogenesis. Taken together these data revealed that PPAR\(\beta/\delta\) is a positive regulator of skeletal muscle myogenesis, which functions through negatively modulating myostatin activity via a mechanism involving Gasp-1.

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† This article contains supplemental Figs. S1 and S2 and Table S1.
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The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; RXR, retinoid-X receptor; qPCR, quantitative real-time PCR; hGASP-1, human GASP-1 protein; rhGASP-1, recombinant hGASP-1; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance; sActRIIB, soluble activin receptor type IIB; MyoD, myogenic differentiation factor 1; SBE, Smad binding element; CM, conditioned medium; DR-1, direct repeat-1; Mstn, myostatin; EDL, extensor digitorum longus; H&E, hematoxylin and eosin.

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PPARβ/δ Regulates Postnatal Myogenesis via Gasp-1

nists, tissue-specific PPARβ/δ knockdown, or PPARβ/δ overexpressing mouse models have confirmed an array of functions for PPARβ/δ in adipose tissue, skin, and muscle, as well as in response to cancer and inflammation. Using overexpressing transgenic mice and agonists, PPARβ/δ has been shown to influence skeletal muscle metabolism. Specifically, Luquet et al. (17) have demonstrated that muscle-specific overexpression of PPARβ/δ results in hyperplasia of muscle fibers with increased oxidative capability. Similarly, constitutive overexpression of VP6-PPARβ/δ in muscles results in a skeletal muscle fiber type switch from glycolytic to slow oxidative. As a result, increased fatty acid oxidation, reduced fat accumulation in adipose tissue, and a lean phenotype is reported in mice (18). In addition, pharmacological activation of PPARβ/δ by GW0742 increases angiogenesis, enhances oxidative myofiber number, and improves myonuclear accretion in vivo (19), all features observed in response to exercise (20–22). Furthermore, pharmacological activation of PPARβ/δ may act as a potential therapeutic in preventing the dramatic muscle wasting observed during muscular dystrophy (23). Specifically, activation of PPARβ/δ results in increased utrophin A transcript levels in mdx mice (a model of Duchene muscular dystrophy), which is a protein that can functionally compensate for the loss of dystrophin in mdx mice and as such helps to maintain the sarcolemmal integrity of degenerating muscle fibers. A very recent study reports that postnatal activation of PPARβ/δ results in an effect on muscle metabolism to that observed following activation of myostatin (24), a TGF-β superfamily member that is a potent negative regulator of myogenesis (25). Activation of PPARβ/δ by GW501516 decreases myostatin activity via PF-879 at concentrations that result in reduced fat mass, improved muscle triglyceride and free fatty acid concentration, and clearly demonstrates that there is some degree of similarity between PPARβ/δ activation and myostatin inhibition, at least during postnatal growth. Given the loss of muscle associated with PPARβ/δ activation and skeletal muscle growth, we attempted to delineate the mechanism(s) through which PPARβ/δ regulates muscle growth. We report here for the first time that activation of PPARβ/δ, through the addition of L165041, enhances myogenesis in C2C12 myoblasts via an increase in both myoblast proliferation and differentiation. Consistent with this, loss of PPARβ/δ results in reduced proliferation of primary myoblasts and defective differentiation. Microarray analysis revealed the Gasp-1 (growth and differentiation factor-associated serum protein-1) gene as a potential target of PPARβ/δ. Subsequent expression analysis confirmed up-regulation of Gasp-1 following activation of PPARβ/δ and also revealed enhanced association of Gasp-1 with myostatin in response to PPARβ/δ activation. Importantly, Gasp-1 has been shown previously to be a potent antagonist of myostatin (27, 28), which is a well characterized potent negative regulator of myoblast proliferation and differentiation (29, 30) as well as muscle stem cell (satellite cell) activation and self-renewal (31). Therefore, we propose that PPARβ/δ positively regulates myogenesis through a mechanism that results in Gasp-1-mediated inhibition of myostatin activity.

EXPERIMENTAL PROCEDURES

Animals—PPARβ/δ-null mice (mixed genetic background of Sv129/C56BL/6) were kind gifts from Prof. Walter Wahli (University of Lausanne, Lausanne, Switzerland). PPARβ/δ-null mice were maintained at 20 °C with a 12-h light-dark cycle. mdx mice were obtained from the Animal Resources Centre, Canning Vale, Western Australia, Australia. wild type mice (C57BL/6) were purchased from the Center for Animal Resources, National University of Singapore (NUS-CARE), Singapore. All animal procedures were reviewed and approved by the Institute Animal Ethics Committee, Singapore.

Cell Culture—Mouse C2C12 myoblasts (32) were obtained from American Type Culture Collection (Manassas, VA) and maintained as described previously (33). Human primary myoblasts (isolated from a 15-year-old healthy subject) (34, 35), kind gifts from Drs. Vincent Moully and Gillian Butler-Browne, were maintained as described previously (36, 37). Primary myoblasts were isolated from PPARβ/δ-null mice as described previously (38). To induce differentiation, C2C12, human, and PPARβ/δ-null primary myoblasts were plated at a density of 25,000 cells/cm² in differentiation medium consisting of DMEM supplemented with horse serum and 1% penicillin/streptomycin. Myoblast proliferation assay and differentiation of C2C12 and primary myoblasts were conducted as described (L165041; catalog No. L2167), Wy14643 (Wyeth, catalog No. G5668), and PPARγ agonists were purchased from Sigma-Aldrich, recombinant human GASP-1 (rhGASP-1) protein was purchased from R&D Systems (catalog No. 2070GS; Minneapolis, MN). The generation of soluble activin type IIB receptor (sActRIIB) protein was described previously (39). Assessment of proliferation assay with conditioned medium (CM) involves the collection of CM from cells treated with either L165041 or 0.02% DMSO without serum for 24 h. After collection, CM was supplemented with 10% FBS and 1% penicillin/streptomycin. The serum-supplemented CM was then used to treat myoblasts for the assessment of proliferation rate.

Quantitative Real-time PCR (qPCR)—Total RNA from cells and tissue was isolated using TRIzol reagent (Invitrogen). Synthesis of cDNA, qPCR, and subsequent data analysis were performed as described previously (38). The gene-specific primers used in this manuscript are listed in supplemental Table S1.

Protein Isolation and Immunoblotting—Cell and tissue samples were collected in lysis buffer (50 mM Tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, Complete protease inhibitor mixture (Roche Applied Science), 1 mM PMSF, and 2 mM NaF) followed by centrifugation at 12,000 rpm for 10 min at 4 °C to remove cell debris. Protein quantification, gel electrophoresis, and target protein detection were performed as published previously (38). For GASP-1 and myostatin immunoprecipitation studies, CM was collected, as described above following L165041 treatment. Prior to use, the collected CM was concentrated using Amicon-Ultra centrifugal filters (catalog No. UFC900324, Millipore, Billerica, MA). Immunoprecipitation studies were performed as described previously (40). Briefly, 1 ml of CM and straight DMEM was preclereased using 50
μl of protein A-agarose (Invitrogen) for 1 h at 4 °C. Immunoprecipitation of human GASP-1 (hGASP-1) was performed by incubating the precleared CM and DEMEM with either anti-hGASP-1 or anti-IgG for 2 h at 4 °C. Precleared protein A-agarose was added for 1 h at 4 °C followed by centrifugation to pellet the immunoprecipitated complexes. Pellets were washed four times with cold PBS, resuspended in 50 μl of 1 × NuPAGE sample buffer (Invitrogen), and boiled for 10 min. Immunoprecipitated samples were then subjected to Western blot analysis to detect myostatin levels. The antibodies used in this study are as follows: rabbit polyclonal anti-MyoD (C-20) (sc-304, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit polyclonal anti-myogenin (M-225) (sc-576, Santa Cruz Biotechnology); mouse monoclonal anti-MyHC, all types (MF-20 C, Developmental Studies Hybridoma Bank, Iowa City, IA); mouse monoclonal anti-PPARγ (F-10) X (sc-74517, Santa Cruz Biotechnology); mouse monoclonal anti-hGASP-1 (MAB2070, R&D Systems); rabbit polyclonal anti-human myostatin antibody (HPA021681, Sigma-Aldrich); rat monoclonal anti-myostatin antibody (sc-74041, Santa Cruz Biotechnology); and purified mouse monoclonal anti α-tubulin antibody (T-9026, Sigma-Aldrich).

Microarray Analysis—C2C12 myoblasts were cultured in differentiation medium for 72 h followed by a further 1, 2, 4, 6, 8, 12, or 24 h with or without 10 μM PPARβ/δ agonist (Sigma-Aldrich). Total RNA was isolated using TRIzol reagent (Invitrogen). RNA was then column-purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s guidelines. Cells were then incubated with the transfection mix (Promega) or 10 μM M Wy14643 for a further 24 h. Luciferase assays were performed using the Dual-Luciferase assay system as per the manufacturer’s protocol (Promega). Relative luciferase activity in each of the extracted protein samples was measured in triplicate using the Fluoroskan Ascent microplate fluorometer and luminometer (catalog No. 5210460, Thermo Fisher Scientific).

Chromatin Immunoprecipitation (ChIP) Assay—C2C12 myoblasts were transfected with pGL3-Gasp-1 promoter and incubated for 48 h. Following incubation, the myoblasts were treated without (DMSO) or with 10 μM L165041, 30 μM GW1929, or 10 μM Wy14643 for a further 24 h. Luciferase assays were performed using the Dual-Luciferase assay system as per the manufacturer’s protocol (Promega). Relative luciferase activity in each of the extracted protein samples was measured in triplicate using the Fluoroskan Ascent microplate fluorometer and luminometer (catalog No. 5210460, Thermo Fisher Scientific).

Transient Transfection and Luciferase Assay—Human myoblasts (36C15Q) were plated at a density of 10,000 cells/cm² in 24-well plates. Following an overnight attachment period, human myoblasts were transfected with the 1.5-kb Gasp-1 promoter-luciferase reporter construct (pGL3-Gasp-1), the 1-kb Gasp-1 promoter-luciferase deletion construct (pGL3-Gasp-1 del), or the mutated Gasp-1 promoter-luciferase reporter construct (mut-pGL3-Gasp-1), the mutated GST-1 promoter-luciferase vector (mut-pGL3-Basic) using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions. Cultures were transfected with the transfection mix (Promega, Madison, WI) and pGL3-Basic luciferase vector (Promega). The proximal 1.5-kb Gasp-1 promoter region was amplified from genomic DNA isolated from wild type mice with the following primer pair: forward, 5’-GCT AGC TTC CAG GGA CAG AA-3’; and reverse, 5’-AAG CTT CCG ACT TTA GGC TGT AC-3’. A 1-kb truncated promoter fragment was also amplified using the following primer pair: forward, 5’-GCT AGC TTC CAG GGA CAG AA-3’; and reverse, 5’-AAG CTT CCG ACT TTA GGC TGT AC-3’. Positive sequence-verified clones were selected and subcloned into the pGL3-basic luciferase vector system for gene reporter studies. In addition, the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used in the construction of a mutated Gasp-1 promoter reporter construct, where the DR-1 site was mutated from AGGGCTTTTACCC to TCCCCATTAAACCC. The sequences of the DR-1 site at —480 to —482 were introduced using the following oligonucleotides: forward, 5’-CTC GAC GCT TTT AAC CCC TTC CA G-3’; and reverse, 5’-CTG GAA GGG GTT AAA GGG gag CTG GAG-3’. The mutated Gasp-1 promoter reporter construct was further verified by sequencing to ensure that the mutation was present prior to experimentation.

Gas1 Promoter Analysis and Cloning—The entire list of mouse known gene promoter sequences (from University of California, Santa Cruz) was extracted from the evolutionary conserved regions database. The available 1.5-kb proximal Gasp-1 promoter sequence was obtained and subjected to in silico analysis for the identification of conserved transcription factor binding sites using the vRista 2.0 online tool. PCR primers were designed with restriction enzymes sites compatible with both the pGEM-T Easy cloning vector (Promega, Madison, WI) and pGL3-Basic luciferase vector (Promega). The proximal 1.5-kb Gasp-1 promoter region was amplified from genomic DNA isolated from wild type mice with the following PCR primers: forward, 5’-GCT AGC TTC CAG GGA CAG AA-3’; and reverse, 5’-AAG CTT CCG ACT TTA GGC TGT AC-3’. A 1-kb truncated promoter fragment was also amplified using the following primer pair: forward, 5’-GCT AGC TTC CAG GGA CAG AA-3’; and reverse, 5’-AAG CTT CCG ACT TTA GGC TGT AC-3’. Positive sequence-verified clones were selected and subcloned into the pGL3-basic luciferase vector system for gene reporter studies. In addition, the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used in the construction of a mutated Gasp-1 promoter reporter construct, where the DR-1 site was mutated from AGGGCTTTTACCC to TCCCCATTAAACCC. The sequences of the DR-1 site at —480 to —482 were introduced using the following oligonucleotides: forward, 5’-CTC GAC GCT TTT AAC CCC TTC CA G-3’; and reverse, 5’-CTG GAA GGG GTT AAA GGG gag CTG GAG-3’. The mutated Gasp-1 promoter reporter construct was further verified by sequencing to ensure that the mutation was present prior to experimentation.
through incubation with 50 μl of protein A-agarose (Invitrogen) for 30 min at 4 °C. The nuclear extracts were then centrifuged at 12,000 rpm for 5 min at 4 °C and incubated overnight with 5 μg of anti-PPARβ/δ, anti-PPARγ, or anti-IgG antibody at 4 °C. Following overnight incubation, 50 μl of protein A-agarose was added for 2 h at 4 °C followed by centrifugation to pellet the immunoprecipitated complexes. Pellets were washed twice with 1 ml of high salt lysis buffer followed by four washes with wash buffer (100 mM Tris, pH 8.0, 500 mM LiCl, 1% Nonidet P-40, and 1% deoxycholate). Pellets were then resuspended in 400 μl of elution buffer (1% SDS and 0.1 mM NaHCO₃) and incubated for 2 h at 67 °C with occasional mixing to reverse formaldehyde cross-linking. Beads were subsequently removed by centrifugation at 12,000 rpm for 10 s, and the supernatant was further incubated at 67 °C overnight. Samples were then centrifuged for 3 min at 10,000 rpm, and phenol/chloroform/isoamyl alcohol (25:24:1) was added to the supernatants, after which the samples were vortexed and centrifuged for 3 min at 14,000 rpm with the aqueous phase collected. DNA was subsequently purified and concentrated using the QIAquick PCR purification kit (Qiagen). The following sets of primers were used for PCR: Gasp-1 forward primer, 5′-CGT GGC TGA TCA CAG ACG TA-3′; Gasp-1 reverse primer, 5′-GTG GGG AAA GGG AAA CAA AC-3′; β-actin forward primer, 5′-CCA GAA TGC AGG CCT AGT AA-3′; and β-actin reverse primer, 5′-CGA GAG AGA AAG CGA GAT TA-3′. The GASP-1 gene promoter was extracted from the transcriptional Regulatory Element Database (TRED) with accession 72793. The antibodies used for CHIP are as follows: rabbit polyclonal anti-PPARβ/δ (sc-7197X, Santa Cruz Biotechnology); mouse monoclonal anti-actin (E-8), Santa Cruz Biotechnology; rabbit polyclonal anti-PPARγ (H-9251, Santa Cruz Biotechnology); mouse monoclonal anti-PPARγ (H-9252, Santa Cruz Biotechnology); goat anti-human IgG antibody at 4 °C. Following overnight incubation, 50 μl of elution buffer was added to the beads, and the beads were washed twice with 1 ml of high salt lysis buffer followed by addition of CM collected from L165041 or control (DMSO) to human myoblast cultures. After 8 h of infection, the medium was replaced and the human myoblasts were allowed to differentiate for a further 72 h, after which total RNA and total protein was harvested for analysis of hGASP-1 expression levels.

**Smad3 Reporter Assay**—The activity of Smad3 was assessed using a Smad3 reporter assay. Briefly, C2C12 myoblasts were transfected with a Smad binding element reporter construct (SBE-4x-Luc (Addgene plasmid 16495), which contains four repetitive Smad3 binding elements linked to the luciferase reporter gene. C2C12 myoblasts were also transfected with the Renilla luciferase vector (pRL-CMV) as an internal control. Cells were transfected 48 h post-electroporation at 110 volts and 500 ohms resistance using the Pulser Mxcell electroporation system (Biorad) and harvested for luciferase assay after which the cells were washed twice with 1 ml of PBS. Cells were then plated for 24 h. SBE-4x-Luc-transfected cells were treated with 2 μg/ml sActRIIB and dialysis buffer as control; rhGASP-1 (1, 2, 5 μg/ml sActRIIB and dialysis buffer as control); human myostatin (Mstn) CM (1:2 or 1:4 dilution) of either control-sActRIIB or dialysis buffer as control; and human myostatin (Mstn) CM (1:2 or 1:4 dilution) from either control-sActRIIB or dialysis buffer as control. Cells were transfected with a Smad3 reporter assay. Briefly, C2C12 myoblasts were transfected with either control shRNA or rhGASP-1 shRNA lentiviral particles, consisting of the pCMV-dR8.2 dvpr, pCMV-VSVG (Addgene plasmid 8454), and the envelope plasmid pCMV-VSVG (Addgene plasmid 8454), were produced. Lentivirus-mediated Knockdown of hGASP-1—Individual shRNA constructs specifically designed to target the human GASP-1 gene (hGASP-1) were purchased from Open Biosystems (RHS4533-NM_175575; Open Biosystems, Huntsville, AL). hGASP-1 shRNA lentiviral particles, consisting of the packaging plasmid pCMV-dR8.2 dvpr (Addgene plasmid 8455) and the envelope plasmid pCMV-VSVG (Addgene plasmid 8454), were produced. Lentiviral Production and Infection—The pCMV-dR8.2 dvpr, hGASP-1 shRNA, or empty plKO.1 and pCMV-VSVG vectors were transfected into 293T cells using the calcium phosphate precipitation technique (Invitrogen). Briefly, 1 million cells/ml were seeded in 6-well plates and, after an overnight attachment period, were transfected with 5 μg of the plasmids in a 2:2:1 ratio (pCMV-dR8.2 dvpr: pCMV-VSVG/hGASP-1 shRNA/plKO.1). After 16 h of transfection, the medium was replaced with fresh proliferation medium, and the cells were incubated for a further 60 h. After 60 h of incubation, the supernatant was collected as a source of viral particles. The viral particles were then tested for infection efficiency by adding 10–100 μl of virus together with 8 μg/ml hexadimethrine brome (Sigma-Aldrich) to human myoblast cultures. After 8 h of infection, the medium was replaced and the human myoblasts were allowed to differentiate for a further 72 h, after which total RNA and total protein was harvested for analysis of hGASP-1 expression levels.

**Statistics**—The data from myoblast proliferation analysis are presented here as means ± S.E. of eight replicates, and an average was taken from three independent experiments. Total myotubes were counted in 12 random images/coverslip, and the mean myotube number ± S.E. of three coverslips/treatment was calculated from three individual experiments. The mononucleated and multinucleated nuclei number was calculated in 20 random images/coverslip, and the mean percentage fusion index ± S.E. from three coverslips/treatment was calculated. Individual myotube area was assessed for all myotubes present in 12 random images taken from three coverslips/treatment. All variations were compared using one-way ANOVA, and values of \( p \leq 0.05 \) were deemed significant.
RESULTS

Activation of PPARβ/δ via L165041 Agonist Treatment Enhances Myogenesis—Treatment of C2C12 myoblasts or murine primary myoblasts with L165041 (10 μM), a subtype-selective, high affinity ligand for PPARβ/δ, resulted in a significant increase in myoblast numbers when compared with control-treated cells (DMSO) (Fig. 1A and supplemental Fig. S1B). The L165041-mediated increase in C2C12 myoblast proliferation was observed as early as 12 h after the addition of L165041 and was maintained up to 96 h (Fig. 1A). Treatment of C2C12 or murine primary myoblasts with L165041 during differentiation also resulted in an observable increase in myotube formation (Fig. 1B and supplemental Fig. S1C), with an ~55 and 52% increase in the myotube number detected at 48 and 72 h, respectively, following the addition of L165041, as compared with control-treated cells (Fig. 1C). Although we observed an increased myotube number, we found no appreciable change in either the myotube fusion index or the myotube area between cells treated with L165041 and control-treated C2C12 myoblasts (Fig. 1, D and E). However, we did observe an overall increase in the percentage of myotubes, with the average myotube area at 10,000–250,000 μm² during differentiation (Fig. 1E). Next we analyzed the expression of critical myogenic regulatory factors involved in the normal progression of myogenic differentiation. Subse-

FIGURE 1. PPARβ/δ regulates myoblast proliferation and differentiation. A, proliferation analysis of C2C12 myoblast cultures grown under proliferating conditions in the absence (Control (DMSO)) or presence of the PPARβ/δ agonist L165041 (10 μM) for 96 h as monitored by a methylene blue assay. B, representative images of H&E-stained differentiating myoblasts across a differentiation time course (24–96 h) in L165041 and control-treated myoblasts. Scale bars, 100 μm. C, quantification of myotube number from 12 random images/cover slip (n = 3) from three independent experiments. D, quantification of fusion index in C2C12 myotubes cultures at 48 and 72 h of differentiation in the absence (Control) or presence of L165041. The graph shows the mean percentage fusion index ± S.E. over three coverslips/treatment. E, frequency distribution of myotube area (μm²) at 96 h of differentiation in L165041 and control-treated myotubes as calculated from 12 random images/cover slip (n = 3). F and G, qPCR analysis of MyoD (F) and myogenin (G) mRNA expression during differentiation (0–96 h) in L165041 and control-treated C2C12 myoblasts. The graphs represent -fold change normalized to GAPDH. Data are mean ± S.E. (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001.
sequent qPCR and Western blot analysis of differentiating C2C12 cells revealed increased mRNA expression (Fig. 1, F and G) and protein levels (supplemental Fig. S1A) of both MyoD and myogenin in L165041-treated cells. Furthermore, MyHC protein levels were elevated significantly in differentiating C2C12 myoblasts at 48 and 72 h of differentiation following treatment with L165041 (supplemental Fig. S1A). These data are consistent with the enhanced differentiation and increased myotube number observed following treatment with L165041.

**Primary Myoblasts Derived from PPARβ/δ-null Mice Have Reduced Proliferation and Defective Myogenic Differentiation**—Because L165041-mediated activation of PPARβ/δ resulted in enhanced proliferation and differentiation of C2C12 and primary myoblast cultures *in vitro*, we next studied the myogenic potential of primary myoblast cultures derived from *PPARβ/δ*-null mice. Consistent with the results obtained following treatment with L165041, the absence of PPARβ/δ resulted in reduced myoblast proliferation (Fig. 2A) as well as reduced myogenic differentiation (Fig. 2, B and C). Specifically, loss of PPARβ/δ resulted in the formation of fewer myotubes, with a visible reduction in myotube size and branching, when compared with wild type controls (Fig. 2D). Subsequent quantification revealed a decreased myotube area, with a ~55% decrease in the number of large myotubes (4,000–22,000 μm²) in cultures derived from *PPARβ/δ*-null mice when compared with wild type controls (Fig. 2D). Furthermore, we found a
reduced myotube fusion index during early differentiation (48 h) in the primary cultures derived from PPARβ/δ-null mice when compared with wild type controls (Fig. 2E), suggesting that the reduced myotube number observed in the absence of PPARβ/δ may result from impaired myoblast fusion during early myogenic differentiation.

Identification of Novel Downstream Targets of PPARβ/δ in Skeletal Muscle—Next we sought to determine the molecular mechanism(s) through which L165041-mediated activation of PPARβ/δ enhances skeletal muscle myogenesis. To this end we performed microarray analysis on RNA collected from L165041-treated and control-treated (DMSO) C2C12 myotubes across a differentiation time course. The results of the microarray are summarized in a heat map (Fig. 3A, left panel); genes that were significantly (p < 0.05) up-regulated (Table 1) or down-regulated (Table 2) by more than 1.5-fold were selected. Importantly, genes that had been identified previously as targets of PPARβ/δ in muscle, such as Abca1, Abcg1, Angpt1, Adfp, Pdk4, Ucp3, Cpt1b, and Ppargc1a (42–45), were similarly up-regulated following the addition of L165041 (Table 1). From the list of significantly up-regulated genes, we selected 24 genes (supplemental Table S1) to validate using qPCR, the results of which are summarized in a heat map (Fig. 3A, right panel). Microarray analysis and subsequent confirmation through qPCR revealed the *Gasp-1* gene as a novel PPARβ/δ target in muscle (Fig. 3A, right panel). *Gasp-1* is a secreted protein, which has been shown to interact directly with mature and Latency Associated Peptide for mediating myostatin signaling, resulting in inhibition of myostatin signaling. Importantly, loss of myostatin function, much like what we observed (Fig. 3A), results in an increase in *Gasp-1* expression in C2C12 myoblasts during early myogenic differentiation.

**PPARβ/δ Regulates *Gasp-1* Expression**—We further confirm PPARβ/δ regulation of *Gasp-1* expression by treating C2C12 myoblasts and differentiating myotubes with L165041 and monitored *Gasp-1* expression. Subsequent qPCR results revealed a significant increase in *Gasp-1* expression in C2C12 myoblasts following 8-, 12-, and 24-h treatment with L165041 (Fig. 3B). Similarly, an 8- and ~12-fold induction of *Gasp-1* expression was observed following L165041 treatment at 72 and 96 h of differentiation, respectively (Fig. 3C). Elevated *Gasp-1* protein levels were detected at all differentiating time points (24–96 h) following treatment with L165041 (Fig. 3E). As *Gasp-1* is a secreted protein, we next addressed whether PPARβ/δ activation increases *Gasp-1* protein secretion. Human myoblasts were treated with L165041 for a period of 24 h, after which CM was collected and subjected to Western blot analysis. Consistent with increased *Gasp-1* expression, we found that L165041-mediated PPARβ/δ activation resulted in enhanced hGASP-1 protein secretion in vitro (Fig. 3D). It is noteworthy to mention that although L165041 treatment resulted in an increase in *Gasp-1* expression, the addition of either PPARγ agonist GW1929 or PPARα agonist Wy14643 failed to significantly alter *Gasp-1* expression in both myoblasts and myotubes when compared with control-treated cultures (supplemental Fig. S2, A and C), suggesting that PPARβ/δ, but not PPARγ or PPARα, induces *Gasp-1* mRNA expression in skeletal muscle. However, in the same samples we do see a significant increase in the expression of the PPARγ target gene *adiponectin* and the PPARα target gene *FABP3* (supplemental Fig. S2, B and D), suggesting that PPARγ and PPARα are activated in our system in response to treatment with GW1929 and Wy14643, respectively. Further evidence for PPARβ/δ regulation of *Gasp-1* is observed in PPARβ/δ-null mice. Significantly reduced *Gasp-1* expression was detected in skeletal muscle tissues isolated from PPARβ/δ-null mice (Fig. 4A), and moreover,
significantly reduced Gasp-1 expression was observed in both slow twitch muscles (soleus) and fast twitch muscles (extensor digitorum longus (EDL)) (Fig. 4A). Furthermore, a significant reduction in Gasp-1 expression was also observed in differentiating primary myoblast cultures derived from PPARβ/δ-null mice (Fig. 4B). Microarray and subsequent expression analysis collectively confirm that PPARβ/δ positively regulates Gasp-1 gene expression; as such, we suggest that Gasp-1 represents a novel muscle-specific downstream target of PPARβ/δ. Previously published work has revealed that PPARβ/δ expression is greater in atrophying muscle tissue isolated from mdx mice, a mouse model of Duchenne muscular dystrophy (23). Based on this observation, we next wanted to ascertain whether increased endogenous PPARβ/δ expression, as seen in mdx mice, would induce Gasp-1 expression in vivo. In agreement with the L165041 agonist studies described herein above, we observed an increase in both PPARβ/δ and Gasp-1 expression in EDL muscle isolated from mdx mice (Fig. 4, C and D).

A Consensus PPAR Binding Motif (DR-1) Mediates Up-regulation of the Gasp-1 Promoter in Response to PPARβ/δ Agonist Treatment—To further investigate the mechanism of Gasp-1 transactivation by PPARβ/δ, we performed in silico analysis of the 1.5-kb upstream sequence of the Gasp-1 gene promoter. Subsequent sequence analysis identified a putative PPRE, specifically a DR-1 motif (Fig. 4E) within the proximal 1.5-kb region of the Gasp-1 promoter, which has high sequence homology between mouse and human (Fig. 4F). Importantly, the DR-1 sequence we identified in the Gasp-1 promoter is consistent with a consensus DR-1 sequence that has been predicted previously to be specific for PPARβ/δ (47). In studying the role of the DR-1 motif in PPARβ/δ-mediated activation of Gasp-1, C2C12 myoblasts were transfected with either a prox-
TABLE 2
List of genes down-regulated in the microarray data with L165041 agonist treatment when compared with control-treated cells at all time points

| GenBankTM accession No. | Gene symbol | Description | Fold change of genes listed (p<0.05) |
|-------------------------|-------------|-------------|-------------------------------------|
| XM_097304               | Abch7       | ATP-binding cassette, sub-family B (MDR/TAP) | 1.22 3.11 1.26 1.26 1.31 2.87 3.22 |
| NM_029277               | Arhgap12    | Rho GTPase-activating protein 12              | 1.05 2.22 1.10 1.15 1.11 2.31 2.24 |
| NM_175335               | Arhgap20    | Rho GTPase-activating protein 20              | 1.70 1.25 1.35 2.21 2.27 2.29 4.40 |
| NM_029466               | Arl5b       | ADP-ribosylation factor-like 5B               | 1.25 2.21 1.15 1.12 1.39 2.17 2.30 |
| NM_080708               | Bmp2k       | BMP2 inducible kinase                         | 1.53 1.33 1.24 1.48 2.44 2.44 2.13 |
| NM_178939               | Car12       | Carbonic anhydrase 12                         | 2.32 1.84 3.08 4.36 7.15 2.62 1.93 |
| NM_030558               | Car15       | Carbonic anhydrase 15                         | 5.90 4.78 12.16 13.82 6.90 5.07 1.62 |
| NM_139305               | Car9        | Carbonic anhydrase 9                          | 3.09 1.37 2.86 4.94 3.91 5.62 2.58 |
| NM_007699               | Casp4       | Caspase 4, apoptosis-related cysteine peptidase | 1.30 2.05 2.35 3.72 3.95 2.38 1.75 |
| NM_007679               | Cebpδ       | CCAAT/enhancer-binding protein (C/EBP), δ     | 1.35 2.12 3.29 4.07 4.38 8.11 7.36 |
| NM_009890               | Ck25b       | Cholesterol 25-hydroxylase                    | 1.29 1.23 2.74 5.35 4.46 3.03 1.87 |
| NM_010828               | CITED2      | CITED2 protein 500, family 26, subfamily a, polyomavirus peptidase | 1.23 1.77 3.27 3.04 3.66 6.16 4.55 |
| NM_198415               | Cnk1t2      | Creatine kinase, mitochondrial 2              | 2.79 1.21 1.67 4.07 3.01 4.26 1.57 |
| NM_007811               | Cyp26a1     | Cytochrome P450, family 26, subfamily a, polypeptide 1 | 1.00 1.17 1.50 2.22 3.06 2.05 1.66 |
| NM_020010               | Cyp54       | Cytochrome P450, family 51                    | 1.07 1.10 2.16 2.77 2.76 4.43 1.85 |
| NM_025869               | Dusp26      | Dual specificity phosphatase 26               | 1.97 1.15 1.75 3.66 3.46 13.01 5.32 |
| NM_026268               | Dusp6       | Dual specificity phosphatase 6                | 1.05 1.01 1.74 2.16 2.39 3.69 4.81 |
| NM_028748               | Dusp8       | Dual specificity phosphatase 8                | 1.05 2.14 2.00 2.41 4.48 6.67 3.95 |
| NM_177076               | Fbx13       | F-box and leucine-rich repeat protein 13      | 1.04 1.02 1.52 1.82 2.08 10.41 14.66 |
| NM_027968               | Fbxo30      | F-box protein 30                              | 1.26 2.09 1.70 1.21 1.46 2.70 2.56 |
| NM_080428               | Fbxw7       | F-box and WD-40 domain protein 7              | 1.23 1.28 1.87 2.04 2.97 2.29 |
| NM_008046               | Fst         | Follistatin (Fat)                             | 1.15 1.15 2.08 2.44 2.74 3.05 |
| NM_008073               | Gabrg2      | γ-Aminobutyric acid (GABA-A) receptor, subunit γ2 | 1.65 2.08 1.89 3.14 1.97 |
| TC1651824               | Igf1        | Insulin-like growth factor 1 (Igf1), transcript variant 1 | 1.85 2.76 8.51 10.64 |
| NM_010518               | Igf8p5      | Insulin-like growth factor-binding protein 5   | 1.31 1.29 2.79 2.56 2.43 |
| XM_620516               | Mex3b       | PREDICTED: ring finger and KH domain-containing 3 | 2.11 5.17 5.43 |
| NM_030612               | Nfkbi       | Nuclear factor of κ light polypeptide enhancer in B-cells  | 1.01 1.15 1.23 1.38 2.51 2.82 5.66 5.12 |
| NM_025436               | Sc4mol      | Sterol-C4-methyl oxidase-like (Sc4mol), mRNA  | 1.05 1.06 1.24 1.33 1.60 2.10 3.91 4.06 |
| NM_023214               | Slc30a7     | Solute carrier family 2                       | 1.00 1.17 2.26 2.26 2.49 1.87 |
| NM_027052               | Slc38a4     | Solute carrier family 1                       | 1.00 1.17 2.26 2.26 2.49 1.87 |
| NM_016917               | Slc40a1     | Solute carrier family 14                      | 1.00 1.17 1.24 1.33 1.62 2.19 2.40 |
| NM_028746               | Slc7a13     | Solute carrier family 7                       | 1.00 1.17 2.16 2.06 3.06 4.15 2.19 |
| NM_032118               | Srebf2      | SREBP-2, mRNA                                  | 6.24 3.94 3.31 10.21 7.99 2.59 1.99 |
| NM_145375               | Tmem5f1     | Transmembrane protein 5 (Tmem5f1)             | 1.39 1.38 1.24 1.60 2.10 3.91 4.06 |
| NM_138655               | Tmem21      | Transmembrane protein 21 (Tmem21)             | 3.42 7.46 7.55 4.20 1.76 35.75 30.66 |
| NM_001025606            | Tmem171     | Transmembrane protein 17 (Tmem171)            | 1.10 1.10 2.16 2.76 3.06 4.15 2.19 |
| NM_133758               | Usp47       | Ubiquitin-specific peptidase 47 (Usp47), mRNA | 1.13 1.13 1.24 1.41 1.33 2.60 2.53 2.42 |
| NM_133857               | Usp53       | Ubiquitin-specific peptidase 53 (Usp53), mRNA | 1.21 1.24 1.34 1.47 2.14 2.14 3.54 7.05 |
| NM_172271               | Slc6a17     | Solute carrier family 6 (neurotransmitter transporter), member 17 | 1.06 1.61 1.31 3.44 1.64 5.63 3.37 |
| NM_144852               | Slc7a4      | Solute carrier family 7                       | 1.30 2.73 1.05 1.39 1.56 1.30 5.29 |
| NM_023719               | Ttnip       | Thio-redoxin-interacting protein (Ttnip)      | 1.28 2.20 2.04 3.55 2.27 20.35 1.72 |
| NM_009464               | Ulp3        | Uncoupling protein 3 (mitochondrial, proton carriers) | 2.23 1.66 5.39 1.09 7.18 5.75 25.36 |

We further assessed whether PPARβ/δ binds to the DR-1 site in the Gasp-1 promoter construct following L165041 treatment. After treatment cells were collected and subjected to chromatin immunoprecipitation, after which DNA was isolated and purified.
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FIGURE 4. Ligand-mediated activation of PPARβ/δ induces Gasp-1 promoter-reporter luciferase activity. A, qPCR analysis of Gasp-1 mRNA expression in EDL, gastrocnemius (GAS), soleus, and quadriceps (Quad) muscles from PPARβ/δ-null and wild type mice. Data are mean ± S.E. (n = 3). The graphs represent -fold change normalized to GAPDH expression. B, qPCR analysis of Gasp-1 mRNA expression in differentiating primary myoblasts isolated from 10-week-old PPARβ/δ-null and wild type mice. The graph represents -fold change normalized to GAPDH expression. Data are mean ± S.E. (n = 3). C and D, qPCR analysis of PPARβ/δ (C) and Gasp-1 (D) mRNA expression in EDL muscle isolated from 10-week-old mdx and wild type mice. Data are mean ± S.E. (n = 3). The graphs represent -fold change normalized to GAPDH expression. E, localization of the consensus DR-1 site present within the 1.5-kb proximal Gasp-1 promoter region. F, homology between the mouse and human DR-1 motif present within the 1.5-kb proximal Gasp-1 promoter region. G, top, schematic representation of reporter constructs used for luciferase analysis. Bottom, assessment of promoter-luciferase reporter activity in C2C12 myoblasts transfected with the empty vector control (pGL3-basic), the 1.5-kb proximal Gasp-1 luciferase construct (pGL3-Gasp-1), or the 1-kb DR-1 deletion construct (pGL3-Gasp-1 del) following 24-h treatment with DMSO (Control), L165041 (10 μM), or Wy14643 (10 μM). H, top, sequences highlighting the DR-1 site (bold) in both wild type (wt-Gasp-1) and mutated Gasp-1 promoters (mut-Gasp-1). Arrows indicate the mutated base pairs. Bottom, assessment of promoter-luciferase reporter activity in C2C12 myoblasts transfected with empty vector control (pGL3-basic), pGL3-Gasp-1, or mut-pGL3-Gasp-1, following treatment for 24 h with DMSO (Control), L165041 (10 μM), GW1929 (30 μM), or Wy14643 (10 μM). Promoter-reporter luciferase activity was normalized to Renilla luciferase and expressed as -fold change relative to the empty vector control (pGL3-basic). Each bar represents the mean values ± S.E. from four independent experiments. I, agarose gel image revealing the interaction of PPARβ/δ with the DR-1 site of the Gasp-1 promoter in the absence (-) or presence (+) of L165041 as assessed through ChIP (upper panel). Analysis of PPARβ/δ interaction with the β-actin promoter in the absence (-) or presence (+) of L165041 was also performed as a negative control (lower panel). The relative amounts of both the Gasp-1 and β-actin promoters in the input were also assessed and are indicated. Both isotype-specific IgG and no antibody (No Ab) controls are shown. *, p < 0.05; **, p < 0.01.
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fied. As seen in Fig. 4I, we observed binding of PPARβ/δ to the DR-1 site specific to the Gasp-1 promoter, which was further enhanced upon treatment with L165041 (Fig. 4J). Importantly, no binding of PPARβ/δ to the control β-actin promoter was observed (Fig. 4J). In contrast to the above results, we observed no interaction between PPARγ or PPARα and the DR-1 site found in the Gasp-1 promoter (supplemental Fig. S2, F and G, respectively). Similar to PPARβ/δ, no binding of PPARγ or PPARα to the control β-actin promoter was observed (supplemental Fig. S2, F and G, respectively). These data further confirm that PPARβ/δ, but not PPARγ or PPARα, specifically binds to the DR-1 site located in the Gasp-1 promoter region.

Activation of PPARβ/δ Enhances Myogenesis through Modulating Myostatin Activity—Myostatin is a secreted growth factor that acts as potent negative regulator of skeletal muscle growth through targeting and inhibiting both myoblast proliferation and differentiation (29, 30). As mentioned earlier, Gasp-1 is a secreted protein that has been demonstrated previously to bind and inhibit myostatin activity (27, 28). Therefore, if PPARβ/δ-mediated induction of Gasp-1 is associated with inactivation of myostatin, we reasoned that treatment with L165041 would increase the levels of secreted Gasp-1, inhibit myostatin activity, and increase myoblast proliferation. In agreement, we found elevated levels of Gasp-1 in CM isolated from L165041-treated cells (Fig. 5A) as well as enhanced interaction between Gasp-1 and myostatin following treatment with L165041, as measured through co-immunoprecipitation analysis (Fig. 5A). Importantly, treatment of myoblasts with CM isolated from wild type and PPARβ/δ-null mice. As seen in Fig. 5E, the addition of CM from L165041-treated cells resulted in a significant 24% reduction in SBE-4x-Luc activity. However, the addition of CM from PPARβ/δ-null primary myoblast cultures significantly increased SBE-4x-Luc activity by 35%. As a positive control we also treated SBE-4x-Luc-transfected myoblasts with sActRIIB myostatin antagonist, and as expected we observed a significant (42%) reduction in SBE-4x-Luc activity upon addition of sActRIIB, consistent with inhibition of myostatin function (Fig. 5F). A recent study has reported that fenofibrate-mediated activation of PPARα results in decreased Mstn mRNA expression (49). Therefore, we next analyzed the consequence of PPARβ/δ activation or deletion on the expression of Mstn. However, unlike the decreased Mstn expression observed following activation of PPARα, we found no significant change in Mstn mRNA expression upon L165041 treatment (Fig. 5G). Similarly, myostatin protein levels remained unchanged in gastrocnemius muscle tissue collected from PPARβ/δ-null mice (Fig. 5H).

Exogenous rhGASP-1 Promotes Myoblast Proliferation and Enhances Myotube Formation—Given that PPARβ/δ-mediated induction of Gasp-1 is associated with enhanced myogenesis and inhibition of myostatin activity, we next assessed whether rhGASP-1 to human myoblast cultures influences myoblast proliferation and/or differentiation. As expected, rhGASP-1 resulted in a dose-dependent increase in myoblast proliferation, with a significant 19.2% increase in the number of large myotubes observed following treatment with 1 μg/ml rhGASP-1 protein when compared with control-treated cells (0.01% BSA). Treatment with rhGASP-1 resulted in enhanced myogenic differentiation (Fig. 6A), with a 29.7% increase in myotube number observed upon treatment of SBE-4x-Luc-transfected C2C12 myoblasts when compared with the control following 72 h of differentiation (Fig. 6C). In addition, we also found an increase in the myotube area, consistent with myotube hypertrophy (Fig. 6D), following the addition of rhGASP-1. In fact, a 19.2% increase in the number of large (3,000–33,000 μm²) myotubes was detected following treatment with rhGASP-1 (Fig. 6D). Clearly, these data suggest that the addition of exogenous rhGASP-1 promotes myogenesis and induces myotube hypertrophy. To ascertain, whether rhGASP-1 protein treatment results in reduced myostatin activity, we next assessed SBE-4x-Luc reporter activity in the presence of rhGASP-1 protein. As expected, the addition of Mstn protein to SBE-4x-Luc-transfected C2C12 myoblasts resulted in a maximal ~5.4-fold increase in SBE-4x-Luc reporter activity (Fig. 6E), which is consistent with enhanced myostatin activity. However, in contrast, treatment of SBE-4x-Luc-transfected C2C12 myoblasts with increasing concentrations of rhGASP-1 protein (0.5, 1, and 2 μg/ml) resulted in a dose-dependent decrease in SBE-4x-Luc reporter activity, with 2 μg/ml treatment resulting in a ~7-fold decrease in SBE-4x-Luc reporter activity when compared with the untreated control (Fig. 6E). Taken together, these data indicate that the addition of exogenous rhGASP-1 protein can significantly interfere with myostatin signaling, which is consistent with the increased myoblast proliferation and differentiation observed in response to rhGASP-1 treatment.
shRNA-mediated Knockdown of hGASP-1 Negatively Regulates Myogenesis—To further confirm the role of hGASP-1 in myogenesis, we next generated cell lines stably overexpressing a lentivirus-based shRNA designed to specifically target and repress hGASP-1 expression. Subsequent analysis revealed a significant reduction in hGASP-1 expression both at the mRNA (~80%) and protein (~75%) level (Fig. 7A and supplemental Fig. S2E). Next we assessed myoblast proliferation and differ-
entiation in the hGASP-1 knockdown cells. As seen in Fig. 7B, lack of hGASP-1 resulted in reduced myoblast proliferation with a significant 28.1 and 24.6% reduction in myoblast number detected at 72 and 96 h of proliferation, respectively (Fig. 7B). Furthermore, knockdown of hGASP-1 resulted in an observable reduction in myoblast formation (Fig. 7C) with a significant 57.3% reduction in myoblast number at 96 h of differentiation when compared with control shRNA-transfected cells (Fig. 7D). Taken together, these data suggest that hGASP-1 plays an important role during the normal progression of myogenesis, specifically through regulation of both myoblast proliferation and differentiation. To analyze the effect of GASP-1 knockdown on myostatin activity, we analyzed SBE-4x-Luc reporter activity in hGASP-1 shRNA-transfected C212 myoblasts. In agreement with the above results, we observed a ~4.4-fold increase in SBE-4x-Luc reporter activity in response to Mstn protein treatment (Fig. 7E). Furthermore, and consistent with enhanced myostatin activity, we detected a ~2.8-fold induction in SBE-4x-Luc reporter activity in hGASP-1 shRNA-transfected myoblasts. These data further confirm that GASP-1 is a potent inhibitor of myostatin activity.

**DISCUSSION**

Pharmacological activation of the muscle-specific PPARβ/δ isoform promotes muscle development, myonuclear accretion, et al. D. Knockdown of the hGASP-1 gene leads to inhibition of myoblast proliferation and differentiation. A, qPCR analysis of hGASP-1 expression in human myoblasts infected with lentivirus containing either control shRNA or shRNA designed to specifically target and repress hGASP-1. The corresponding graph represents -fold change of hGASP-1 expression normalized to GAPDH. Data are mean ± S.E. (n = 3). B, proliferation analysis of human myoblasts infected with lentivirus containing either control shRNA or hGASP-1 shRNA for 48 h as monitored by methylene blue assay. C, representative images of H&E-stained 72-h differentiated human myotube cultures infected with lentivirus containing either control shRNA or hGASP-1 shRNA. Scale bars, 100 μm. D, quantification of myotube number in 72-h differentiated human myoblasts infected with lentivirus containing either control shRNA or hGASP-1 protein. E, assessment of SBE-4x-Luc reporter activity in C212 myoblasts grown for 24 h in the absence (−) or presence (+) of 1:4 (−/+) or 1:2 (+/+) diluted CM from Mstn protein-secreting CHO cells. SBE-4x-Luc reporter activity was also assessed following treatment without (−) or with increasing concentrations (0.5, 1, and 2 μg/ml) of rhGASP-1. The corresponding graph represents the -fold change in luciferase activity normalized to Renilla luciferase. Each bar represents the mean ± S.E. of triplicate samples from two independent experiments. *, p < 0.05; **, p < 0.01.

FIGURE 7. Knockdown of the hGASP-1 gene leads to inhibition of myoblast proliferation and differentiation. A, qPCR analysis of hGASP-1 expression in human myoblasts infected with lentivirus containing either control shRNA or hGASP-1 shRNA for 48 h as monitored by methylene blue assay. C, representative images of H&E-stained 72-h differentiated human myotube cultures infected with lentivirus containing either control shRNA or hGASP-1 shRNA. Scale bars, 100 μm. D, quantification of myotube number in 72-h differentiated human myoblasts infected with lentivirus containing either control shRNA or hGASP-1 shRNA. E, assessment of SBE-4x-Luc reporter activity in myoblasts transfected with (−) or with (+) hGASP-1 shRNA grown for 24 h in the absence (−) or presence (+) of 1:2 diluted CM from Mstn protein-secreting CHO cells. The corresponding graph represents the -fold change in luciferase activity normalized to Renilla luciferase. Each bar represents the mean ± S.E. of triplicate samples from two independent experiments. *, p < 0.05; **, p < 0.01.
and satellite cell proliferation and restores sarcolemmal integrity in dystrophic mouse models (17, 19, 23, 50, 51), strongly supporting a role for PPARβ/δ in regulating postnatal muscle growth and development. However, no study has yet clearly revealed the molecular mechanism(s) through which PPARβ/δ regulates skeletal muscle growth. Using a selective PPARβ/δ ligand (L165041) and the PPARβ/δ-null mouse model, we show here for the first time that PPARβ/δ positively regulates postnatal myogenesis through a mechanism involving transcriptional activation of Gasp-1 and reduced activity of the Gasp-1 downstream target myostatin.

Microarray analysis, with subsequent verification by qPCR, revealed a pattern of gene expression changes similar to that observed previously upon ligand-mediated activation of PPARβ/δ (44). Specifically, the addition of L165041 resulted in increased expression of genes involved in lipid transport and storage (Abca1, Abcg1, and Adip), glucose and fatty acid oxidation (Pdk4 and Cpt1b), energy uncoupling, mitochondrial biogenesis (Ucp3 and Ppargc1a), and angiogenesis (Angplta4). One of the novel and significantly up-regulated genes identified following L165041 treatment was Gasp-1 (WFIKK2), which is a secreted protein that has been reported previously to function as a specific antagonist of myostatin. Subsequent qPCR and Western blot analysis confirmed up-regulation of Gasp-1 expression in both myoblast and myotube cultures following activation of PPARβ/δ. In addition, the activation of PPARβ/δ also resulted in enhanced levels of secreted and thus active Gasp-1 protein into conditioned medium. Importantly, the up-regulation of Gasp-1 was specific for PPARβ/δ, as activation of PPARα or PPARγ did not alter Gasp-1 levels in both myoblast and myotube cultures (Supplemental Fig. S2, A and C). Furthermore, regulation of Gasp-1 was observed in positively regulating postnatal skeletal muscle growth. These data, together with previously published reports (19, 23, 51), strongly support a role for PPARβ/δ in positively regulating postnatal skeletal muscle growth. However, in contrast to the results presented here, previously published data from Dressel et al. (44) describe that GW501516-mediated activation of PPARβ/δ does not affect myogenic differentiation of C2C12 myoblasts. However, it is noteworthy to mention that Dressel et al. (44) treated C2C12 myoblasts with GW501516 only after 96 h of differentiation, whereas here we activated PPARβ/δ with L165041 treatment immediately upon initiation of differentiation. Therefore, we propose that timely activation of PPARβ/δ during the early initiation stages of myogenic differentiation, rather than after terminal differentiation, may be required to promote enhanced differentiation. Moreover, Dressel et al. (44) neither assessed myoblast proliferation nor studied myogenesis using the PPARβ/δ-null mouse model we have described here. In agreement with the results described above, we observed significantly reduced Gasp-1 mRNA expression in differentiating primary myoblast cultures as well as fast and slow muscle tissues isolated from PPARβ/δ-null mice. Taken together these data confirm Gasp-1 as a downstream target of PPARβ/δ, further supporting the conclusion that PPARβ/δ regulates Gasp-1 expression at the transcriptional level during postnatal muscle growth.

Previously published studies have revealed that Gasp-1 family proteins are able to bind to and block the function of myostatin (27, 28). Similarly, in the current report we have presented several lines of evidence that support Gasp-1 regulation of myostatin in response to PPARβ/δ activation. In addition to increased Gasp-1 secretion (as mentioned above), immunoprecipitation studies revealed that there is more interaction between Gasp-1 and myostatin, despite there being no change in Mstn mRNA expression, upon L165041-mediated activation of PPARβ/δ. Furthermore, and consistent with reduced myostatin activity, the addition of CM from L165041-treated cells resulted in enhanced myoblast proliferation, which was reversed upon the addition of exogenous anti-hGASP-1 antibody. We also observed decreased SBE-4x-Luc reporter activity in response to treatment with L165041 CM, similar to that observed following sActRIIB-mediated blockade of myostatin. Moreover, we observed impaired myoblast proliferation and increased SBE-4x-Luc reporter activity following treatment with PPARβ/δ-null myoblast CM, which is consistent with both the reduced Gasp-1 expression observed in PPARβ/δ-null mice and enhanced myostatin activity. We further suggest that enhanced myostatin activity in PPARβ/δ-null mice is not due to alterations in myostatin expression, as we observed no change in Mstn mRNA between wild type and PPARβ/δ-null mice. Taken together these data support the notion that PPARβ/δ is able to post-transcriptionally regulate myostatin activity via a mechanism involving Gasp-1. As agreement with PPARβ/δ blockade of myostatin function, we observed enhanced myoblast proliferation, increased number/size, and a dose-dependent increase in luciferase reporter activity in response to treatment with active Gasp-1 protein. Furthermore, shRNA-mediated knockdown of Gasp-1 resulted in reduced myoblast proliferation and differentiation, and increased myostatin activity. However, we have described for the first time that PPARβ/δ is able to regulate both myoblast proliferation and differentiation, which we suggest is through modulation of myostatin activity. These data, together with previously published reports (19, 23, 51), strongly support a role for PPARβ/δ in positively regulating postnatal skeletal muscle growth. However, in contrast to the results presented here, previously published data from Dressel et al. (44) describe that GW501516-mediated activation of PPARβ/δ does not affect myogenic differentiation of C2C12 myoblasts. However, it is noteworthy to mention that Dressel et al. (44) treated C2C12 myoblasts with GW501516 only after 96 h of differentiation, whereas here we activated PPARβ/δ with L165041 treatment immediately upon initiation of differentiation. Therefore, we propose that timely activation of PPARβ/δ during the early initiation stages of myogenic differentiation, rather than after terminal differentiation, may be required to promote enhanced differentiation. Moreover, Dressel et al. (44) neither assessed myoblast proliferation nor studied myogenesis using the PPARβ/δ-null mouse model we have described here. In agreement with the results presented in the current report, a recent study by Angione et al. (51) reports that treatment of primary myoblasts with GW501516 stimulates myoblast proliferation as assessed through measuring the proliferating cell marker Ki67. Furthermore, a study by Han et al. (52) implicates ligand-mediated activation of PPARβ/δ in skeletal muscle regeneration. Specifically, treatment of C2C12 myoblasts with CM collected from GW501516-treated endothelial progenitor cells resulted in increased myoblast proliferation as well as enhanced C2C12 myoblast survival during serum starvation (52). In addition, systemic administration of GW501516 to a mouse hind limb.
ischemia model resulted in increased regenerating muscle fibers, with characteristic centrally formed nuclei (52). It is interesting to surmise that the increased proliferation observed following GW501516 treatment might also be due to the regulation of circulating growth factors such as myostatin; however, further work will need to be performed to confirm this. Taken together these data further confirm a role for PPARβ/δ in postnatal skeletal muscle growth and repair. It is important to mention that treatment of C2C12 myoblasts with L165041 resulted in increased myoblast and myotube number without effecting the myotube fusion index or size. However in contrast, the absence of PPARβ/δ resulted in reduced myoblast proliferation and differentiation together with impaired myotube fusion and reduced myotube size. Thus, we propose that the L165041-mediated increase in myotube formation may be due to the enhanced myoblast number observed as opposed to enhanced myoblast fusion. However the reduced fusion index and myotube size observed in PPARβ/δ-null mice is consistent with the increased myostatin activity in these mice, and in fact, enhanced myostatin signaling has been shown to promote myotubular atrophy and cachexia-like muscle wasting in vitro and in vivo (41, 53, 54).

We propose that upon stimulation with either exogenous (L165041) or endogenous PPARβ/δ ligands (present following exercise or during muscle wasting), PPARβ/δ becomes activated (Fig. 8). Once activated PPARβ/δ regulates target gene expression, including Gasp-1, via interaction with the functional PPRE (DR-1) located within the Gasp-1 proximal promoter region. Whether or not PPARβ/δ regulates Gasp-1 promoter activity in an RXR-dependent or -independent manner remains unclear, and as such, further work will need to be done in verification. Nonetheless, up-regulation of Gasp-1 gene expression results in enhanced secretion of Gasp-1 protein, which is then able to bind to, and regulate, the activity of Myostat...
1-interacting proteins such as myostatin. Subsequent Gasp-1 interaction with myostatin blocks myostatin downstream signaling, resulting in increased postnatal muscle growth and development (Fig. 8). In conclusion, these data suggest that PPARβ/δ agonists, such as L165041, may not only have therapeutic potential in muscle metabolism but may also be a novel class of therapeutics that have utility in regulating muscle growth and repair.

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Supplemental data

Supplementary Figure legends

Fig. S1

**PPARβ/δ activation regulates primary myoblast proliferation and differentiation.** (A) Western Blot analysis of MyoD, myogenin (*Upper panel*) and MyHC (*Lower panel*) protein expression in C2C12 myoblasts at 24h, 48h, 72h and 96h of differentiation following treatment with (+) or without (-) L165041 (10µM). Corresponding graphs represent optical density values for MyoD (*left*), myogenin (*middle*) and MyHC (*Right*). α-tubulin expression was analyzed to ensure equal loading of samples. (B) Assessment of Wild type primary myoblast proliferation, at regular 24h intervals, following treatment with DMSO (Control) or with L165041 for 72h, as monitored by methylene blue assay. (C) Representative images of H&E stained 24h and 48h differentiated Wild type primary myotube cultures following treatment with DMSO (Control) or with L16504. Scale bars, 100μm. \( p<0.05 \) (*) and \( p<0.01 \) (**).

Fig. S2

**PPARβ/δ, but not PPARγ or PPARα, induces Gasp-1 expression.** (A) qPCR analysis of *Gasp-1* mRNA expression in myoblasts (*left*) and myotubes (*right*) treated with DMSO (Control) or with the PPARγ agonist GW1929 (30µM) for 24h. Corresponding graph represents fold change of *Gasp-1* expression normalized to *GAPDH*. Data are mean ± S.E.M (n=3). (B) qPCR analysis of *Adiponectin* mRNA expression in myoblasts (*left*) and myotubes (*right*) treated with DMSO (Control) or with the PPARγ agonist GW1929 (30µM) for 24h. Corresponding graph represents fold change of *Adiponectin* expression normalized to *GAPDH*. Data are mean ± S.E.M (n=3). (C) qPCR analysis of *Gasp-1* mRNA expression in myoblasts (*left*) and myotubes (*right*) treated with DMSO (Control) or with the PPARα agonist Wy14643 (10µM) for 24h. Corresponding graph represents fold change of *Gasp-1* expression normalized to *GAPDH*. Data are mean ± S.E.M (n=3). (D) qPCR analysis of *FABP3* mRNA expression in myoblasts (*left*) and myotubes (*right*) treated with DMSO (Control) or with the PPARα agonist Wy14643 (10µM) for 24h. Corresponding graph represents fold change of *Gasp-1* expression normalized to *GAPDH*. Data are mean ± S.E.M (n=3). (E) Western Blot analysis of hGASP-1 expression in human myoblasts infected with lentivirus containing either Control shRNA or hGASP-1 shRNA. Corresponding graphs represent optical density values for hGASP-1 protein. α-tubulin expression was analyzed to ensure equal loading of samples. Agarose gel images revealing the interaction of PPARγ (*F*) and PPARα (*G*) with the DR-1 site of the *Gasp-1* promoter in the absence (-) or presence (+) of GW1929 or Wy14643 respectively, as assessed through ChIP (*Upper panels*). Analysis of PPARγ (E) and PPARα (G) interaction with the β-actin promoter, in the absence (-) or presence (+) of GW1929 or Wy14643 respectively, was also performed as a negative control (*Lower panels*). The relative amounts of both the *Gasp-1* and β-actin promoters in the input were also assessed and are indicated. Both isotype-specific IgG and no antibody (No Ab) controls are shown. \( p<0.05 \) (*) and \( p<0.01 \) (**).
### Supplementary table

Table S1. List of genes selected for further validation through qPCR analysis. The table shows gene symbols, accession numbers and gene-specific forward and reverse primer sequences.

| Gene symbol | Accession Number | Forward Primer 5'-3' | Reverse Primer 5'-3' |
|-------------|------------------|----------------------|----------------------|
| Abca1       | NM_013454        | AAAACCGCAGACATCCTTCAG| CATACCGGAACCTCGGTACC|
| Abcg1       | NM_009593        | CTTTCTACCTGTACCAGAGG| GGCCAATTCTGGATAAGG   |
| Acsbg1      | NM_053178        | ATGCCACCGTTCTCAGAG   | GAGCTGTCTGGAGTTGCTCT|
| Adfp        | NM_007408        | GACCTTGTTCCCTCGGTAT  | CACACCAAATTTGGCTCT   |
| Adipoq      | NM_009605.4      | TGTTCCTCTTAACTTCGCTCA| CCAACCTGGAAAGGTCCCT |
| Ahrr        | NM_009644        | ACATACCGCAGAGAGAGAGA| GGTCACGTCTGTATTGAGGC|
| Ak5         | NM_001081277     | GACACAGCAGGGAGCTCAG  | TTTCCTCCAGGCTACACAC |
| Aldh3a1     | NM_007436        | AATATCAGTACATGTAAGACG| GAGAGCCCTTTAATCGGTG |
| Angpt4      | NM_020581        | CACGAGGTACGACGGATAGA | TGACAAGCTGCCACAGGGA|
| Arl4D       | NM_025404        | CGGCAAGATGGACTGTTCTT| CCAGCTCTTCTCCACCTCT|
| Cpt1b       | NM_009948.2      | CCCATGTGTCTCCTACAGATG| CACGTGTGTGCTGTCCGGA|
| FABP3       | NM_010174        | CCCCTCAGCTCACCGACCAT| CAGAAAAATCCCCAACCACAG|
| Gasp-1      | NM_181819        | GGGAATATGAGTGCTTCTTCTGAC| CTGCCCTTCCACACAG   |
| Gpi1        | L09104           | TCAAGCTGCAGCAACTTGGTC| CAGAGAGCCCTTTAATCGGT|
| Hsp90aa1    | NM_010480        | TGTTGCGGTACTACATGCTCAA| CCAACAGGGACTGCTGAAA|
| Lrp8        | NM_053073        | CACGAGACGGTACGTGCTGAT| CCAACAGGGACTGCTGAAA|
| Mc3r        | NM_008561        | TCCGATGCTGCGAGGAGGTGC| CCAACAGGGACTGCTGAAA|
| Mcd         | NM_019966.2      | TGCTGCTGCAGGTGCTGTCTT| CCAACAGGGACTGCTGAAA|
| Mlt5        | AK053809         | CGGTAACGTGCTGCTGCTGCT| CCAACAGGGACTGCTGAAA|
| Pdk4        | NM_013743.2      | CGGTAACGTGCTGCTGCTGCT| CCAACAGGGACTGCTGAAA|
| Ppargc1a    | NM_008904.2      | TGCTGCTGCTGCTGCTGCTGCT| CCAACAGGGACTGCTGAAA|
| Slc27a1     | NM_011977        | GATGACGAGAAGTCTGACG  | CCAACAGGGACTGCTGAAA|
| Slc9a17     | NM_172289        | GATGACGAGAAGTCTGACG  | CCAACAGGGACTGCTGAAA|
| Tnfaip5     | NM_032679        | GATGACGAGAAGTCTGACG  | CCAACAGGGACTGCTGAAA|
| Ucp3        | NM_011977        | GATGACGAGAAGTCTGACG  | CCAACAGGGACTGCTGAAA|

**WITHDRAWN June 1, 2016**
Figure: S1

A. 

| Time  | 0h | 24h | 48h | 72h | 96h |
|-------|----|-----|-----|-----|-----|
| L165041  | + | - | - | + | + |
| IB: MyoD  |  |  |  |  |  |
| IB: myogenin   |  |  |  |  |  |
| IB: α-tubulin |  |  |  |  |  |

IB: MyHC

B.

C.

Control

L165041

Optical density values (MyoD)

Optical density values (myogenin)

Optical density values (MyHC)

Absorbance at 605 nm

24h diff

48h diff

**WITHDRAWN**

June 1, 2016
