Sharp-1 regulates TGF-β signaling and skeletal muscle regeneration

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Running title: Sharp-1 regulates postnatal myogenesis

Key words: TGF-β, fibrosis, regeneration, muscle, degeneration

JCS Advance Online Article. Posted on 19 December 2013

Summary

Sharp-1 is a basic helix-loop-helix (bHLH) transcriptional repressor which is involved in a number of cellular processes. Our previous studies have demonstrated that Sharp-1 is a negative regulator of skeletal myogenesis and blocks differentiation of muscle precursor cells by modulating MyoD activity. In order to understand its role in pre- and post-natal myogenesis, we assessed skeletal muscle development, and freeze-injury induced regeneration in Sharp-1 deficient mice. We show that embryonic skeletal muscle development is not impaired in the absence of Sharp-1, but post-natally, the regenerative capacity is compromised. Although the initial phases of injury induced regeneration proceed normally in Sharp-1-/- mice, during late stages, the mutant muscle exhibits necrotic fibers, calcium deposits, and fibrosis. TGF-β expression, as well as pSmad 2/3 levels are sustained in the mutant tissue, and treatment with decorin, a TGF-β blocker improves histo-pathology of Sharp-1-/- injured muscles. *In vitro*, Sharp-1 associates with Smad3, and its overexpression inhibits TGF-β and Smad3-mediated expression of extracellular matrix genes in myofibroblasts. These results demonstrate that Sharp-1 regulates muscle regenerative capacity, at least in part, by modulation of TGF-β signaling.

Introduction

Regeneration is a feature of postnatal skeletal muscle and occurs to replace damaged myofibers following exercise or injury (Hawke and Garry, 2001; Huard et al., 2002; Charge and Rudnicki, 2004). Regeneration of muscle is dependent on satellite cells, and can be divided into distinct phases, which include inflammation, tissue formation, and tissue remodeling. During the inflammatory phase, the damaged muscle tissue is infiltrated by immune cells, and activated macrophages play a key role in the removal of necrotic tissue. This is followed by tissue formation that is dependent on the proliferation and differentiation of satellite cells. Many growth factors and cytokines that are mitogenic for satellite cells have been implicated in this process (Husmann et al., 1996). The progeny of activated satellite cells called muscle precursor cells undergo multiple rounds of proliferation, withdraw from the cell cycle, and differentiate to form myotubes characterized by the presence of a centrally located nuclei (Huard et al., 2002; Charge and Rudnicki, 2004). Further growth and fusion results in the formation of myofibers. During tissue remodeling, fibroblasts present at the site of injury produce an initial extracellular matrix (ECM) of type I and type III collagens. Differentiation of fibroblasts into myofibroblasts augments their contractile activity (Border and Ruoslahti, 1992; Border and Noble, 1994; Grinnell, 1994). Myofibroblasts also produce ECM further contributing to tissue remodeling. Once the tissue is repaired, the contractile activity of myofibroblasts is terminated, and cells are removed by apoptosis. Myofibroblast persistence and excessive fibroblast proliferation can result in the formation of scar tissue (fibrosis) that is a sign of incomplete regeneration, and is often accompanied by elevated TGF-β levels and collagen deposition (Desmoulière et al., 1993; Serini et al., 1996; Serini et al., 1998). In myopathies such as Duchenne Muscular Dystrophy (DMD), the regeneration process is compromised, and muscle tissue is replaced by dysfunctional scar (fibrotic) tissue. TGF-β 1 and 2 levels are elevated in muscular dystrophies and have been shown to cause fibrosis in dystrophic muscles (Bernasconi et al., 1995; Bernasconi et al., 1999; Mutakami et al., 1999; Zhu et al., 2007). Interestingly inhibition of TGF-β signaling not only prevents fibrosis, but also improves regeneration in the mdx mutants that are widely studied as a mouse model for DMD (Lefaucheur and Sébille, 1995; Cohn et al., 2007) indicating that regulation of fibrosis presents a key step in the pathology of DMD.

Members of the TGF-β superfamily are multifunctional cytokines that regulate diverse physiological processes including development, homeostasis, wound healing, differentiation,

apoptosis and cell cycle arrest (Barnard et al., 1990; Moses et al., 1990). In general, TGF-βs inhibit proliferation of most cells and induce apoptosis of epithelial cells. In contrast, TGF-β stimulates fibroblast cells to proliferate and produce ECM that results in a fibrotic response in tissues. TGF-β signals through heteromeric transmembrane type I and type II receptors (Wrana et al., 1992; Heldin et al., 1997). In presence of TGF-β ligand, the receptor activated Smads i.e. Smad2 and Smad3, are phosphorylated, bind to the common Smad (Smad4) and translocate into the nucleus (Wrana et al., 1992; Massagué et al., 1997). Nuclear Smad protein complexes bind specific DNA sequence motifs and recruit coactivators such as CBP/p300 and participate in transcriptional regulation of target genes (Massagué et al., 1997) including those encoding ECM proteins. Excessive TGF-β-induced deposition of ECM at the site of injury can lead to fibrosis (Serini et al., 1996; Serini et al., 1998). While several pathways are implicated in regulation of the distinct steps in muscle regeneration, the molecular mechanisms governing regeneration and repair are not fully understood.

Sharp-1/Dec2, a basic helix-loop-helix transcription factor plays complex roles in cellular differentiation, apoptosis, cell cycle arrest, tumor progression and circadian rhythms (Yamada and Miyamoto, 2005; Sun et al., 2007a). Our previous studies have shown that overexpression of Sharp-1 in myoblasts or preadipocytes blocks their ability to undergo terminal differentiation (Azmi et al., 2004; Gulbagci et al., 2009; Ling et al., 2012; Wang et al., 2013). Here, we demonstrate that Sharp-1 plays a role in skeletal muscle regeneration via regulation of TGF-β signaling. In response to injury, Sharp-1 null mutants exhibit a defect at late stages of regeneration. Sustained TGF-β expression, increased pSmad2/3 levels, and smooth muscle actin (SMA^+) positive myofibroblasts are evident in Sharp-1-/- regenerating tissue. Inhibition of TGF-β signaling ameliorates muscle pathology and degeneration in Sharp-1-/- mice. We demonstrate that Sharp-1 directly regulates TGF-β signaling and antagonizes Smad3-dependent expression of collagens and tissue inhibitor of metalloproteinase 1 (TIMP1) likely via interaction with Smad3 and inhibition of its transcriptional activity. Taken together, our studies indicate that Sharp-1 is essential to limit TGF-β and Smad3 signaling in myofibroblast cells that impacts skeletal muscle regeneration.

Results

Skeletal muscle development in Sharp-1-/- mice

We have previously demonstrated that Sharp-1 is expressed in skeletal muscles during embryonic development as well as in adult tissues, and its overexpression inhibits myogenesis through regulation of MyoD activity (Azmi and Taneja, 2003; Azmi et al., 2004; Ling et al., 2012; Wang et al., 2013). To further examine the role of endogenous Sharp-1 we analyzed embryonic and postnatal myogenesis in wild type (WT) and littermate Sharp-1 null (Sharp-/-) mutants (Rossner et al., 2008). No overt difference in myogenin expression was apparent in WT and Sharp-1 null embryos at embryonic (E) day 16.5 (Fig 1A). Histological analysis of quadriceps muscles from adult WT and Sharp-1-/- mice also did not reveal obvious defect in muscle architecture (Fig 1B) or in the mean cross section area (CSA) (Fig 1C, right panel) indicating that the development of skeletal muscles occurs normally in the absence of Sharp-1. Moreover, no changes in the number of $Pax7^{+}$ satellite cells was noted between WT and Sharp-1-/- mutants (Fig 1D & E).

Skeletal muscle regeneration is impaired in Sharp-1 null mutants

We then examined the regenerative potential of Sharp-1 null mutants in response to freeze injury. Quadriceps muscles from 2-3 month old WT and Sharp-1-/- mice were injured (Sun et al., 2007b) and regeneration was analyzed histologically. Hematoxylin & Eosin (HE) staining of injured muscle sections revealed extensive myofiber damage in both WT and Sharp-1-/- mice at D2, with no marked histological differences apparent at this stage (data not shown). Five days after injury (D5), regeneration was evident in both WT and Sharp-1-/- muscles with the presence of newly formed myotubes containing central nuclei (Fig 2A). Ten and sixteen days (D10 and D16 respectively) post injury, regeneration in WT mice was evident by presence of centrally nucleated newly formed myotubes. In contrast to WT muscles, the mutant tissue revealed overt signs of necrosis and degeneration (Fig 2A). Indeed, mutant quadriceps muscles stained positively with alizarin red indicating calcification, a hallmark of dystrophic tissues (Fig

2B). In addition, fibrosis was detectable in the mutant regenerating muscles by Masson's Trichrome staining (Fig 2C).

To examine the mechanisms underlying defective regeneration in Sharp-1-/- muscle, we first analyzed the expression of MyoD, a marker of proliferating myoblasts. MyoD levels were upregulated during regeneration in both WT and Sharp-1-/- mutants as seen by western blot analysis (Fig 3A). Immunostaining of D10 injured tissue revealed many $MyoD^+$ cells in the mutant tissue compared to WT muscle. Moreover, several smooth muscle actin positive (SMA^+), cells were also apparent in Sharp-1-/- muscle that did not co-localize with $MyoD^+$ cells indicating presence of myofibroblasts in the mutant tissue (Fig 3B). We then examined differentiation using embryonic MHC (eMHC) antibody. Immunostaining of injured WT and Sharp-1-/- muscles at D5 revealed a higher number of $eMHC^+$ myotubes in the mutant tissue that correlated with a higher regeneration index (Fig 3C &D). Together these results suggest the compromised regeneration at late stages (D10/D16) is not due to a differentiation defect, but may reflect an additional role for Sharp-1 in late steps of tissue remodeling.

Increased TGF-β signaling in Sharp-1-/- injured muscles

On activation by ligand, TGF-β receptors induce phosphorylation of Smad2 and Smad3 (pSmad2/3), which form a heterotrimeric complex with Smad4 and then translocate to the nucleus (Wrana et al., 1992). Estimation of pSmad2/3 levels thus serves as a measure of TGF-β signaling. Since Sharp-1-/- mutants exhibited fibrosis, we examined TGF-β expression levels by western blot analysis in WT and Sharp-1-/- muscles at various time points after injury (Fig 4A). In WT mice, TGF-β expression was strongly induced upon injury with elevated levels apparent at D2, and the levels started to decline at D5 and D10. In contrast, TGF-β expression was sustained at high levels at D5 and D10 in Sharp-1 null mutants. To examine whether the increased TGF-β resulted in increased signaling, we analyzed pSmad2/3 levels by immunostaining (Fig 4B). Interestingly, in correlation with increased TGF-β levels, pSmad2/3 levels were also higher in Sharp-1-/- injured muscles at D5 and D10 compared to WT muscles (Fig 4B). TGF-β induces differentiation of myofibroblasts that play a critical role in fibrosis (Desmoulière et al., 1993; Li et al., 2004). Consistent with sustained high levels of TGF-β signaling, the mutant tissue exhibited increased number of SMA^+ cells at D5 and D10 after

injury (Fig 4C) which was in contrast to WT tissue where few SMA^+ myofibroblasts were apparent at any stage.

Inhibition of TGF-β signaling ameliorates muscle necrosis

Previous studies have demonstrated that blocking TGF-β can improve muscle pathology in mdx mice (Cohn et al., 2007) and one such blocker which has been successfully used against fibrosis in mice is the proteoglycan, decorin (Isaka et al., 1996; Li et al., 2007). To assess whether the enhanced TGF-β signaling underlies muscle necrosis in Sharp-1-/- mice, we injected decorin in Sharp-1-/- muscle and performed histological analysis three days later. The contralateral muscle was injected with PBS. Decorin injected injured muscles from Sharp-1-/- mice showed a considerable decrease in degenerating myofibers and significant increase in regenerating myofibers (Fig 5A). Quantification of the damaged area indicated reduced pathology in decorin injected muscles relative to uninjected muscle (Fig 5B). To further confirm that decorin indeed led to reduced TGF-β signaling, uninjected and injected injured muscles were immunostained with pSmad2/3 antibody (Fig 5C). As expected, pSmad2/3 staining was higher in uninjected tissue compared to one injected with decorin. Moreover, consistent with inhibition of TGF-β activity, SMA^+ cells were also reduced in decorin treated muscle (Fig 5D).

Sharp-1 regulates TGF-β signaling

To examine the mechanisms that might account for de-regulated TGF-β expression and signaling, we first investigated whether Sharp-1 interacts with components of the pathway. The interaction of Sharp-1 with Smad3, a key mediator of TGF-β response was analyzed. Myc-Sharp-1 was transfected in cells and lysates were harvested in the absence and presence of TGF-β treatment. An interaction of Sharp-1 with endogenous pSmad2/3 and total Smad2/3 was apparent in the presence of TGF-β (Fig 6A). To define the domains in Sharp-1 that associate with Smad3, various Sharp-1 deletion mutants (Ling et al., 2012) were transfected alone or together with Smad3. Immunoprecipitation assays indicated that full length Sharp-1, and the deletion mutants Sharp-1ΔC, lacking the C-terminal region, and Sharp-1ΔO, devoid of the orange domain, interacted with Smad3. However no association was seen with the Sharp-1-bHLH mutant, which lacks the bHLH domain indicating that the region essential for interaction with Smad3 resides between amino acids 173-265 of Sharp-1 (Fig 6B). Moreover, Sharp-1 co-

localized with Smad3 in the presence of TGF-β (Fig 6C). To determine if Sharp-1 directly interacts with Smad3, we performed GST-pull down assays. Equivalent amounts of GST-Sharp-1 or GST protein alone were incubated with ^{35}S labeled Smad3. Sharp-1 directly interacted with Smad3, while as expected, no interaction of Smad3 was apparent with GST protein (Fig 6D).

We next investigated the effect of Sharp-1 expression on TGF-β and Smad3-dependent transcriptional responses using 3TP-Lux reporter, which contains a TGF-β response element from plasminogen activator inhibitor-1 (PAI-1) promoter and has been widely used to monitor TGF-β and Smad signaling (Wrana et al., 1992). Co-expression of full length Sharp-1 resulted in repression of Smad3-induced reporter activity. In contrast, the Sharp-1-bHLH mutant, which failed to interact with Smad3, was significantly less effective in repression of Smad3-dependent transcriptional activity (Fig 7A). Conversely, reporter activity was increased in cells transfected with Sharp-1 siRNA (siSharp-1) relative to control cells transfected with scrambled siRNA (siRNA) validating that endogenous Sharp-1 regulates Smad3 activity (Fig 7B). To examine the mechanisms underlying the inhibitory effect of Sharp-1 on Smad3 activity, we investigated whether Sharp-1 impacts the ability of Smad3 to bind to a Smad binding element (SBE). Flag-Smad3 and Flag-Smad4 were transfected in the absence or presence of Myc-Sharp-1. Nuclear extracts were incubated with a radioactive SBE probe. A complex containing Smad3/4 was evident on SBE, that was supershifted with anti-Flag, but not with anti-Myc antibody (Fig 7C). Together, these data demonstrate that Sharp-1 does not directly bind to SBE, nor does it alter binding of Smads to SBE.

Myofibroblasts which are heterogenous in origin, play a key role in tissue remodeling and are characterized by synthesis of extracellular matrix (ECM) proteins and expression of SMA. Differentiation of fibroblast and other cells to myofibroblasts requires TGF-β signaling which results in expression of ECM genes such as collagen1A1 (COL1α1), collagen1A2 (COL1α2), and collagen3A1 (COL3α1) in a Smad3-dependent manner. In addition, TGF-β inhibits ECM degradation by increasing expression of matrix metalloprotease inhibitors such as tissue inhibitor of metalloprotease (TIMP-1). Given the increased TGF-β signaling and SMA^+ cells in Sharp-1 injured muscle, and the ability of Sharp-1 to antagonize Smad3 activity, we sought to determine whether Sharp-1 regulates TGF-β induced ECM gene expression in myofibroblasts. NIH3T3 fibroblast cells that inducibly express Sharp-1 (Liu et al., 2010) were treated with TGF-β. As expected, the expression of collagens and TIMP-1 was induced by TGF-β (Fig 7D).

Interestingly, induction of Sharp-1 with doxycycline significantly inhibited TGF-β mediated expression of COL1α1, COL3α1 and TIMP-1 (Fig 7D). Similar results were seen when cells were treated with TGF-β for 48hr (data not shown). The basal expression of COL1α1, and COL3α1 were suppressed by Sharp-1 even in the absence of exogenous TGF-β indicating an impact of Sharp-1 on endogenous Smad3 activity. Moreover, chromatin immunoprecipitation assays showed that both Sharp-1 and Smad3 were present promoters of plaminogen activator inhibitor PAI-1 which plays a significant role in fibrosis, and SMA, in a TGF-β responsive manner (Fig 7E). Taken together, these results provide evidence that Sharp-1 antagonizes TGF-β signaling likely via association with Smad3, and inhibition of its transcriptional activity in myofibroblast cells.

Discussion

The results of this study demonstrate a novel role for Sharp-1 in tissue remodeling and muscle regeneration via its ability to limit TGF-β signaling in myofibroblasts. While embryonic myogenesis does not appear to be overtly perturbed in Sharp-1-/- embryos, differentiation of myogenic precuror cells in response to injury is enhanced. Interestingly however, despite productive differentiation, Sharp-1-/- mice show a regeneration defect at late stages characterized by myonecrosis, proliferating myogenic and non-myogenic cells, calcification, and fibrosis.

Postnatal myogenesis involves the interplay of many growth factors and cytokines, which act as positive or negative regulators of regeneration (Husmann et al., 1996; Charge and Rudnicki, 2004). One such negative regulatory factor is TGF-β. TGF-β has been reported to inhibit myoblast proliferation and myogenic differentiation by inhibition of MyoD (Massague et al., 1986; Allen and Boxhorn, 1987; Liu et al., 2001). Moroever, TGF-β also induces differentiation of fibroblasts into myofibroblasts in injured skeletal muscle (Li et al., 2004). The resulting overproliferation of myofibroblasts underlies deposition of extracellular matrix proteins resulting in fibrosis and hindering complete regeneration of muscle tissue.

TGF-β expression and signaling are sustained at high levels in the mutants during regeneration. Intriguingly however, myogenic differentiation is augmented at early stages in Sharp-1 mutant tissue as seen by the increased number of $eMHC^{+}$ cells. This may likely occur as Sharp-1 is a potent inhibitor of MyoD activity and myogenic differentiation (Azmi et al., 2004; Morosetti et al., 2006; Ling et al., 2012; Wang et al., 2013). Its absence therefore would presumably result in increased MyoD activity that may counter inhibitory effects of TGF-β at early stages in Sharp-1-/- mutants. Sustained TGF-β levels at a step subsequent to myogenic differentiation, likely result in increased SMA^{+} myofibroblasts and fibrosis seen in the mutant tissue. Previous studies have demonstrated that TGF-β neutralizing antibodies and blockers can be used as potential antifibrotic agents (Isaka et al., 1996; Li et al., 2007). Blockade of TGF-β with decorin indeed resulted in nearly 35% decrease in myonecrosis in Sharp-1-/- mice. Therefore, enhanced TGF-β expression and signaling accounts at least in part, for defective regeneration in Sharp-1-/- muscle.

Consistent with our findings, recent studies have shown an inverse correlation between Sharp-1 expression and TGF-β activity in breast and prostate cancer (Montagner et al., 2012; Sato et al., 2012). However, whether Sharp-1 directly inhibits TGF-β signaling, and the

mechanisms underlying its inhibitory effect have not been reported. TGF-β signaling and Smad3 play a central role in fibrosis by enhancing expression of ECM genes such as collagens, decreasing expression of MMPs and increasing expression of MMP inhbitors (Flanders, 2004). Our data demonstrates that Sharp-1 directly interacts with Smad3, and inhibits its transcriptional activity. This antagonism is not due to direct competition for DNA-binding as Sharp-1 does not bind to the Smad binding sites, nor does it impact binding of Smads to DNA. It is plausible that Sharp-1 may recruit co-factors such as HDAC1 (Garriga-Canut et al., 2001), Sirt1 (Fujimoto et al., 2007) or G9a (Ling et al., 2012; Wang et al., 2013) to block Smad3 transcriptional activity in a cell type specific manner.

Fibrosis poses a major obstacle to therapy not only in muscle diseases, but several other tissues as well. While the role of TGF-β signalling in fibrosis is well established, much remains to be understood with regard to the mechanisms that lead to inappropriate TGF-β signaling and development of fibrosis. Our studies provide novel insights into the role of Sharp-1 in controlling TGF-β signaling in myofibroblasts, and development of anti-fibrotic therapies.

Material and methods

Regeneration of skeletal muscle: Sharp-1-/- mice have been described (Rossner et al., 2008). Freeze-crush injury induced regeneration was performed as described (Sun et al., 2007b). At least 4 mice were analyzed per time point (2, 5, 10, and 16 days after injury). All animal protocols followed institutional guidelines. For TGF-β blockade *in vivo*, quadriceps muscles were injured as described above. Seven days after injury, 20 ul (50 μg) of decorin (Sigma, St. Louis, MO, USA) was injected at 6 sites as described (Li et al., 2004). PBS (in 0.1% BSA) was injected as a control in the contralateral injured muscle. Muscles were collected three days after decorin injection and analyzed histologically.

Antibodies: Anti-MyoD and anti-pSmad2/3 antibodies used for immunohistochemistry were from SantaCruz, Dallas, Texas, USA; anti-TGF-β was from Novocastra, Buffalo Grove, IL, USA; anti β-actin from Sigma, St. Louis, MO, USA; anti-EF1α from Upstate Biotechnology Inc., Lake Placid, NY USA; anti-Pax7 and anti-eMHC from Developmental Studies Hybridoma Bank; anti-SMA from Sigma, St. Louis, MO, USA, anti phospho-Smad2/3 (Ser465/467) antibody from Millipore, Billerica, MA, USA; anti-mouse and anti-rabbit alexafluor secondary antibodies from Molecular Probes, Eugene, OR, USA; anti-mouse and anti-rabbit HRP conjugated secondary antibodies from Sigma, St. Louis, MO, USA.

Histology and immunohistochemistry*:* Serial cross-sections (8 μm thick) were collected along the entire length of embedded muscle tissues, and one out of every ten slides was stained with hematoxylin and eosin (HE) for identification of the largest damaged area. Sections from both genotypes with comparable damaged area were used for histology and immunohistochemistry. Immunohistochemistry was performed as described previously (Sun et al., 2001) and visualized using the VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Staining without primary antibody served as a negative control. For immunofluorescence, paraffin-embedded sections were incubated with appropriate primary antibody followed by detection with secondary antibody from Alexafluor and analysed under fluorescence microscope. Masson's Trichrome staining was performed using a kit (Diagnostics Biosystem, Pleasanton, CA, USA). For alizarin red staining, sections were deparaffinized and stained for 30 seconds in 2% alizarin red solution followed by washes in acetone, acetone:xylene (1:1), xylene and mounted. Calcium deposits were viewed as orange-red staining.

Morphological analysis: Damaged area and cross section area (CSA, μm^2) were measured using ImageJ software (version 1.36b, NIH). For measuring CSA, quadriceps from 4 mice (3 month old) of each genotype were analysed and at least 500 myofibers per muscle were measured. The extent of pathology with or without decorin treatment was determined as a ratio of the necrotic area of the injured muscle to the total area of the injured muscle section.

Regeneration index: Regeneration was assessed by counting the number of $eMHC^+$ fibers normalized to the total number of nuclei in 5-10 randomly selected fields as described previously (Sun et al., 2007b).

Satellite cell number: Paraffin sections of quadriceps muscle from two-month old mice were stained with anti-Pax7 antibody and counter stained with hematoxylin. Both myonuclei and $Pax7^+$ nuclei were counted from several random fields for each animal, and the percentage of satellite cells was calculated. At least 500 nuclei were counted for each animal, and the data presented are an average of four animals.

Immunofluorescence analysis: Paraffin embedded sections were deparaffinised in histoclear. After antigen retrieval sections were incubated in blocking buffer for 1hr, followed by staining with primary antibodies to SMA and MyoD and detection with alexa flour dye conjugated secondary antibodies. Sections were mounted in DAPI containing mounting medium, Vectashield H-1200, (Vector Laboratories, Burlingame, CA, USA) and imaged using Zeiss AX10 microscope. To determine localization of Sharp-1 and Smad3, C2C12 cells were seeded in chamber slides at ~30% confluence. Cells were transfected with Myc-Sharp-1, Flag-Smad3 alone or together. 24hr after transfection, cells were fixed in 4% paraformaldehyde, treated with 0.5 % Triton-X 100 and washed with PBS. After blocking (10% BSA in PBS), slides were incubated with Myc and Flag antibodies followed by fluorescence conjugated secondary antibodies. Cells were mounted in DAPI containing mounting medium from Vectastain, Vector Laboratories, Burlingame, CA, USA and viewed under a fluorescence microscope.

Plasmids, cell culture, luciferase assays and Sharp-1 knockdown: Flag-Smad2, Flag-Smad3, and Flag-Smad4 were kindly provided by Rik Derynck; 3TP-Lux was kindly provided by Jeff Wrana; Myc-Sharp-1 and GST-Sharp-1 have been described (Ling et al., 2012). C2C12 and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% and 10% fetal bovine serum (FBS) respectively. For differentiation, C2C12 cells were cultured in

DMEM with 2% horse serum. NIH3T3 cells were maintained in DMEM containing 10% bovine serum (BS). Sharp-1 inducible NIH3T3 cells have been described and were treated with 2μg/ml doxycycline (DOX) for 24 and 48 hr to induce Sharp-1 (Liu et al., 2010). Transient transfections were performed using Lipofectamine Plus or Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). For reporter assays, cells were transfected with the reporter 3TP-Lux and various plasmid constructs as indicated in the figures along with 5ng of Renilla Luciferase as an internal control. Empty expression vector was added to normalize the amount of total DNA. 48 hr post-transfection, luciferase assays were performed using the dual luciferase system (Promega, Fitchburg, WI, USA). All transfections were performed in triplicates and repeated at least twice. Values are reported as means with standard deviation (SD). To knockdown Sharp-1, C2C12 cells were transfected with 100nM siRNA specific for mouse Sharp-1 (Qiagen, Valencia, CA), or with control scrambled siRNA using Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY, USA). 24 hr post-transfection, siRNA knockdown cells were transfected with the reporter and plasmid constructs and luciferase activity was measured as described above. For all experiments, TGF-β was used at 5ng/ml.

Co-immunoprecipitation (Co-IP) and GST-pull down assays: Co-IP and GST-pull down assays were done as described (Sun and Taneja, 2000). Briefly, for Co-IP assays, cells were transfected with Myc Sharp-1. 24hr later, cells were treated for 1hr with 5ng/ml TGF-β before lysing. Control cells were not treated with TGF-β. Sharp-1 was immunoprecipated using Myc-agarose beads (Sigma, St. Louis, MO, USA) and probed for endogenous pSmad2/3 and Smad3. To map the interaction domains between Sharp-1 and Smad3, cells were transfected with full length Myc-Sharp-1 and deletion mutants Sharp-1 bHLH, Sharp-1 ΔO or Sharp-1 ΔC (Ling et al., 2012) together with Flag-Smad3. 48hr later, cells were washed twice with cold PBS, lysed in 50 mM Tris-HCl pH8.0, 50 mM NaCl, 1mM EDTA, 0.1% Triton X-100, 0.5 mM PMSF and protease inhibitors (Roche Applied Science , Mannheim, Germany). Equal amounts of total protein were loaded for western blotting. Lysates were incubated with Flag-agarose beads (Sigma, St. Louis, MO, USA) and analyzed by western blotting using anti-Myc antibody (1:1000 Sigma, St. Louis, MO, USA). For GST-pull down assays, Smad3 was translated *in vitro* and labeled with ^{35}S-methionine using the TNT-coupled reticulocyte lysate system (Promega, Fitchburg, WI, USA). ^{35}S-labeled Smad3 was incubated with purified GST-Sharp-1 or GST in binding buffer. Samples were run on SDS gels and detected by autoradiography (Sun and Taneja,

2000).

Electrophoretic Mobility Shift Assay (EMSA): Flag-Smad3, Flag-Smad4 and Myc-Sharp-1were transfected and 48 hr later, cells were stimulated with TGF-β (5ng/ml) for 1hr. Nuclear extracts prepared from transfected samples were incubated with ^{32}P-labeled SBE probe (5'-CTCTATCAATTGGTCTAGACTTAACCGGA) (Luo et al., 1999). Binding reactions contained 40,000 cpm probe, 1 μg of poly(dI-dC), 10mM HEPES, pH 7.9, 80mM KCl, 0.1 mM EDTA, 5mM MgCl2, 0.5 mM dithiothreitol, 10% glycerol and increasing amounts of nuclear extract (+ 1.5μg; ++ 3μg). The reaction was incubated in room temperature for 20min and fractionated on 5% polyacrylamide gels. For supershift, antibodies were added and incubated for 10min following incubation with radiolabeled probe.

Chromatin Immunoprecipitation assay: C2C12 cells at 80% confluency were cultured in differentiation medium for 2 days in the absence or presence of 5ng/ml TGF-β. Cells were fixed with 1% formaldehyde and ChIP assays were performed using Millipore ChIP kit (Billerica, Massachusetts). 2ug of anti-Sharp-1 and anti-Smad3 antibodies (SantaCruz, Dallas, Texas USA) were used. DNA was amplified by QRT-PCR ((Roche Applied Science , Mannheim, Germany) with primers specific to mouse PAI-1 promoter (-600 to -800bp) containing TGFβ/SMAD responsive regions. Primer sequences for mouse PAI-1 are: 5' CACAAAGAGCGAGCCCTCAG-3' and 5'-CCAGAGGGCATGAAATGTGC-3'. Primers for SMA promoter have been described previously (Elberg et al., 2008).

Quantitative real time PCR (Q-PCR). Q-PCR was done as described (Ling et al., 2012). Briefly, RNA was reverse transcribed using iSCRIPT (Biorad, Hercules, California, USA) and Q-PCR was performed using Roche Sybr green in LC480 (Roche Applied Science , Mannheim, Germany). Mouse Sharp-1 was amplified using the following primers: Forward 5'-AACACTGGGGCATTTGGAGA-3' and Reverse 5'-TGGACCGGCGATTTCAGAG-3'. Primers for TIMP-1, COL1α1 and COL3α1 have been described previously (Meng et al., 2010; and Uezumi et al., 2011).

Statistical analysis: Student *t*-test was used to perform statistical analysis and *p* values of <0.05 were considered significant [* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$]

Acknowledgements:

We thank J Massagué and R Derynck for reporter constructs and Flag-Smad expression vectors.

This work was supported in part by MOE Academic Research Fund (to RT).

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Figure Legends:

Figure 1: Skeletal muscle development in Sharp-1-/- mutants.

(A) Embryonic myogenesis in WT (+/+) and Sharp-1-/- (-/-) embryos at E16.5 was analyzed by immunostaining with anti-myogenin antibody followed by fluorescence microscopy. (B) Quadriceps muscles from 2 month old WT (+/+) and Sharp-1-/- (-/-) mice (n=4) were analyzed histologically by H&E staining. (C) H&E stained sections of quadriceps muscles were used to determine the cross-section area of myofibers in both genotypes (n=4). The mean fiber size was not significantly different between WT and Sharp-1-/- mice. Data are mean ± standard error. (D) 3 week old WT (+/+) and Sharp-1-/- (-/-) muscles were immunostained with anti-Pax7 antibody to detect satellite cells. Sections were counterstained with hematoxylin. (E) Pax7 stained muscle sections were used to analyse the percentage of satellite cells from four independent WT (+/+) and Sharp-1-/- (-/-) mice. Scale bar: 100 μm.

Figure 2: Altered regenerative response upon freeze injury in Sharp-1-/- mice.

(A) Histological analysis of muscle regeneration in WT (+/+) and Sharp-1-/- (-/-) mice at D5, D10 and D16 following injury. At D5, newly formed myotubes with central nuclei are evident in both WT and mutant muscles. At later stages (D10 and D16 after injury), muscle degeneration is apparent in Sharp-1-/- mutants. (B, C) Alizarin red staining (B), and Masson's Trichrome staining (C), at D16 after injury revealed the presence of calcium deposits and collagen deposition (blue staining) respectively in Sharp-1-/- muscles. Scale bar: 100 μm.

Figure 3: Myogenic differentiation is not altered in Sharp-1-/- muscles.

(A) Western blotting was performed with protein lysates from WT (+/+) and Sharp-1-/- (-/-) uninjured (D0) and injured tissues (D2, D5 and D10 after injury) to detect MyoD expression. EF1α was used as an internal control. (B) Sections of quadriceps muscles from WT (+/+) and Sharp-1-/- (-/-) mice at D10 after injury were immunostained with anti-MyoD and anti-SMA antibodies. (C) Sections of quadriceps muscles from WT (+/+) and Sharp-1-/- (-/-) mice at D5 after injury were stained with anti-eMHC antibody. (D) Regeneration index was calculated by determining the percentage of $eMHC^{+}$ myotubes in WT and Sharp-1-/- mice (n=4). Data are mean±standard error.

Figure 4: Increased TGF-β and pSmad2/3 levels in Sharp-1-/- regenerating tissue.

(A) Western blotting was performed with protein extracts from WT and Sharp-1-/- regenerating muscle (n=4) after 2, 5 and 10 days of injury and analyzed using anti-TGF-β antibody. EF1α was

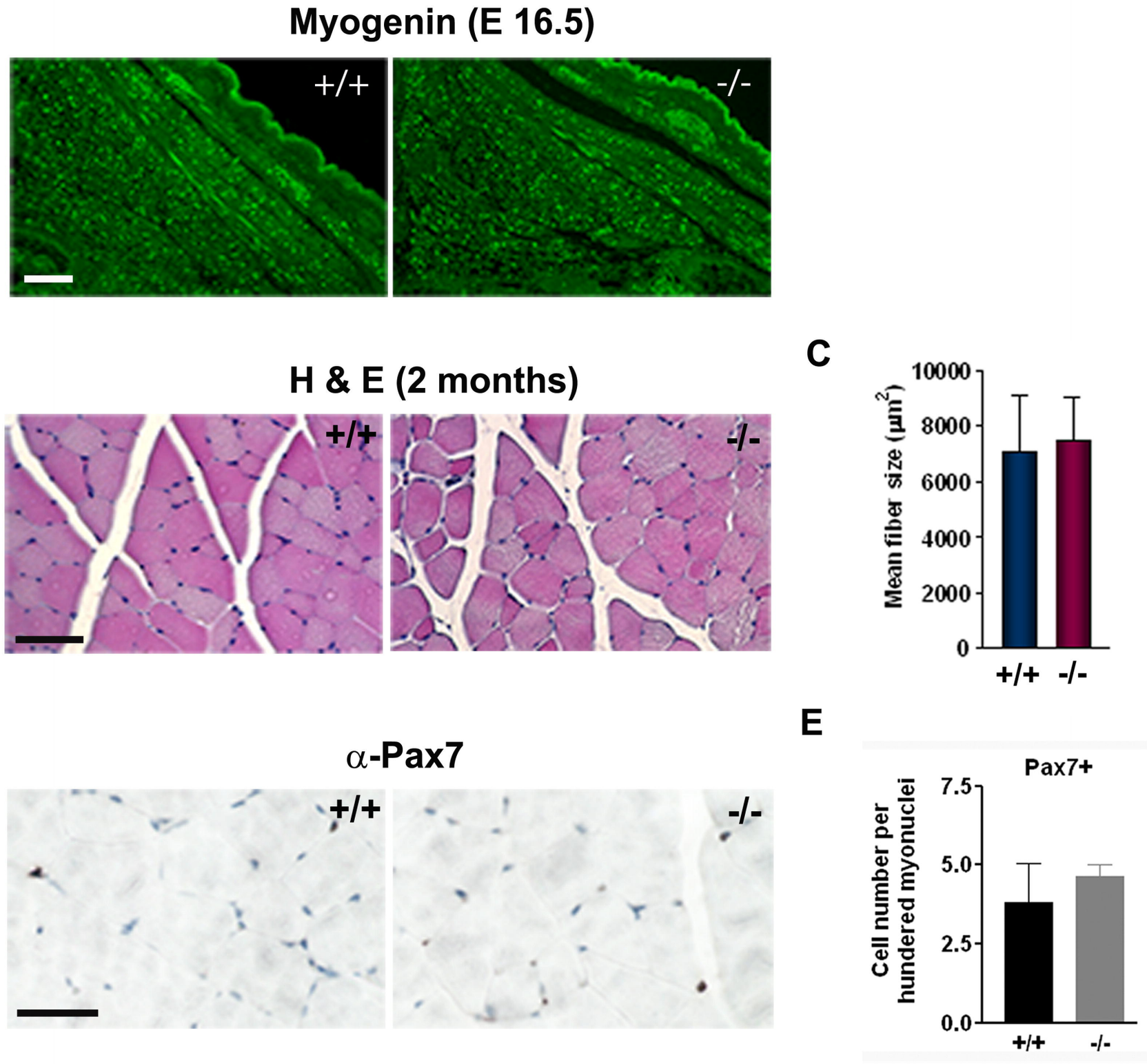
used as an internal control. (B-C) Sections of injured muscles from WT and Sharp-1-/- mice were immunostained with antibodies against pSmad2/3 (B), and SMA (C) at D5, and D10 after injury.

Figure 5: Inhibition of pSmad signaling reduces muscle necrosis in Sharp-1-/- mutants. (A) Decorin, or the control vehicle (PBS) was injected in Sharp-1-/- muscles seven days after injury, and cross sections were analyzed at D10 by H&E staining. Decorin injected muscles show a striking reduction of muscle pathology compared to the contralateral injured muscles (n=4) that were injected with PBS. (B) The percentage of pathology in the absence and presence of decorin was determined as a ratio of the necrotic area to the total injured area of the muscle. Results shown are representative from 4 sets of mice. Data are mean ± SD. (C-D) Control and decorin injected sections were immunostained with anti-pSmad2/3 (C) and anti-SMA (D) antibodies which revealed reduced pSmad2/3 signaling in decorin injected muscles (n=3). Bar: 100 μm.

Figure 6: Sharp-1 interacts with Smad3. (A) C2C12 cells were transfected with expression vectors for Myc-Sharp-1. Cells were left untreated or treated with TGF-β1 for 1hr. Lysates were immunoprecipitated with anti-myc agarose beads and immunoblotted with anti-pSmad2/3, Smad3 and myc antibodies. Input shows expression of Smad3 and Sharp-1 in the lysates. (B) Cells were transfected with plasmids expressing Flag-Smad3 and Myc-Sharp-1, Myc-Sharp-1 bHLH, Myc-Sharp-1 ΔO or Myc-Sharp-1 ΔC. 48 hr after transfection, lysates were immunoprecipitated with flag agarose beads followed by western blot with anti-Myc antibody. Input shows expression of Smad3 and Sharp-1 in lysates. β actin was used as an internal control. *NS refers to a non-specific IgG band. (C) C2C12 cells co-transfected with Flag-Smad3 and Myc-Sharp-1 were analyzed using anti-Flag and anti-Sharp-1 antibodies. Nuclei were stained with DAPI. (D) Equivalent amounts of GST-Sharp-1 or GST alone were incubated with ^{35}S labeled *in vitro* translated Smad3. 10% of input was run on the gel as a control.

Figure 7: Sharp-1 inhibits TGF-β signaling in myofibroblasts. (A) Cells were transfected with reporter p3TP-Lux (200 ng) together with Smad3 (100ng) in the absence and presence of Sharp-1 (25ng) and Sharp-1 bHLH (25ng) as indicated. Cells were harvested 48 hr after transfection, and assayed for luciferase activity. Error bars indicate mean ± SD. (B) Cells were transfected siSharp-1 and control scrambled siRNA at final concentration of 100nM. 24hr later,

Sharp-1 cells were transfected with p3TP-Lux (200 ng) with Smad3 (100ng) as indicted in figure. 24hr later, luciferase activity was assayed. Error bars indicate mean ± SD. (C) ^{32}P-labeled SBE oligonucleotide was incubated with nuclear extracts prepared from non-transfected cells (lanes 1, 2), increasing amount of Flag-Smad3/4 (lanes 3, 4), and increasing amount of Smad3/4 and Myc-Sharp-1 (lanes 5, 6). Supershift assays were done with anti-Flag (lane 7) and anti-Myc-antibodies (lanes 7 and 8). (D) NIH3T3 cells were left uninduced or induced with doxycycline (DOX) and treated with TGF-β for 24 hr. The expression of COL1α1, COL3α1 and TIMP-1 was analyzed by Q-PCR. (E) ChIP assays were performed to determine occupancy of Sharp-1 and Smad2/3 at the PAI-1 and SMA promoters in the absence and presence of TGF-β. Error bars indicate mean±SD.



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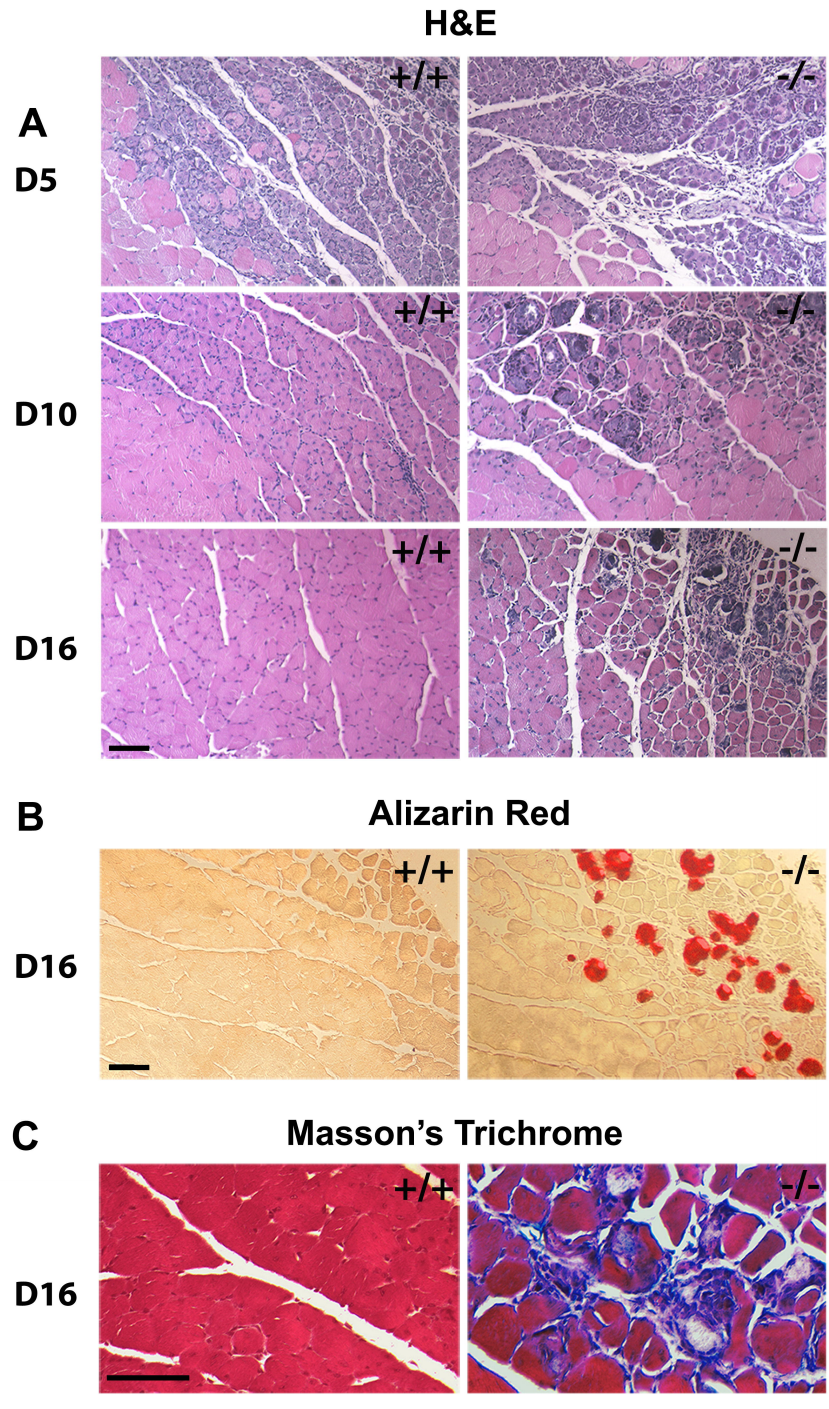

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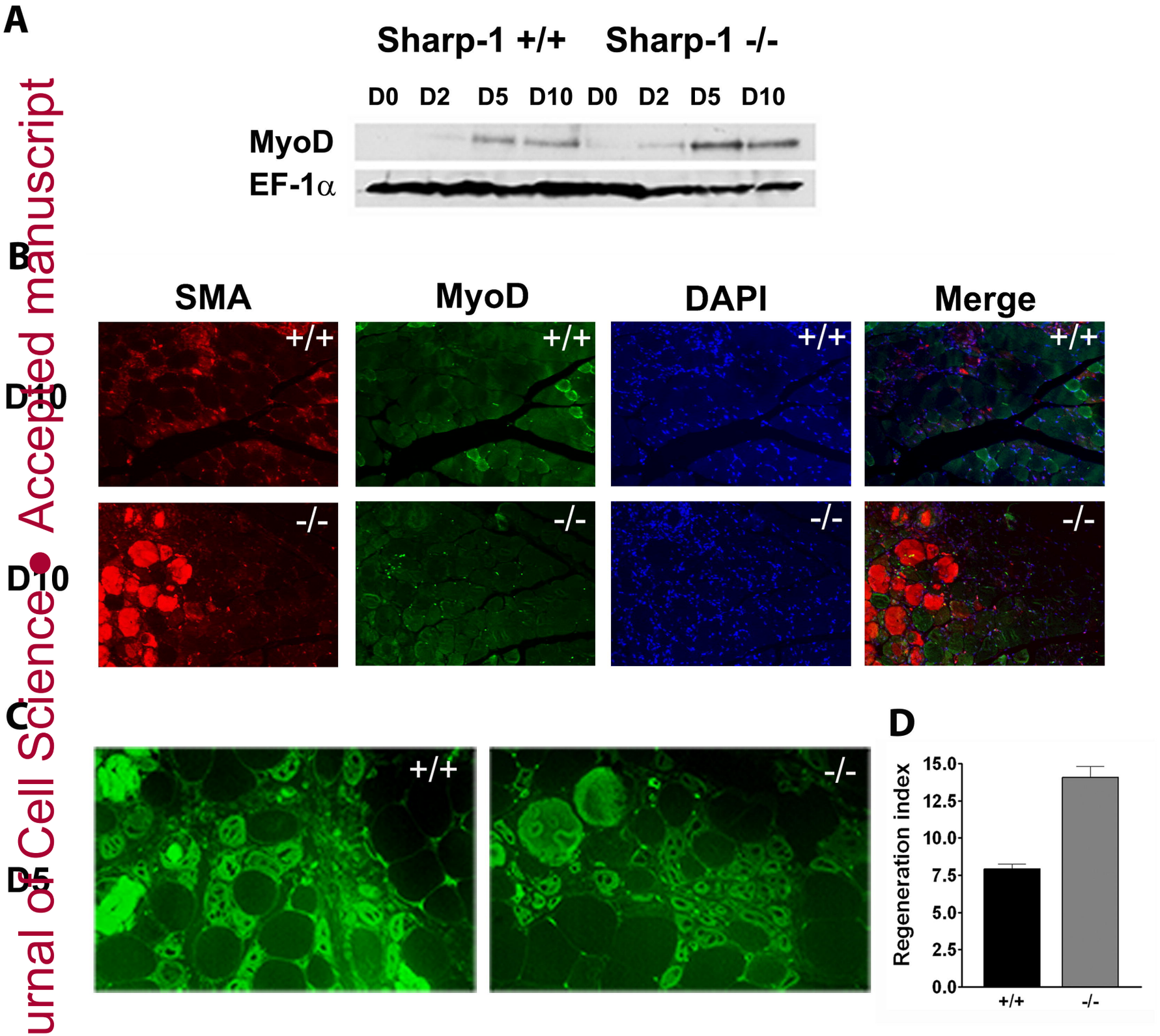

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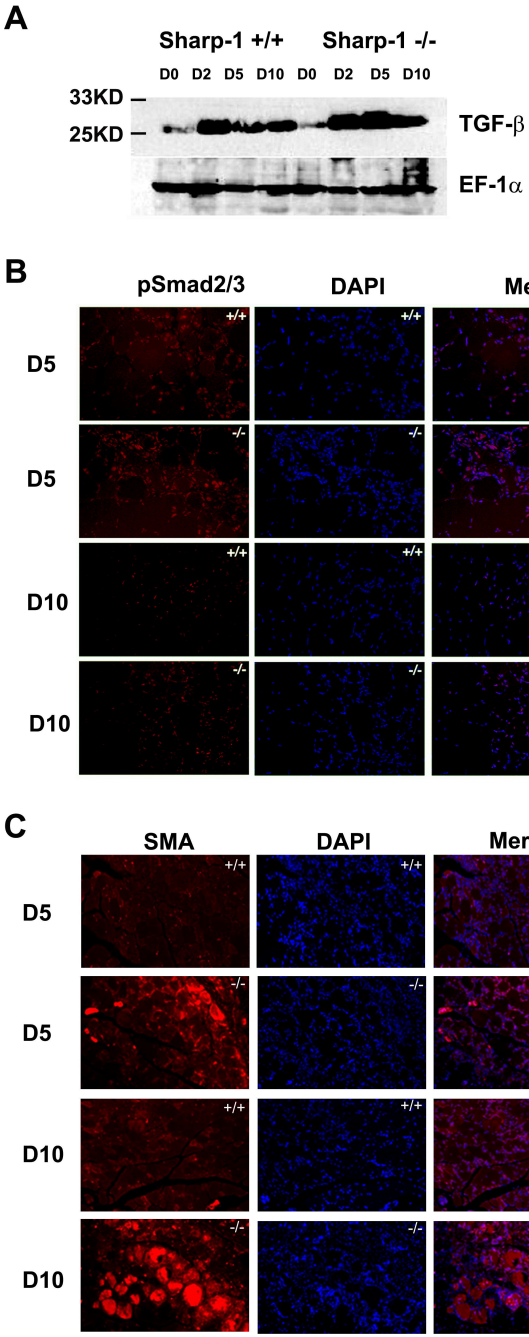

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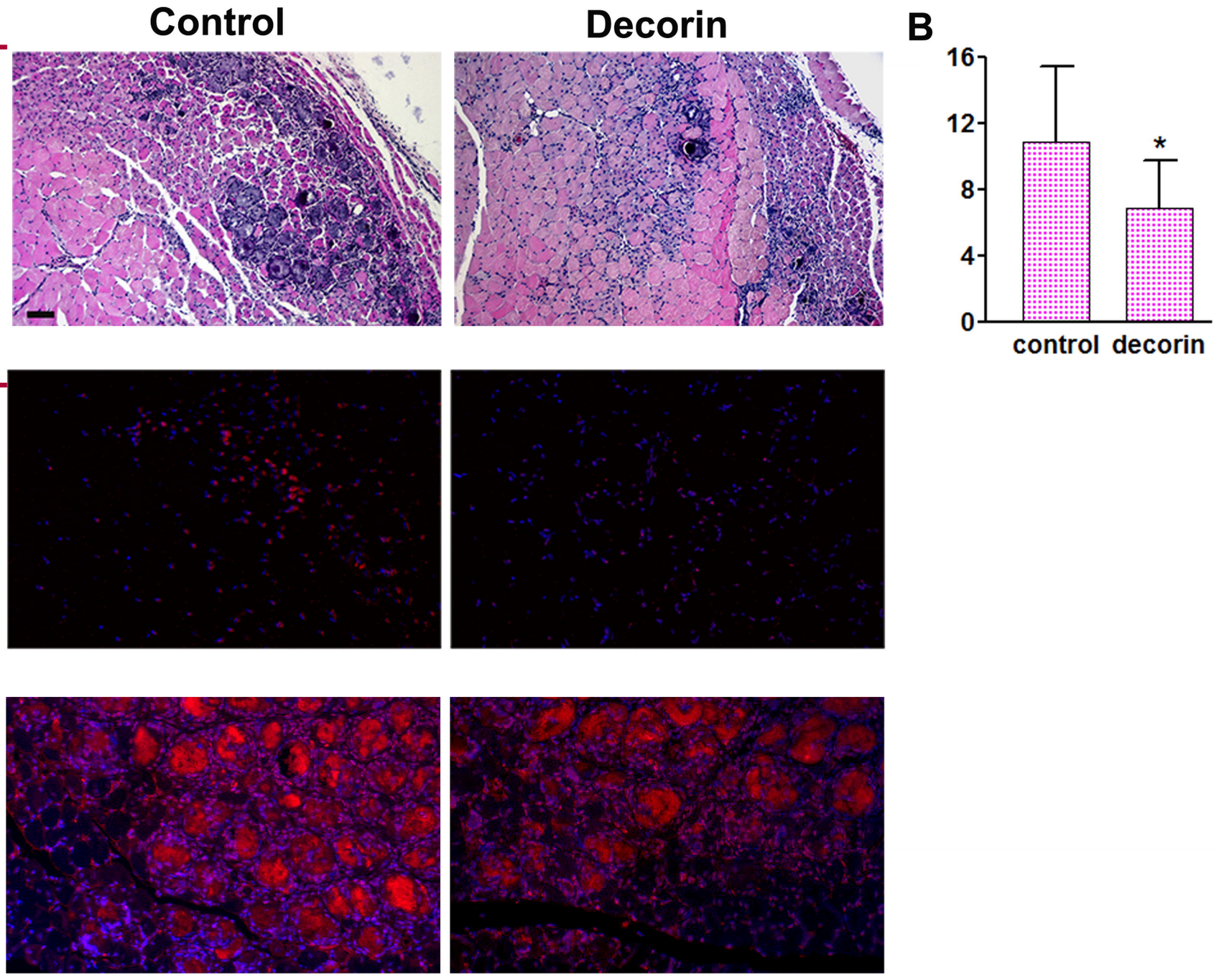

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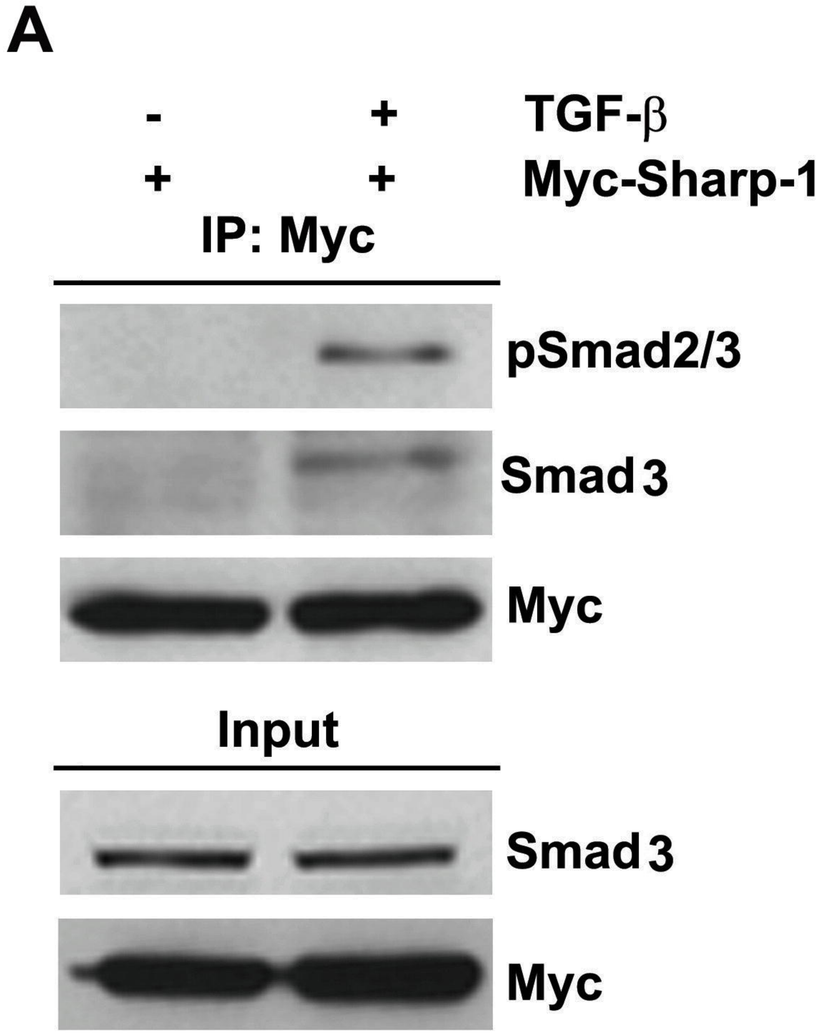
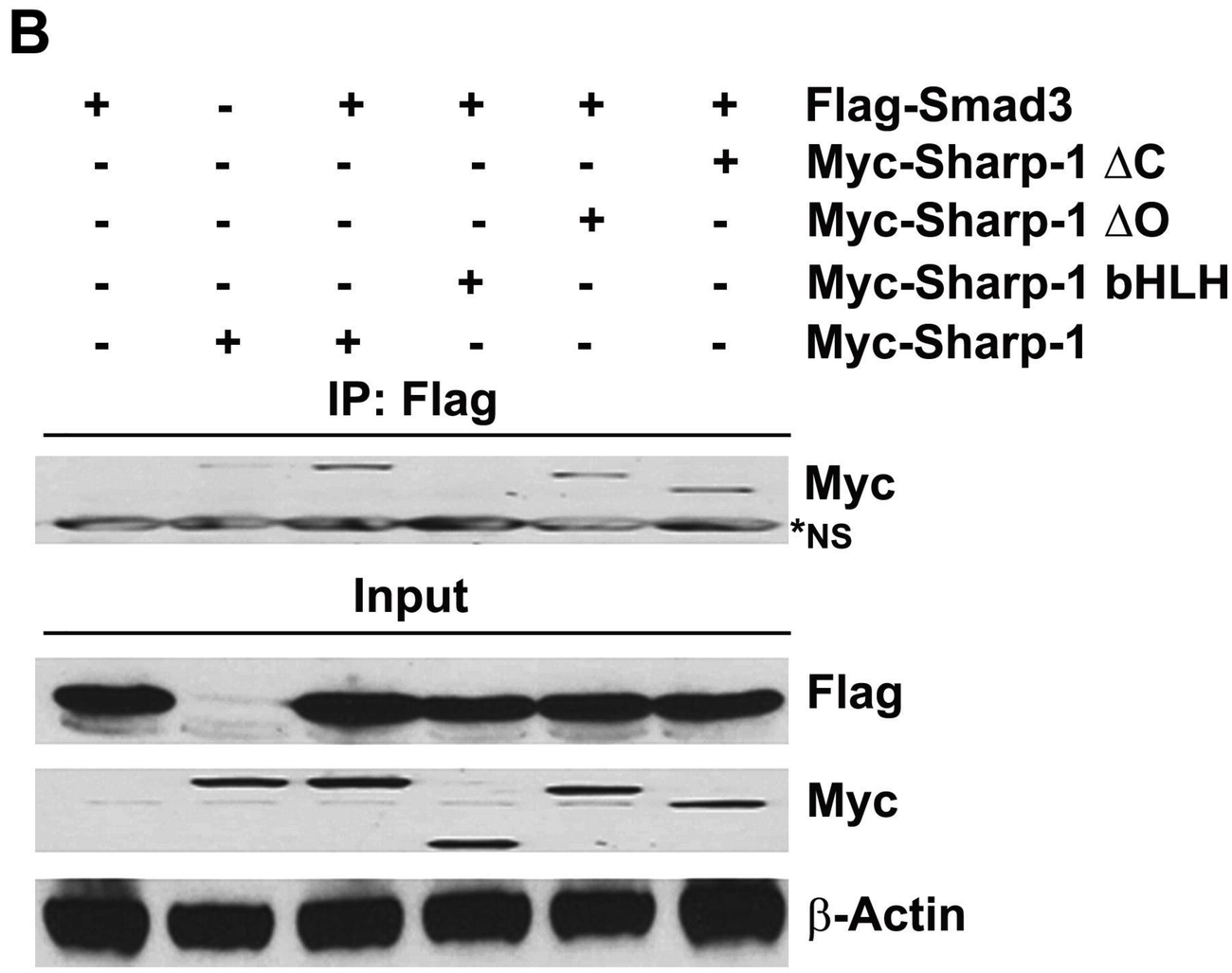
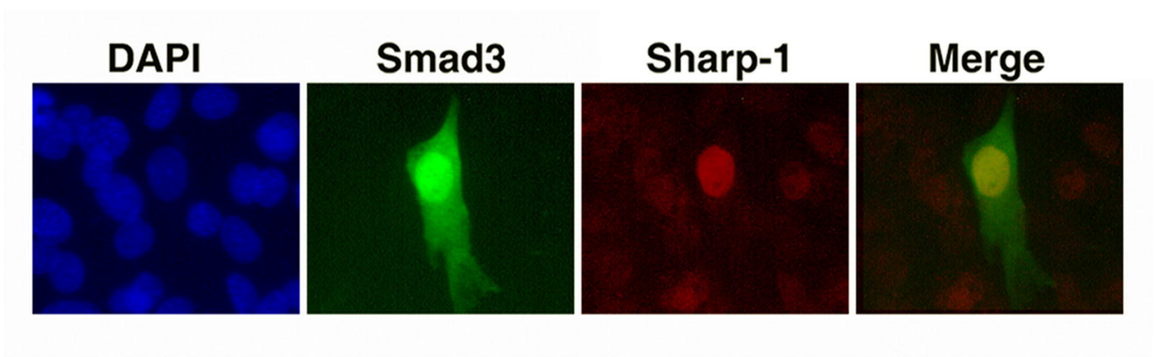






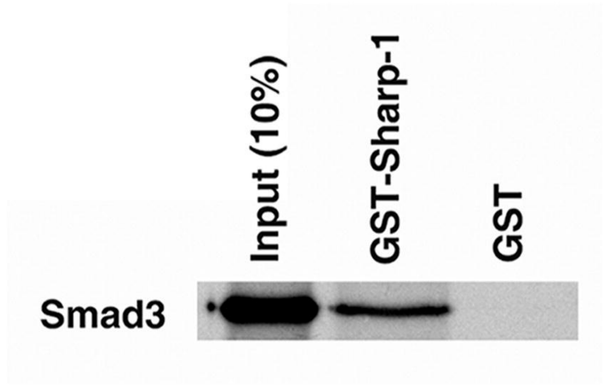
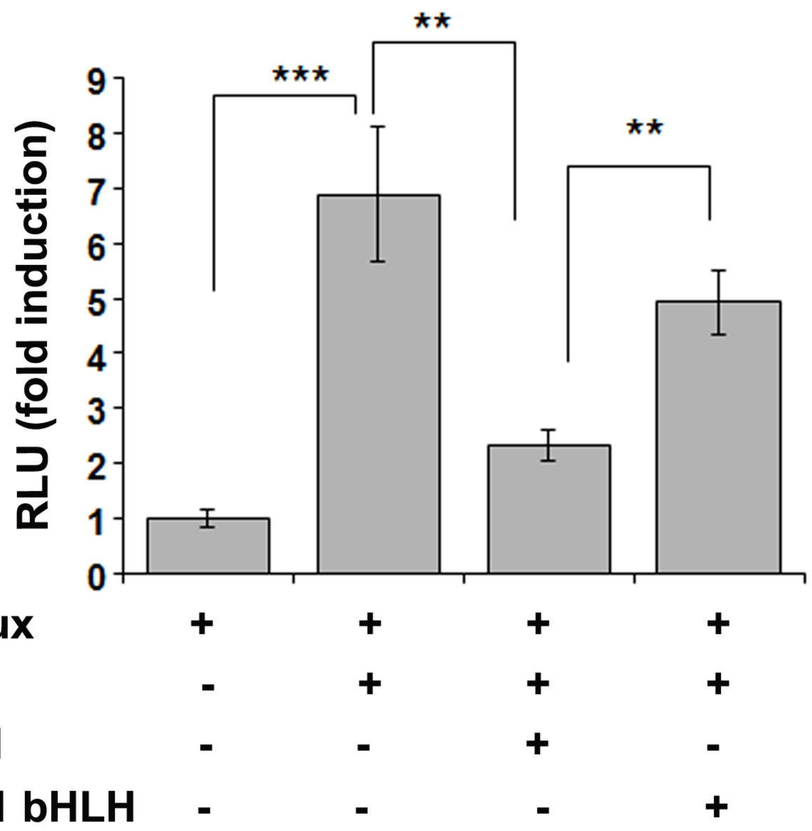


Fig 6.

A



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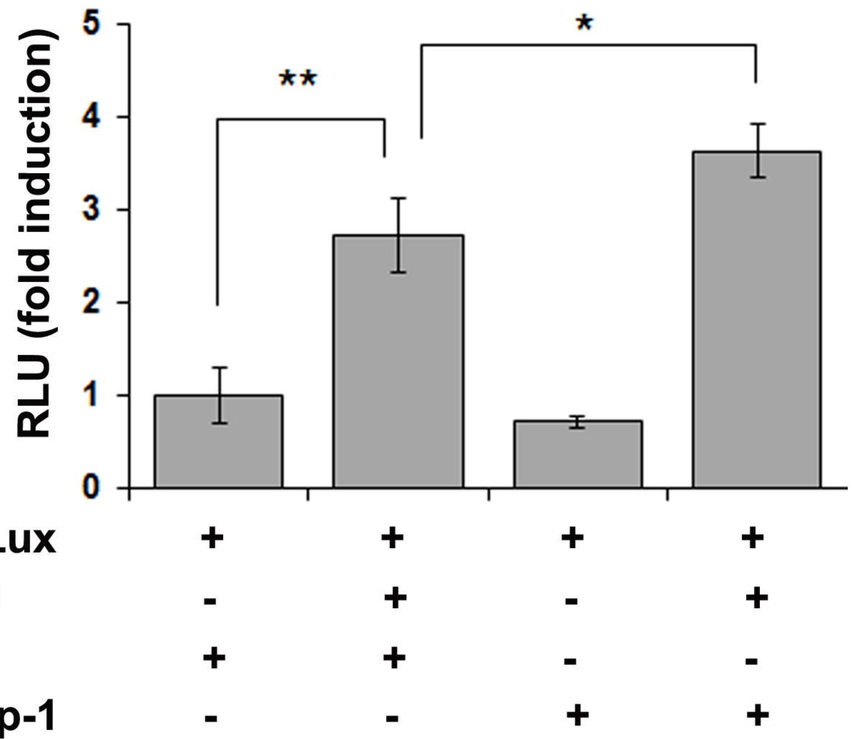

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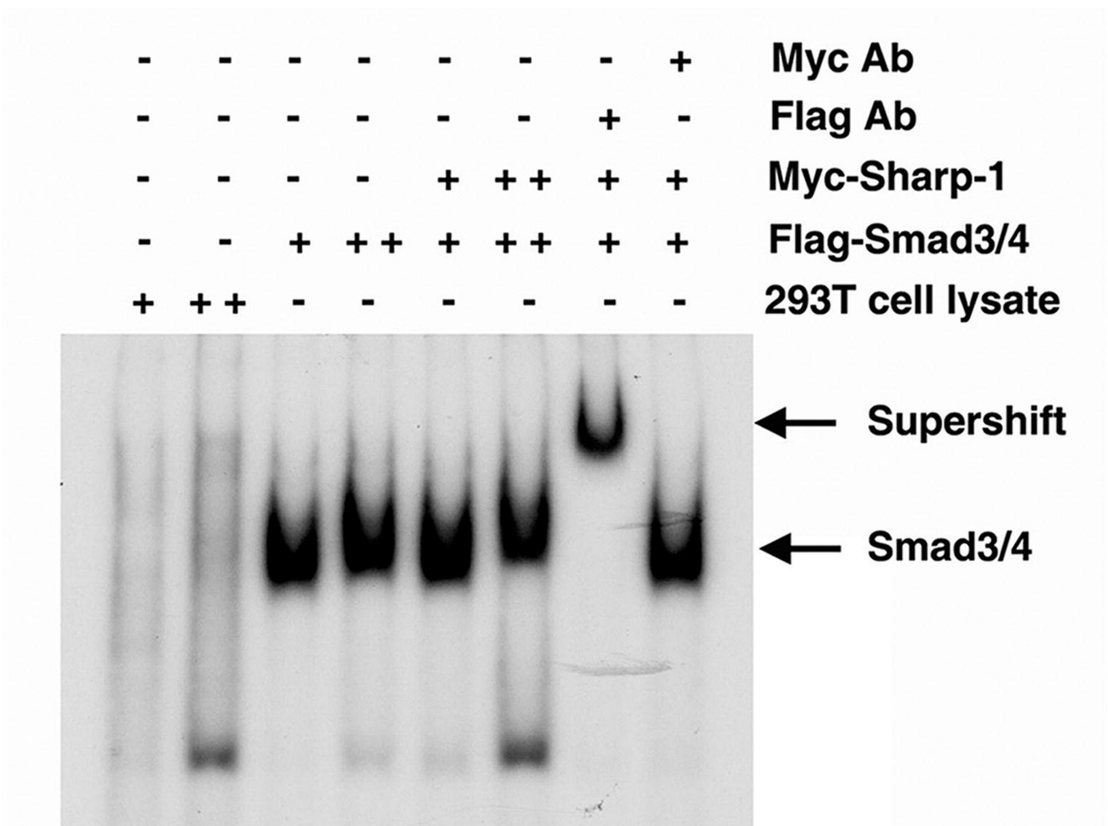

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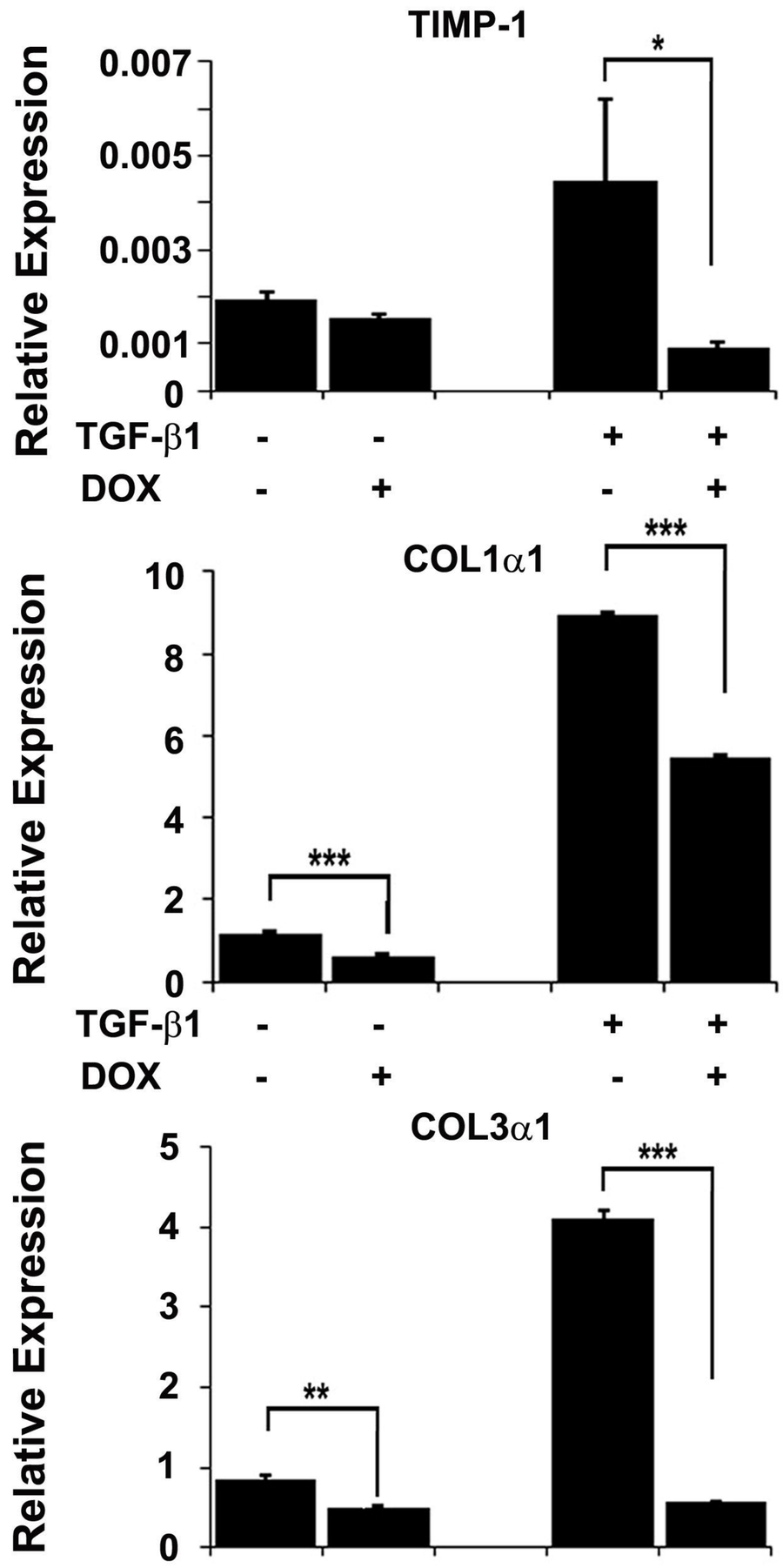

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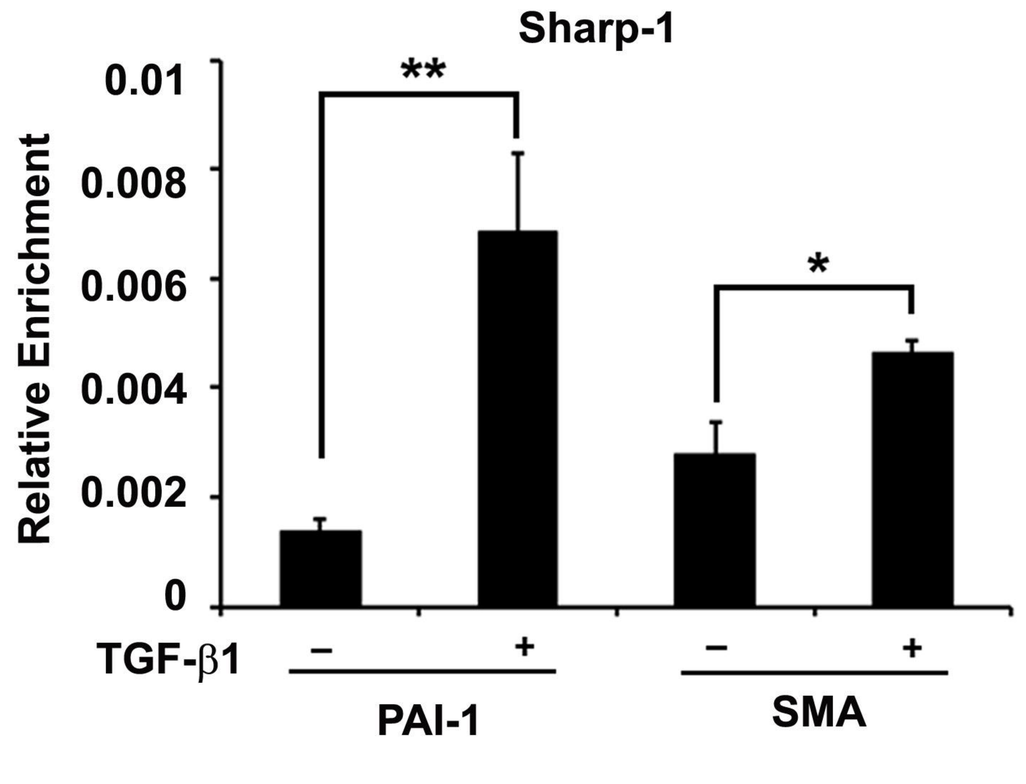

Fig 7.