Supporting Information

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SI Experimental Procedures

Cell Isolation, Treatments, and FACS Analysis. Briefly forelimbs and hind limbs were removed from neonatal mice and dissociated by enzymatic digestion using 2 mL of dispase (grade II, 2.4 U/mL; Roche) and collagenase B (1%; Roche), supplemented with $CaCl_2$ to a final concentration of 2.5 mM/mg tissue. Myoblasts were purified and grown in DMEM–Glutamax-I (Invitrogen) supplemented with 20% FBS, 10% horse serum (HS), 1% chicken embryo extract (CEE), 1% penicillin-streptomycin, 1% insulin-transferrin-selenium X, 10 ng/mL basic FGF, and 5 ng/mL insulin-like growth factor-1 (Invitrogen).

Tibialis anterior (TA) muscles were digested as described above, and unfixed cells were treated with anti-Cripto Allophycocianin (APC)-conjugated (R&D Systems) and anti-F4/80 FITC-conjugated (BD Biosciences) antibodies, along with the appropriate mouse IgG isotype control. Determination of cell surface expression of Cripto and F4/80 antigens was performed by means of a cytofluorimetric analysis assay using the FACSARIA cell-sorting system (BD Biosciences) and analyzed by means of DIVA software (BD Biosciences).

Immunohistochemistry. Muscles sections were treated as mentioned in *Experimental Procedures* for immunofluorescence and then incubated overnight with primary anti-Cripto (1) antibodies (7 μg/mL), followed by incubation with the secondary antibody goat anti-rabbit HRP-conjugated antibody (1:300; DakoCytomation) or rat anti-mouse F4/80 antibody (1:50; Serotec), followed by rabbit anti-rat biotinylated antibody (1:300; DakoCytomation) and streptavidin HRP-conjugated antibody (1:100; PerkinElmer). Slides were incubated using a Betazoid DAB Chromogen kit (Biocare Medical) following the manufacturer's instructions, and nuclei were labeled with hematoxilin (Dako) and mounted with Aquatex mounting agent (Merck).

Macrophage Quantitation in Regenerating Muscles. The number of infiltrated cells was counted in all the fields in the regeneration area using ImageQuant software (QWin; Leica) and is expressed as the mean number ± SEM per square millimeter.

ELISA. Ninety-six–well plates were coated with 0.5 ng/mL homemade anti-Cripto antibodies (2) in PBS (pH 7.5) overnight at 4 °C and were washed three times with PBS-Tween. Unbinding sites were blocked with 1% PBS-BSA (180 μL per well) for 2 h at room temperature (RT). After washing three times, the mouse sera (100 μL) or proteic muscles extract (300 μg) was added and incubated overnight at 4 °C. The plates were incubated with 1 μg/mL anti-Cripto biotinylated antibodies (R&D Systems) in PBS-Tween for 1 h at 37 °C and then for 1 h at RT. Finally, the plates were incubated for 1 h at RT with avidin/streptavidin complex conjugated with HRP (Vectastain elite ABC kit; Vector Laboratories). The plates were then developed with *o*-phenylenediamine peroxidase substrate (Sigma–Aldrich), and the absorbance was read at 490 nm on a Benchmark microplate reader (Bio-Rad Laboratories).

Mouse Models of Less Severe Muscle Damage. For the CTX model, muscle damage was induced as previously described (*Experimental Procedures*) by injection of 10 μL of cardiotoxin (10^{-5} M in PBS; Latoxan) in the TA muscle from 8-wk-old BALB/c mice.

Limb ischemia was induced by unilateral right ligation of the femoral artery and vein, distal from the branching site of the caudal femoral artery, and of the cutaneous vessels branching from the caudal femoral artery, sparing the femoral nerve.

Quantitative RT-PCR. Total RNA from the cells and/or skeletal muscles was extracted with a TRIzol Kit (Life Technologies) according to the manufacturer's instructions. Muscle tissue was homogenized with TissueLyser (Qiagen), and RNA was extracted with TRIzol and purified with an RNeasy mini kit (Qiagen). RNA was reverse-transcribed to cDNA with a QuantiTect Reverse Transcription Kit (Qiagen). cDNA samples synthesized from 1 μg of total RNA were subjected to PCR amplification or quantitative RT-PCR with the primers listed in Table S1.

Single-Fiber Culture Assays. Single myofibers were prepared from the extensor digitorum longus (EDL) muscles from 6-wk-old C57/Bl6 or Myf5nLacZ/$^{+}$ mice (3, 4) as described (5). Briefly, muscles were dissected and digested in 2% (wt/vol) collagenase type 1 (Sigma–Aldrich) in DMEM (Gibco) for 1.5 h in a 35 °C water bath. Myofibers were isolated by gentle trituration of the muscle using a customized heat-polished Pasteur pipette. Individual intact myofibers were washed by serially transferring them through three dishes of warmed DMEM supplemented with 4 mM L-glutamine (Sigma–Aldrich) and a 1% penicillin and streptomycin solution (Sigma–Aldrich). They were then plated on matrigel-coated wells of a 24-well plate in proliferating medium (20% FBS, 10% HS, and 1% CEE in DMEM) or in HS-coated, round-bottomed Eppendorf tubes and incubated with or without soluble recombinant mouse Cripto (see above) for 72 h in low-activation medium (10% HS and 0.5% CEE in DMEM) and infected at a multiplicity of infection (MOI) of 10 with a lentiviral vector (Lenti-Cripto-Ires-GFP) encoding a membrane-bound form of Cripto (mCripto, Lenti-mCripto) or a control Lenti-GFP (Lenti-Control) overnight in proliferation medium. The Cripto lentiviral vector (in pRRLsin.PPT.CMV.NTRiresGFPpre) was generated and prepared as described previously (6). The final MOI was 5×10^7 transducing unit (TU)/mL.

Immunofluorescence staining on C57/Bl6 fibers was performed after 72 h of treatment using antibodies specific for MyoD (1:50; Dako or Santa Cruz Biotechnology), Pax7 [1:10; Developmental Studies Hybridoma Bank (DSHB)] (7), myogenin (1:50; BD Pharmingen), and Ki67 (1:250; Abcam) (8).

Alternatively, Myf5nLacZ/$^{+}$ myofibers were plated on a feeder layer of mammalian cells expressing membrane-bound Cripto or a mock vector in 24-multiwell plate in proliferating medium (20% FBS, 10% HS, and 1% CEE in DMEM). LacZ staining was performed after 72 h to identify activated satellite cells on the fibers and those that have left the fibers.

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4. Montarras D, et al. (2005) Direct isolation of satellite cells for skeletal muscle regeneration. *Science* 309(5743):2064–2067.
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8. Abou-Khalil R, et al. (2009) Autocrine and paracrine angiopoietin 1/Tie-2 signaling promotes muscle satellite cell self-renewal. *Cell Stem Cell* 5(3):298–309.

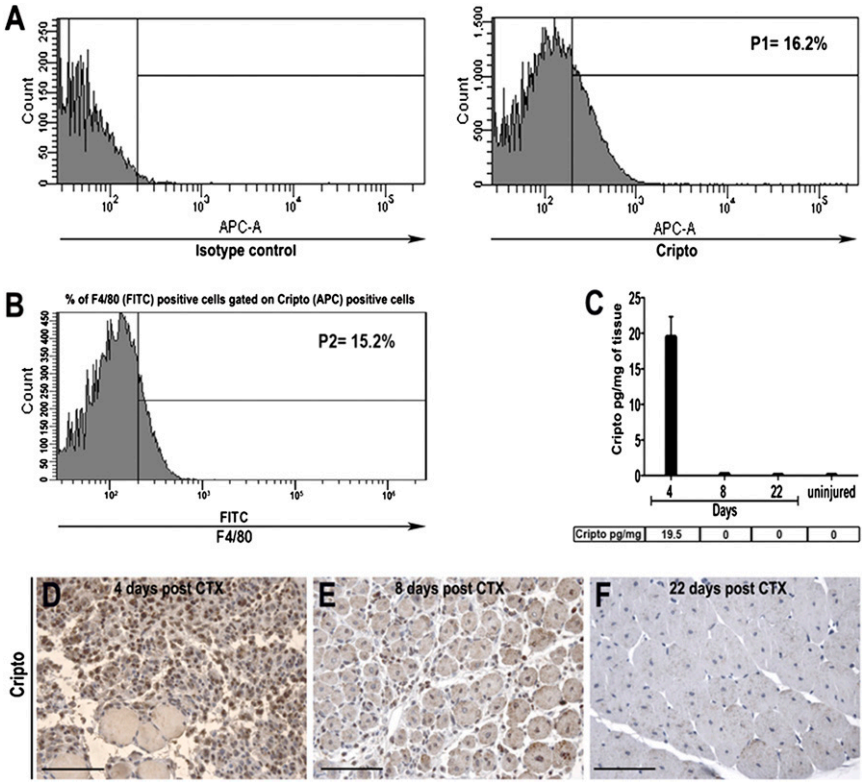


Fig. S1. Cripto expression decreases as the regeneration process proceeds (related to Fig. 1). (*A*) Cripto expression in living cells derived from digested WT muscle at day 4 after cardiotoxin (CTX) injection. (*Left*) FACS histogram plot of cells stained with isotype-matched antibodies as a negative control. (*Right*) Cripto$^+$ cells were gated using Cripto APC-conjugated antibodies [Allophycocyanin-A (APC-A); P1 population], indicating that 16.2% of the cell population expressed Cripto. (*B*) Histogram of F4/80$^+$ (FITC) cells (P2 population) gated on the P1 population (Cripto APC) shows that 15.2% of Cripto$^+$ cells are macrophages. (*C*) ELISA-based assay measuring Cripto protein level in total protein extract of muscles at different time points after CTX injection; the average amount (pg/mg) per muscle is plotted for each group at the indicated time points. Endogenous Cripto protein was detected in muscle tissue extracts at day 4 after injury (~20 pg/mg), whereas it decreased beyond detection levels from day 8 onward. (*D–F*) Representative photos of CTX-injured muscles at indicated days after injury, stained by immunohistochemistry with anti-Cripto antibodies revealed by DAB coloration, show that Cripto expression decreases during regeneration. Muscles are counterstained with hematoxylin. (Scale bars = 50 μm.)

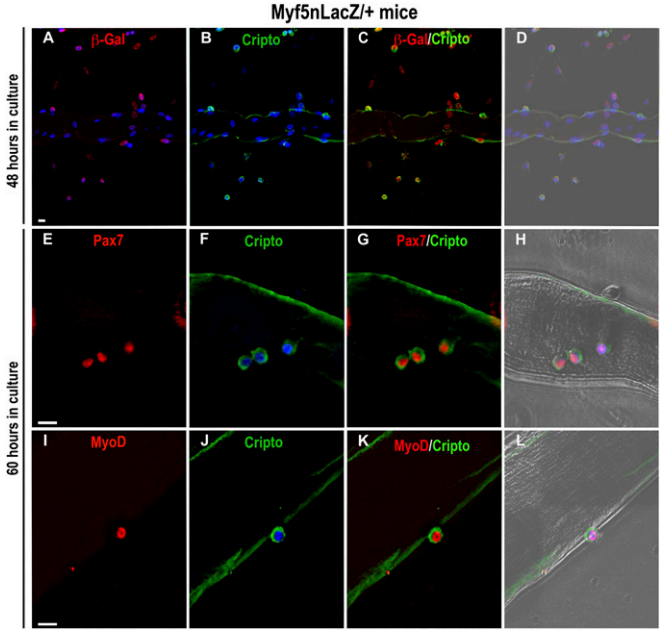
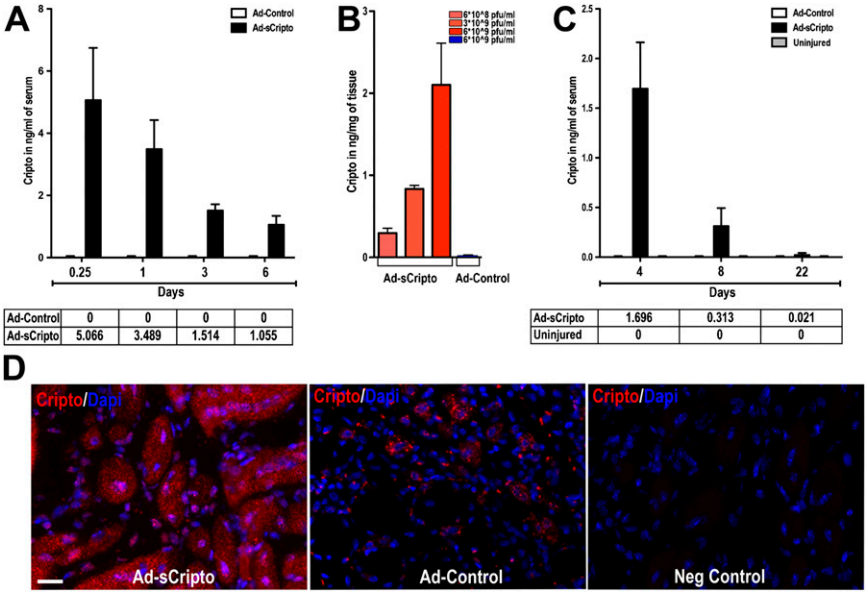


Fig. S2. Cripto expression persisted in proliferating activated satellite cells (related to Fig. 2). Cripto staining on teased myofibers isolated from Myf5-LacZ mice at 48 h (*A–D*) or 60 h in culture (*E–L*) shows coexpression of Cripto with β-gal/Myf5 (*A–C*), Pax7 (*E–G*), and MyoD (*I–K*). All the images are superimposed on a phase-contrast image (*D*, *H*, and *L*). (Scale bars = 50 μm.)



Ad-Control	0	0	0	0
Ad-sCripto	5.066	3.489	1.514	1.055

Ad-sCripto	1.696	0.313	0.021
Uninjured	0	0	0

Fig. S3. Adenovirus-mediated soluble Cripto (sCripto) overexpression in vivo (related to Fig. 4). (*A–D*) sCripto overexpression in cardiotoxin (CTX)-injected skeletal muscles infected with either adenovirus (Ad)-sCripto or Ad-Control. (*A*) ELISA-based assay measuring sCripto protein level in mice serum at different time points after adenovirus delivery; the average amount of serum (ng/mL) is plotted for each group at the indicated time points. (*B*) ELISA-based assay measures sCripto protein level in total protein extract of skeletal muscles harvested 5 d after CTX injection. Injured muscles were injected with increasing concentrations of Ad-sCripto ($6{\cdot}10^8$–$6{\cdot}10^9$ pfu/mL). Adenovirus encoding empty vector has been used as a control (Ad-Control) at the highest concentration ($6{\cdot}10^9$ pfu/mL). Ad-Control 0 vs. Ad-sCripto: 0.23 ± 0.06 ng/mg at $6{\cdot}10^8$ pfu/mL, 0.83 ± 0.04 ng/mL at $3{\cdot}10^9$ pfu/mg, and 2.12 ± 0.5 ng/mg at $6{\cdot}10^9$ pfu/mL. Values are mean ± SEM; $n = 3$ mice per time point. (*C*) ELISA-based assay measures sCripto protein level in the serum: Cripto protein is undetectable in Ad-Control–infected mice, whereas it accumulates in Ad-sCripto–treated mice, decreasing with time. Cripto was undetectable in the serum of uninjured mice. (*D*) Immunofluorescence analysis on skeletal muscle sections at day 8 after CTX injection in the same mice shows Cripto overexpression in Ad-sCripto–treated mice compared with Ad-Control and negative control of immunofluorescence. Nuclei were stained with DAPI. (Scale bars = 50 μm.)

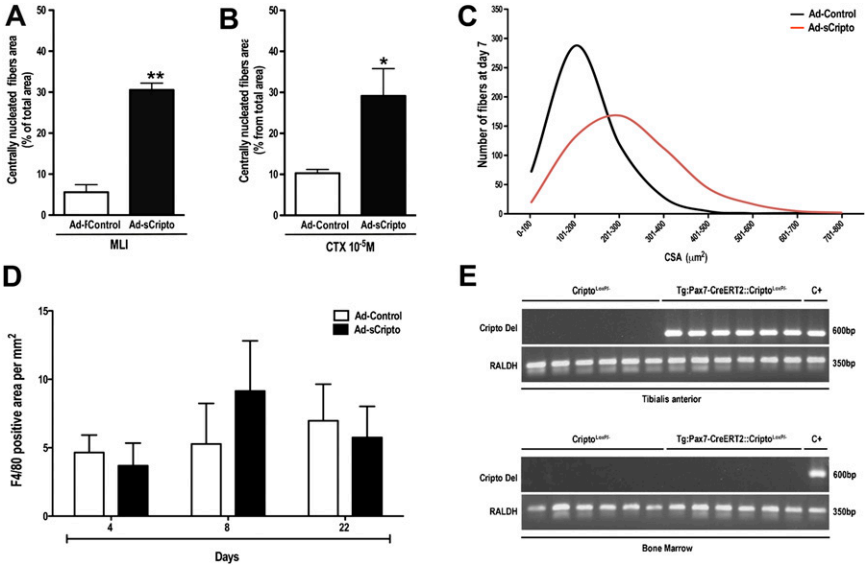


Fig. S4. Cripto overexpression enhances muscle regeneration after injury and does not greatly affect inflammation (related to Figs. 4 and 5). Centrally nucleated myofibers significantly increased in adenovirus (Ad)-soluble Cripto (sCripto)–treated muscles compared with control (Ad-Control) in mouse models of hind limb ischemia [Mild Limb Ischemia (MLI)] 7 d after ligation (*A*) or with administration of cardiotoxin (CTX) (10^{-5} M) 7 d after injury (*B*). Results are expressed as a percentage of the total section area. Values are the mean ± SEM; 5 mice per group; $^{**}P = 0.002$ for MLI; $^{*}P < 0.05$ for CTX. (*C*) Cross-sectional area (CSA) analysis of regenerated fibers in a model of less severe muscle damage (10^{-5} M CTX) shows increased myofiber size in Ad-sCripto–treated mice compared with control mice. (*D*) Quantitative analysis of F4/80$^+$ cell area in mice treated with Ad-sCripto vs. Ad-Control at days 4, 8, and 22 ($3.7 \pm 2\%$ after Ad-sCripto vs. $4.6 \pm 1\%$ after Ad-Control on day 4; $9.1 \pm 4\%$ after Ad-sCripto vs. $5.2 \pm 3\%$ after Ad-Control on day 8; $5.7 \pm 2\%$ after Ad-sCripto vs. $6.9 \pm 3\%$ after Ad-Control on day 22; $n = 5$ mice per group, P = not significant (NS) and $n = 5$ mice per group, P = NS, respectively). Values are mean ± SEM; $n = 5$ mice per time point. (*E*) PCR genotyping of TA muscle and bone marrow DNA from *Tg:Pax7-CreERT2::Cripto*$^{loxP/-}$ and *Cripto*$^{loxP/-}$ mice treated with tamoxifen. *Cripto* Del, *Cripto* floxed allele; RALDH, retinaldehyde dehydrogenase.

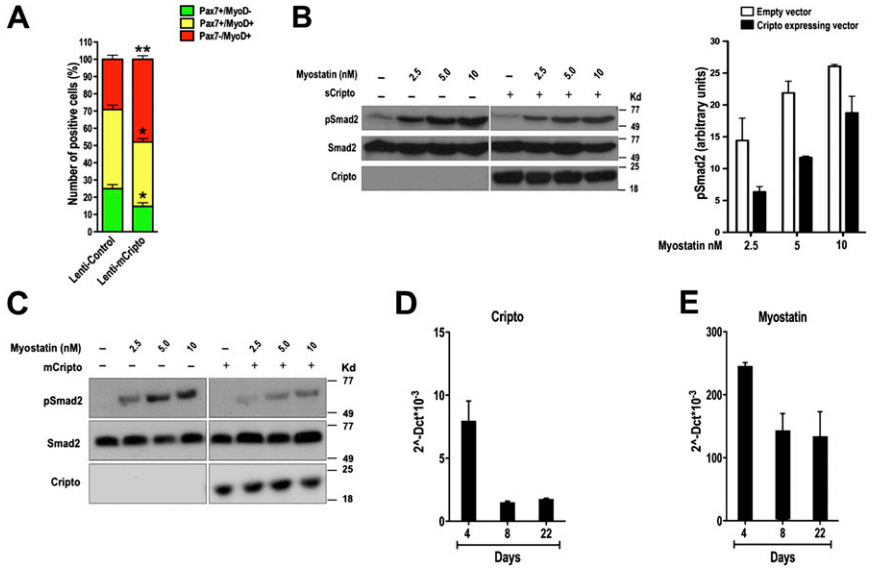


Fig. S5. GPI-anchored membrane Cripto protein (mCripto) promotes myoblast proliferation and antagonizes the myostatin/GDF8 signaling pathway (related to Figs. 6 and 7). (*A*) Coimmunostaining of isolated myofibers infected with lentivirus (Lenti)-mCripto or Lenti-Control after 72 h in culture shows an increased number of $Pax7^{-}/MyoD^{+}$ myogenic cells in Lenti-mCripto fibers compared with control fibers (red bars; $n = 3$ independent experiments; $^{*}P < 0.01$ and $^{**}P < 0.001$ compared with control). (*B* and *C*) Cripto antagonizes the GDF8 signaling pathway. (*B*) (*Left*) Representative Western blot analysis of total lysates of C2C12, transfected with empty vector or soluble Cripto (sCripto)-vector and treated with increasing doses of recombinant GDF8 protein (5–10 nM; R&D Systems). (*Right*) Densitometric analysis is expressed in arbitrary units as the phospho (P)-Smad2/Smad2 ratio, and is representative of two experiments. (*C*) Representative Western blot analysis of total lysates of 293 cells, transfected with empty vector or mCripto-vector and treated with increasing doses of recombinant GDF8 protein (2.5–10 nM; R&D Systems). Anti–P-Smad2, -Smad2 or -Cripto antibodies were used. (*D*) Expression of Cripto. (*E*) Myostatin in TA muscles at days 4, 8, and 22 after cardiotoxin injection, as shown by quantitative RT-PCR. mRNA expression was normalized to β-tubulin expression; data are mean ± SE, $n = 3$ mice per group. See also Table S2.

Table S1. Primer sequences used for genotyping strategies

Genotype	Primer (forward) 5′-3′	Primer (reverse) 5′-3′
Pax7CreERT2	GAATTCCCCGGGGAGTCGCATCCTG	CCACACCTCCCCCTGAACCTGAAAC
LoxPI^{-}	TCTGCACTGGGGCTAAACCTTATG	1.GCCAAGAGCCATGACAGAGATGG 2. AGCGCATGCTCCAGACTGCCTT
Cripto-del	AGCCATCTCACCAGCCTTCA	CATCTGGGACATGCCCACTA
raldh	TATCTGGACAGTGGTTAAGG	CCCAGCCTGCATAATACCTC

This table is related to Fig. 3. *Cripto-del*, *cripto* floxed allele; *raldh*, retinaldehyde dehydrogenase.

Table S2. Primer sequences used for quantitative RT-PCR and PCR analysis

Gene	Primer (forward) 5′-3′	Primer (reverse) 5′-3′
beta-tubulin	GGGAGGTGATAAGCGATGAA	CCCAGGTTCTAGATCCACCA
nmyhc	GAACTTGAAGGAGAGGTCGA	CACCTTCGCCTGTAATTTGTC
cripto	TGTTCGCAAAGAGCACTGTGG	TGAGGTCCTGGTCCATCACTTGAC
myostatin	TGTAACCTTCCCAGGACCAG	TCTTTTGGGTGCGATAATCC

This table is related to Fig. 4 and Fig. S5. *nmyhc*, neonatal myosin heavy chain.