Cripto regulates skeletal muscle regeneration and modulates satellite cell determination by antagonizing myostatin

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Skeletal muscle regeneration mainly depends on satellite cells, a population of resident muscle stem cells. However, our understanding of the molecular mechanisms underlying satellite cell activation is still largely undefined. Here, we show that Cripto, a regulator of early embryogenesis, is a novel regulator of muscle regeneration and satellite cell progression toward the myogenic lineage. Conditional inactivation of cripto in adult satellite cells compromises skeletal muscle regeneration, whereas gain of function of Cripto accelerates regeneration, leading to muscle hypertrophy. Moreover, we provide evidence that Cripto modulates myogenic cell determination and promotes proliferation by antagonizing the TGF-β ligand myostatin. Our data provide unique insights into the molecular and cellular basis of Cripto activity in skeletal muscle regeneration and raise previously undescribed implications for stem cell biology and regenerative medicine.

It is now evident that genes and molecular mechanisms, which have key roles during embryogenesis, are reactivated in the adult during tissue remodeling and regeneration and that when deregulated, they may contribute to cancer progression (1). The cripto gene has emerged as a key player in this complex scenario. Cripto is a GPI-anchored protein and the founder member of a family of signaling molecules, the EGF-CFC proteins, important for vertebrate development (2). Cripto is associated with the pluripotent status of both human and mouse ES cells (ESCs) (3), and it acts as a key player in the signaling networks orchestrating ESC differentiation (4). Intriguingly, it has been recently suggested that Cripto may serve as a regulator to control dormancy of hematopoietic stem cells (5).

Under normal physiological conditions, Cripto is expressed during embryonic development (2), and it has been shown to have activity both as a soluble factor and as a GPI-anchored protein (6–8). Existing models indicate that Cripto can function via different signaling pathways. Cripto plays distinct and opposing roles in modulating the activity of several TGF-β ligands. Indeed, as an obligate coreceptor, Cripto binds Nodal and GDF1/GDF3 and stimulates signaling through the activin receptor complex composed of type I serine-threonine ActRIIB (ALK4) and type II receptor (ActRII/ActRIB) (9–11). Following receptor activation, the intracellular effectors Smad2 and/or Smad3 are phosphorylated and accumulate in the nucleus with Smad4 to mediate transcriptional response (12). In contrast to its coreceptor function, Cripto is able to antagonize signaling of other members of the TGF-β family (i.e., activins and TGF-β). This inhibitory activity of Cripto results in a reduced ability to form an active ActRII/ActRIB receptor complex (13–15).

Despite the well-described role of Cripto in early development and ESC differentiation, the role of this protein in postnatal life remains elusive. To date, de novo expression of Cripto has been associated with several epithelial cancers (16, 17), but its role in other pathological conditions, such as injury or degenerative diseases, has not been investigated. Given the physiological activity of Cripto in the instructive events of embryonic mesodermal commitment and differentiation (4), we hypothesized that Cripto expression might be reactivated in response to injury in mesenchymal tissues, such as skeletal muscles.

Adult skeletal muscle generally has a low cellular turnover rate. However, in response to certain pathological conditions, it undergoes robust regeneration. Regeneration is mainly dependent on satellite cells, a population of resident stem cells that are in a quiescent state during muscle homeostasis. After injury or disease, satellite cells become activated, proliferate, migrate to the site of injury, and either fuse to form multinucleated myotubes or reestablish a self-renewing pool of quiescent satellite cells (18). Quiescent satellite cells express the transcription factor Pax7, which is involved in myogenic specification (19, 20). Following injury, activated satellite cells start proliferating and expressing MyoD, whereas Pax7 expression is progressively reduced. Subsequently, expression of myogenin and MRF4 (muscle regulatory factor 4 or muscle regulatory transcription factor 4) is up-regulated as cells enter their terminal differentiation program. A fraction of activated cells down-regulate expression of MyoD and return to cellular quiescence to maintain a pool of satellite cells (21). A delicate balance between satellite cell proliferation and exit from cell cycle,
differentiation, and fusion is required for the correct muscle regeneration to occur. Although some signaling molecules have been found to play a crucial role in these processes (11), including hepatocyte growth factor (22), insulin-like growth factors (23), myostatin (24), and Wnts (25), the underlying molecular mechanisms of muscle regeneration remain largely undefined.

In the present study, we provide evidence that Cripto is reexpressed in adult skeletal muscle in response to injury and that this response correlates with and regulates muscle regeneration. We also show that Cripto is expressed in activated satellite cells and promotes myogenic cell determination and proliferation by antagonizing TGF-β ligand myostatin.

**Results**

**Cripto Is Expressed During Skeletal Muscle Regeneration and in Myogenic Cells in Vivo and ex Vivo.** To evaluate whether Cripto is activated in adult tissues under pathological conditions, we performed double immunofluorescence analysis for Cripto and laminin on normal adult skeletal muscle both during homeostasis and after cardiotoxin (CTX)-induced injury. Cripto was undetectable in actively dividing proliferating muscle satellite cells and in macrophages (Fig. 1 A–C); in contrast, strong expression of Cripto was observed in the regenerating area both inside and outside of the basement membrane surrounding myofibers (Fig. 1 D–F). Cripto expression was confirmed by flow cytometry (FACS) analysis (Fig. S1A). Notably, expression of cripto progressively decreases during the regeneration process (Fig. S1 C–F). To assess whether different cell types that take part in the regeneration process expressed Cripto, we performed double staining with specific markers. Immunofluorescence analysis revealed that Cripto was expressed in myogenic cells, as indicated by coexpression with MyoD (Figs. 1 G–I). In addition, double staining with F4/80, a macrophage-specific membrane antigen, showed that Cripto was expressed in inflammatory cells (Fig. 1 J–L). FACS analysis on dissociated muscle cells confirmed that at day 4 after CTX injection, 15.2% of Cripto+ cells are F4/80+ (Fig. S1B).

Expression of cripto during muscle regeneration and in satellite cell progeny after activation raised the intriguing possibility that cripto might play a role in regulating myogenic cell behavior. To address this issue in more detail, we used single-myofiber preparations isolated from WT myofibers (26) and performed a time course immunofluorescence analysis for Cripto, Pax7, and MyoD. Immediately after plating at time 0 (T0), Cripto expression was undetectable in Pax7+ and MyoD+ satellite cells (Fig. 2 A–E). Interestingly, Cripto started to be detected, along with Pax7 and MyoD (Fig. 2 F–J′), as early as after 24 h (T24), persisting after 48 h (T48) in culture (Fig. 2 K–O). We then extended our analysis using myofibers isolated from Myf5lacZ+ mice (27), which express a nuclear localized lacZ (lacZ) reporter gene targeted to the Myf5 locus (28). Double staining for Cripto and β-galactosidase (β-gal) showed Cripto expression, along with β-gal expression (Fig. S2 A–D). Moreover, Cripto expression persisted in satellite cells detaching from the fibers after 60 h in culture (Fig. S2 E–I).

Taken together, our data provide evidence that Cripto is expressed in activated satellite cells committed to the myogenic lineage, persisting in proliferating transient amplifying myoblasts.

**Conditional Targeted Deletion of Cripto in Adult Satellite Cells Affects Skeletal Muscle Regeneration.** These results prompted us to evaluate whether Cripto might have a physiological role in skeletal muscle regeneration in vivo, using a loss-of-function approach. Cripto null mutants die during early embryonic development (29); we thus used a Cre-Lox strategy to obtain conditional cripto deletion in adult mice. Moreover, to distinguish between the relative roles of Cripto in inflammatory cell and satellite cell contributions during this process, we generated a unique mouse model for conditional inactivation of cripto in satellite cells, TgPax7-CreERT2:Cripto<sup>−/−</sup> mice, by crossing Cripto<sup>−/−</sup> mice with a tamoxifen-inducible TgPax7-CreERT2 transgenic line (30). TgPax7-CreERT2:Cripto<sup>−/−</sup> adult mice were treated with tamoxifen or vehicle, as a control, once a day for 5 d; at day 4, tibialis anterior (TA) muscles were injected locally with CTX, and the effect on muscle regeneration was evaluated at days 4 and 15 after CTX injection (Fig. 3A). To verify the tissue-specific expression of Cre, we isolated and genotyped the contralateral uninjured TA muscle and the bone marrow of both tamoxifen- and vehicle-treated mice (Fig. 3B). As expected, the cripto-deleted specific band was detected in the contralateral uninjured TA muscle of the tamoxifen-treated mice but not the control mice. Notably, the cripto-deleted band was absent in the bone marrow genomic DNA (Fig. 3B), thus confirming that cripto deletion occurred selectively in skeletal muscle cells. Accordingly, Cripto protein levels decreased in muscle tissue of tamoxifen-treated mice compared with control mice at day 4 after injury, as shown by ELISA assays (Fig. 3C). Using these mice, we stained sections of the CTX-injected TA muscles with H&E to perform morphometric analysis (Fig. 3D). Remarkably, the myofiber cross-sectional area (CSA) was significantly reduced in the tamoxifen-treated mice compared with control mice (Fig. 3 E and F). Given that Cripto is also expressed in macrophages, our data provide direct evidence for a role of Cripto specifically in adult myogenic cells during skeletal muscle regeneration.

**Cripto Overexpression Accelerates Skeletal Muscle Regeneration and Induces Myofiber Hypertrophy in Vivo.** We next investigated whether Cripto might modulate acute skeletal muscle regeneration in vivo, using a gain-of-function approach. To do so, we generated a replication-deficient adenovirus, adenovirus (Ad)-soluble Cripto (sCripto), that can be used to overexpress a biologically active sCraig protein (31) in skeletal muscle. We first evaluated whether sCripto was sufficiently expressed on Ad-sCripto gene transfer by measuring...
Cripto protein levels in both muscles and serum. To this end, TA muscles were injected with CTX, along with either Ad-sCripto or Ad-Control (encoding an empty vector); mice were killed at different time points, and sCripto serum levels were determined using a sandwich ELISA-based assay. As early as 6 h after virus injection, sCripto was detectable in the serum of Ad-sCripto–infected mice (~5 ng/mL), which progressively decreased to reach a level of ~1 ng/mL after 6 d (Fig. S3A); by contrast, sCripto was undetectable in the serum of mice infected with Ad-Control. Finally, dose-dependent Cripto overexpression was also detected in Ad-sCripto–transduced muscles after 5 d, confirming that sCripto was efficiently expressed on Ad-sCripto gene transfer (Fig. S3F).

To analyze the overall effect of sCripto overexpression on muscle regeneration, we triggered skeletal muscle regeneration by injecting high doses of CTX (32) in WT TA muscles infected with Ad-Control or Ad-sCripto. Mice were killed 4, 8, and 22 d after CTX and adenovirus injection. We first verified Cripto overexpression in the serum and skeletal muscles by ELISA and immunofluorescence analysis, respectively (Fig. S3 C and D). Muscle sections were then stained with H&E for the morphological and morphometric analysis (Fig. A4); muscle regeneration was assessed and expressed as a percentage of the area of centrally nucleated fibers compared with the total muscle section area (Fig. 4B) at each time point. At day 4 after injections, we did not find any significant difference between Ad-Control and Ad-sCripto–infected muscles [18.7 ± 3% after Ad-sCripto vs. 19.9 ± 4.5% after Ad-Control; n = 5; P = not significant (NS)]; by contrast, 8 d after the CTX injury, Ad-sCripto–infected muscles clearly exhibited more robust regeneration than control muscles (64 ± 11% after Ad-sCripto vs. 15 ± 1.2% after Ad-Control; n = 5 mice; **P = 0.004; Fig. 4A and B). By day 22 after injury, although the regeneration process was nearly completed in both conditions, muscle regeneration was still significantly improved in Ad-sCripto–treated mice (92 ± 4.6% after Ad-sCripto vs. 76 ± 4.5% after Ad-Control; n = 5 mice; *P = 0.04; Fig. 4A and B). Comparable results were obtained in models of less severe muscle damage [i.e., femoral artery ligation (Mild Limb Ischemia [MLI]) and lower doses of CTX] (Fig. S4 A–C). In accordance with these findings, muscles overexpressing Cripto also showed reduced necrotic areas compared with control muscles (36 ± 11% after Ad-sCripto vs. 85 ± 1.2% after Ad-Control; n = 5 mice; **P = 0.004; Fig. 4C). Moreover, the accelerated regeneration was accompanied by high expression in

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**Fig. 2.** Cripto is expressed in activated/proliferating satellite cells. Cripto staining with Pax7 and MyoD on teased myofibers isolated from C57BL/6 mice at different time points in culture: 0 h (A–E), 24 h (F–J, T24), and 48 h (K–O, T48). The Cripto staining images are superimposed on a phase-contrast image (L, J, and O). (Insets, A–O) Higher magnifications of myofibers. (Scale bars = 25 μm and 50 μm.) See also Fig. S2.

**Fig. 3.** Conditional targeted deletion of cripto in satellite cells impairs muscle regeneration after acute muscle damage. (A) Schematic representation of conditional loss of function of Cripto using Tg:Pax7-CreERT2::CriptoloxP/+ mice. Tamoxifen or control vehicle was injected i.p. in adult mice (1 mo of age) once a day for 5 d. At day 4, regeneration was triggered by CTX injection in TA muscle of both groups, and analysis was performed at the indicated time points (day 4 and day 15). (B) PCR analysis shows tamoxifen-induced deletion of Cripto floxed allele (Cripto Del) only in uninjured contralateral TA muscle (Left) but not in bone marrow (Right), isolated at day 15. Genomic DNA isolated from uninjured TA muscles and bone marrow of tamoxifen-treated CriptoloxP/loxP CAG-CreERT2 mice was used as a positive control (Ct). RALDH, retinaldehyde dehydrogenase. (C) ELISA-based assay of Cripto protein levels in muscle tissue of Tg:Pax7-CreERT2::CriptoloxP/+ mice treated with either sesame oil as a control or tamoxifen at day 4 after injury (18.1 ± 0.3 pg/mg for control vs. 11.05 ± 1.4 pg/mg for tamoxifen). Values are mean ± SEM; n = 3 mice per group; *P = 0.04. (D) Representative H&E-stained sections of CTX-treated muscles at indicated time points. (Scale bars = 50 μm.) CSA analysis of regenerated fibers at day 4 (E) and at day 15 (F) shows smaller myofibers in tamoxifen treated mice vs. control mice at both time points. See also Table S1.
Ad-sCripto muscles of neonatal myosin heavy chain (*nmhyc*), a marker of muscle regeneration in the adult. Expression of *nmhyc*, analyzed by quantitative real-time PCR (qRT-PCR), was significantly increased in Ad-sCripto mice at day 8 (Fig. 4D). Most remarkably, morphometric analysis (CSA) showed that Cripto overexpression increased myofiber size at both 8 and 22 d after the CTX injury (Fig. 4E and F, respectively), and results were confirmed in the model of less severe muscle damage (Fig. S4C).

Taken together, our data indicate that sCripto overexpression accelerates muscle regeneration and induces myofiber hypertrophy following acute skeletal muscle damage. Among the different processes active in muscle healing and regeneration, inflammation plays an important role. Because Cripto was also expressed in macrophages during regeneration (Fig. 1 J–L), we compared the degree of inflammation in TA muscles transduced with Ad-sCripto and Ad-Control. Immunostaining for F4/80 followed by morphometric analysis showed that there was no significant difference in the F4/80+ inflammatory cell area in the two groups (3.7 ± 2% after Ad-sCripto vs. 4.6 ± 1% after Ad-Control on day 4; 9.1 ± 4% after Ad-sCripto vs. 5.2 ± 3% after Ad-Control on day 8; 5.7 ± 2% after Ad-sCripto vs. 6.9 ± 3% after Ad-Control on day 22; n = 5 mice per group; P = NS; Fig. S4C), thus suggesting that Cripto overexpression does not substantially contribute to modulation of the inflammatory process.

**sCripto Rescues Muscle Regeneration in Mice with Conditional Targeted Deletion of cripto in Adult Satellite Cells.** To evaluate whether sCripto was able to recapitulate the function of endogenous membrane Cripto (mCripto) fully in vivo, we investigated whether sCripto rescued muscle regeneration defects in mice with genetic ablation of cripto in adult satellite cells. To this end, Tg:Pax7-CreERT2::Cripto*loxP*/loxP− mice were injected i.p. with tamoxifen once a day for 5 d. At day 4, regeneration was triggered in TA muscles by CTX injection, along with local infection of either Ad-sCripto or Ad-Control, and the effect on muscle regeneration was evaluated at day 4 after injury (Fig. S4). We first verified by PCR analysis that cripto deletion occurred selectively in skeletal muscles of Tg:Pax7-CreERT2::Cripto*loxP*/loxP− mice (Fig. S4E). Accordingly, endogenous Cripto protein levels decreased in muscle tissue of Tg:Pax7-CreERT2::Cripto*loxP*/loxP− mice compared with control Cripto*loxP*/loxP− mice, as shown by ELISA (Fig. 5B). As expected, we found that Cripto protein levels strongly increased in Ad-sCripto–transduced muscles compared with Ad-Control (Fig. 5B). We thus performed morphometric analysis of myofiber size in the different mouse groups. As expected, the myofiber CSA was significantly reduced in Tg:Pax7-CreERT2::Cripto*loxP*/loxP− mice compared with Cripto*loxP*/loxP− control mice. Most remarkably, this reduction was fully rescued by sCripto overexpression in Tg:Pax7-CreERT2::Cripto*loxP*/loxP− mice, and the CSA eventually increased compared with that in Cripto*loxP*/loxP− control mice, thus providing direct evidence that sCripto was able to...
Cripto Promotes Myogenic Cell Proliferation. Results of gain-of-function and loss-of-function experiments suggest that Cripto might play a role in regulating satellite cell function and, eventually, modulate skeletal muscle regeneration. To gain more insight into this issue, we first evaluated whether Cripto would be mitogenic for primary myoblasts in culture. To this end, an enriched population of adult mouse primary muscle precursor cells was isolated and cultured under conditions favoring replication (33), and was treated with recombinant sCripto; cell proliferation was measured by BrdU incorporation. Physiological concentrations of recombinant sCripto increased myoblast proliferation in a dose-dependent manner (Fig. 6A), and addition of anti-Cripto antibodies nearly completely abolished the mitogenic effects of exogenous Cripto (Fig. 6B).

To investigate Cripto activity on satellite cells in a more physiological context and without bias of selection, we used isolated myoblasts in culture, which provide an accessible means to study satellite cells in their native position beneath the basal lamina that surrounds each muscle fiber (26). We first performed immunofluorescence analysis for the proliferation marker Ki67 on freshly isolated myoblasts treated with recombinant sCripto or left untreated as a control. In line with results on primary myoblasts, the number of proliferating Ki67+ cells increased in myoblasts treated with sCripto by 72 h compared with control (211 ± 6% after sCripto vs. 69 ± 3.7% after control; Fig. 6C), thus providing further evidence for mitogenic activity of Cripto.

Finally, given that Cripto is a GPI-anchored membrane protein in its physiological configuration (34), we also evaluated the effect of mCripto. To assess the paracrine/juxtacrine ability of mCripto further, we used single myoblasts isolated from Myf5°/° mice plated on feeder layers of mammalian cells, either control or stably expressing mCripto (34), followed by counting the number of β-gal+-proliferating primary myogenic cells. In keeping with our findings, β-gal+ cells had almost doubled in the presence of mCripto compared with control (44 ± 0.58 vs. 22 ± 5.78, respectively; n = 3 independent experiments; *P = 0.0192; Fig. 6D).

Cripto Modulates Myogenic Cell Determination on Isolated Myoblasts. To gain further insight into the role of Cripto on satellite cells, we performed a time course immunofluorescence analysis for Pax7 and MyoD on isolated myoblasts treated with recombinant sCripto or left untreated as a control. By 48 h, supplementation of sCripto resulted in a reduced number of quiescent Pax7+/MyoD− cells compared with control (Fig. 6E, green bars), thus suggesting that Cripto might promote/accelerate the entry of satellite cells into S phase. Moreover, by 72 h and up to 96 h in culture, the number of Pax7+/MyoD+ cells committed to differentiation progressively increased in sCripto-treated myoblasts at the expense of Pax7+/MyoD− cells (33 ± 5% for sCripto vs. 18 ± 3% of Pax7+/MyoD+ cells for control at 72 h, *P < 0.05; 48 ± 2% for sCripto vs. 23 ± 4% for control at 96 h, **P < 0.005; Fig. 6E, red bars).

We thus decided to assess whether the duration of Cripto signaling was critical for its biological activity. Isolated myoblasts were then cultured in the presence of sCripto for 48 h (0–48 h), washed to remove Cripto, and cultured for the remaining 48 h (i.e., up to 96 h in total). Interestingly, the number of Pax7+/MyoD+ cells increased in control sCripto− mice expressing sCripto (orange line and bar) compared with Ad-Control (green line and bar). Values are mean ± SEM; n = 3 mice/group; *P = 0.02; **P < 0.004. See also Fig. 5A.
MyoD+ cells increased to the same extent as observed for cells treated with Cripto throughout the culture (43 ± 4% for sCripto vs. 23 ± 4% for control after 96 h; **P < 0.005; Fig. 6E, red bars), suggesting that treatment for 48 h is sufficient to induce an effective Cripto response.

Finally, as shown for sCripto (Fig. 6E), immunofluorescence analysis of isolated myofibers infected with mCripto-overexpressing lentivirus revealed an increased number of Pax7−/MyoD+ cells compared with lentivirus (Lenti)-Control–infected fibers at 72 h (41.8 ± 2.1% after mCripto vs. 25.9 ± 2.3% after control; n = 3 experiments; *P < 0.01 and **P < 0.001; Fig. S5A).

Taken together, our data suggest that Cripto plays a dual role, by increasing the proliferation of myogenic cells and by promoting satellite cell progression into the myogenic lineage.

**Cripto Antagonizes the Effect of Myostatin/GDF8 on Satellite Cells in Isolated Myofibers.** Previous findings indicated that Cripto contributes to the modulation of cell proliferation and growth by antagonizing members of the TGF-β superfamily, such as TGF-β itself or activin (15, 35). Myostatin/GDF8 is a TGF-β family member and a strong inhibitor of muscle growth, and it is expressed by quiescent satellite cells (36). To explore the molecular mechanism of Cripto signaling on satellite cells, we investigated whether Cripto may act as an antagonist of myostatin/GDF8 (GDF8). We therefore first measured the ability of GDF8 to activate Smad2 phosphorylation in the absence or presence of Cripto. To this end, 293T cells were transfected with sCripto expressing plasmid or empty control vector and were treated with increasing doses of recombinant GDF8 (Fig. 7A). In line with our hypothesis, GDF8-induced Smad2 phosphorylation was inhibited by sCripto, even at the highest concentrations of GDF8 tested (Fig. 7A). Moreover, sCripto was able to reduce GDF8-induced Smad2 phosphorylation in C2C12 myogenic cells (Fig. S5B).

Remarkably, membrane-anchored mCripto retained its ability to antagonize GDF8 signaling (Fig. S5C). Furthermore, in agreement with the idea that Cripto/GDF8 may regulate satellite cell myogenic commitment, blocking GDF8 activity by adding anti-GDF8 antibodies to the fibers increased the tendency to differentiation of satellite cells, as indicated by an increased number of Pax7−/MyoD+ cells, at different time points (178 ± 6.0 cells for anti-GDF8 vs. 48 ± 2.4 cells for control at 72 h; **P = 0.005; Fig. 7B). Moreover, addition of sCripto to anti-GDF8–treated myofibers did not further increase...
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... cells allowed us to unmask the cellular contribution of Cripto in... to compensate for the lack of Cripto in satellite cells. Notably, although our data do not rule out the possibility that Cripto...

Discussion

The capacity of the skeletal muscle regenerative response is primarily due to a resident population of myogenic stem cells, the satellite cells. It is well known that extrinsic and intrinsic signaling pathways modulate the status of the satellite cell pool (37); however, the molecular mechanisms are not yet fully defined.

Here, we demonstrate that Cripto, a critical signal in embryonic development, is reexpressed in adult skeletal muscles that undergo regeneration and that its activity can modulate skeletal muscle regeneration. We show that Cripto is undetectable in quiescent Pax7+/Myod+ satellite cells but that it accumulates in activated satellite cells, being coexpressed with myogenic lineage markers, such as Pax7, Myf5, and Myod, thus suggesting that Cripto expression occurs concomitantly and/or following activation of satellite cells. Interestingly, Cripto is also expressed in inflammatory cells during regeneration. Notably, in addition to myogenic cells, inflammatory cells, which are recruited to the damaged area, provide an important contribution to muscle regeneration. Indeed, recent studies have shown that factors expressed during the inflammatory process can influence skeletal muscle regeneration by stimulating satellite cell survival and/or proliferation (38, 39). For example, recent data provided evidence that infiltrating inflammatory cell-derived granulocyte colony-stimulating factor enhances myoblast proliferation and facilitates skeletal muscle regeneration, thereby underscoring the importance of inflammation-mediated induction of muscle regeneration (39). Conditional Cripto inactivation in adult satellite cells allowed us to unmask the cellular contribution of Cripto in vivo and provide previously undescribed evidence for a functional role of this protein during muscle regeneration. Notably, although our data do not rule out the possibility that Cripto expressed by infiltrating macrophages would also contribute to this effect, our findings simply indicate that this was not sufficient to compensate for the lack of Cripto in satellite cells.

In line with these findings, we demonstrate that Cripto modulates the different fates of satellite cells and that it is mitogenic for satellite cell-derived myoblasts. To address this issue, we used...
isolated myofibers in culture, which allows investigation of the effect of exogenous factors on satellite cells in their native positions (26), and found that exposure to either sCripto or GPI-anchored Cripto increased the number of Pax7+/MyoD+ committed myogenic cells at the expense of Pax7+/MyoD− cells, suggesting that Cripto promotes/accelerates the entry of satellite cells into S phase and their commitment to differentiation. Moreover, we show that Cripto promotes proliferation both in isolated myofibers and in primary myoblasts in culture. This notion is consistent with previous findings that support a model in which Cripto possesses intrinsic activities as a transacting factor both in cell culture and in vivo (6–8, 31). In line with this idea, sCripto was able to rescue fully the effect on muscle regeneration of the genetic ablation of cripto in adult satellite cells. This represents in vivo evidence in the mouse that sCripto recapitulates the function of GPI-anchored Cripto.

Several molecules have been described that regulate stem cell proliferation and/or differentiation, and eventually muscle regeneration, including those belonging to the TGF-β superfamily (11). Interestingly, in addition to its obligate role as a Nodal/GDF1/GDF3 coreceptor, Cripto can antagonize signaling by activins and TGF-β (13–15). GDF8/myostatin is a member of the TGF-β superfamily that has been implicated in the negative regulation of muscle growth and regeneration (36). Consistent with the idea that Cripto could act as an antagonist of myostatin/GDF8, we show that Cripto and myostatin are expressed in regenerating muscles and, most remarkably, that (i) both secreted and membrane-anchored Cripto is able to attenuate the myostatin/Smad2 signaling pathway; (ii) Cripto antagonizes the antiproliferative effect of myostatin on isolated myofibers, promoting myogenic commitment; and, similarly, (iii) blocking myostatin activity increases the tendency toward differentiation of satellite cells. Myostatin is expressed by quiescent satellite cells and has a functional role in repressing satellite cell proliferation and enhancing self-renewal (40). A number of factors have been discovered that antagonize myostatin activity, such as follistatin (24), recently suggested to induce muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin (41). However, the direct relevance of myostatin for satellite cells is still debated, and controversial models have been proposed regarding which cell types mediate the effects of myostatin on myogenesis and myoblast physiology (43).

Several lines of evidence suggested that the normal function of myostatin in adult muscle is to maintain satellite cells in a quiescent state, acting as a negative regulator of cell activation and proliferation (36, 44, 45). Moreover, studies in chick and mouse embryos pointed to a context-dependent effect of myostatin, controlling the balance between proliferation and differentiation on muscle progenitors (46). In contrast, recent data indicate that postnatal muscle hypertrophy generated by the lack of myostatin is largely due to hypertrophy of individual fibers and not to satellite cell activity (47). Indeed, it has been reported that the addition of recombinant myostatin (100 ng/mL) does not influence satellite cell proliferation in vitro. Notably, in that study, myostatin was added to isolated fibers after 48 h in culture and maintained over the subsequent 24 h (47). Our protocol differs from this in that sCripto and/or myostatin (50–200 ng/mL) was added to myofibers immediately after culture, which might explain the apparent discrepancy. We found that in this experimental setting, myostatin is able to inhibit and/or delay the progression of Pax7+/MyoD− quiescent satellite cells toward Pax7+/MyoD+ myogenic/proliferating cells. Interestingly, this effect is reverted by sCripto and also persists on removal of Cripto after 48 h. Although we cannot rule out the possibility that residual Cripto might remain bound to the fibers/cells, thus explaining the long-lasting effect of the treatment, previous findings in ESCs showed that the transient presence of sCripto in the early time window of differentiation (0–48 h) was sufficient to rescue the cardiac phenotype of cripto−/− ESCs fully at later time points (31).

In conclusion, we identified Cripto as a factor required for efficient repair of skeletal muscles and propose that Cripto regulates satellite cell progression toward the myogenic lineage, at least in part, by counteracting myostatin activity. Although we cannot rule out the possibility that other signaling pathways might also be involved, our intriguing findings are in line with very recent data, which report that overexpression of Cripto antagonized myostatin-induced A3 luciferase activity in 293T cells (48). In contrast to these findings, it has recently been proposed that Cripto may also exert a stimulatory role on myostatin signaling, suggesting that Cripto-mediated myostatin signaling is dose-dependent (49). Although further experiments will be necessary to elucidate the molecular basis of this newly identified Cripto/myostatin interaction, our study indicates that this could represent a novel mechanism for the control of satellite cell decisions necessary for robust skeletal muscle maintenance and repair.

Finally, our findings that Cripto is expressed in both myogenic and inflammatory cells places Cripto within a complex regulatory network that links inflammation and skeletal muscle regeneration, a relationship that remains incompletely understood, and thus opens the way to assess the potential of Cripto as target for the treatment of skeletal muscle injury or disease.

Experimental Procedures

Section Immunostaining. Muscles were freshly frozen and cut in cryostat sections. Slides were fixed in 4% (wt/vol) paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100 (Sigma–Aldrich), and boiled in 10 mM sodium citrate. Primary antibodies used are as follows: anti-Cripto (6–7 μg/mL; 1:50, Santa Cruz Biotechnology; 1:150, Abcam), antilysmatin (1:50; Abcam), anti-MyoD (1:2000; DAKO), F4/80 (1:2000; Serotec), and desmin (1:50, ICN). Appropriate fluorophore-conjugated secondary antibodies, Alexa Fluor 488 and Alexa Fluor 594 (1:300; Molecular Probes) or HRP conjugated (DAKO) and fluorescein-labeled thymidine (PerkinElmer) were used for visualization. Vectashield medium containing DAPI (Vector Laboratories) was used for mounting. Sections incubated without primary antibodies served as controls. Labeling was visualized by epifluorescent illumination using an Axioscope microscope (Carl Zeiss), and images were acquired on an Axiocam camera (Carl Zeiss) or a DFC480 or DFC350FX camera (Leica).

Isolation and Growth of Mouse Primary Myoblasts. Purification of primary myoblast culture was performed as previously described (33, 50). Details are provided in SI Experimental Procedures.

Cell Proliferation Assays. Myoblasts were cultured at 5 × 104 cells per well on 96-well microtiter plates in growth medium for a few hours and then serum starved overnight in DMEM with 0.5% FBS. After washing, cells were cultured in DMEM-FBS-0.5% medium containing soluble recombinant mouse Cripto (sCripto) at 5, 50, 100, 250, or 500 ng/mL (R&D Systems) and a homemade product (6), human basic FGF (10 ng/mL; R&D Systems), or neutralizing antibodies at 4 μg/mL (anti-Cripto, MAB1538; R&D Systems). A BrdU cell proliferation assay kit (Roche) was used following the manufacturer’s instructions. BrdU incorporation was measured by the absorbance of the samples in an ELISA reader at 370 nm (reference wavelength of ~492 nm).

Single-Fiber Culture Assays. Single floating myofibers were prepared from the extensor digitorum longus (EDL) muscles from 6-wk-old C57Bl/6 and Mfys3ermice (27, 51), as described (26, 52). Individual intact myofibers were placed in horse serum (HS)-coated, round-bottomed Eppendorf tubes and incubated without or without mouse sCripto (200 ng/mL; R&D Systems) or myostatin/GDF8 (50, 100, or 200 ng/mL; R&D Systems) in low-activation medium [10% (vol/vol) HS and 0.5% chicken embryo extract (CEE) in DMEM]. Myofibers were treated with sCripto or preincubated for 1 h with either blocking anti-Cripto (MAB1538, 4 μg/mL; R&D Systems) or anti-myostatin/GDF8 (GT15213, 10 μg/mL; Neuromics) antibodies before Cripto addition.

In the anti-GDF8 time course experiment, myofibers were incubated for 48, 72, or 96 h with anti-GDF8 (GT15213, 10 μg/mL) either alone or preincubated for 1 h with GDF8 (200 ng/mL). After 48 or 72 h of treatment, floating fibers were fixed in 4% (wt/vol) PFA, rinsed in PBS, and immediately used for immunostaining. Primary antibodies used are as follows: MyoD (1:50; Dako...
DNA Plasmids, Cell Culture, and Western Blot. Cripto and mCripto were previously described (6, 34). Briefly, mCripto corresponds to the full-length cDNA, whereas sCripto corresponds to cripto cDNA with a STOP codon at nucleotide +156. 293T or C2C12 cells were plated on six-well plates at a density of 2 × 10^5. Twenty-four hours after plating, cells were transfected with 2 μg of DNA (pCDNA3, pCDNA3-sCripto, and pCDNA3-mCripto) using lipofectamine (Invitrogen). Twenty-four hours after transfection, cells were serum-starved for 8 h before treatment. Cells were left untreated or were treated for 30 min with the indicated doses of myostatin/GDF8 protein (R&D Systems). Total protein extracts were prepared and analyzed by Western blot as previously described (56). Anti-phospho-Smad2, Smad2 (Cell Signaling Technology), and Cripto antibodies (R&D Systems) were used as previously described (31).

RNA Preparation and RT-PCR. Total RNAs from the TA muscle were isolated using an RNasy mini kit (Qiagen) according to the manufacturer’s instruction. One microgram of total RNA was used for cDNA synthesis using SuperScript II reverse transcriptase (Life Technologies) and random hexamers. A qRT-PCR assay was performed using SYBR Green PCR master mix (EuroClone). Primers are listed in Table S2.

Statistical Analysis. All values are expressed as mean ± SEM. To determine significance between two groups, comparisons were made using unpaired Student t tests. Analyses of multiple groups were performed using paired Student t tests using Prism version 5.00 for Mac (GraphPad Software). P < 0.05 was considered statistically significant.

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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-I (Invitrogen).

Tibialis anterior (TA) muscles were digested as described above, and unfixed cells were treated with anti-Cripto Allophycocyanin (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. Muscle 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-rabbit 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 Aquatek 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 home-made 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–Alrich), 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 μg/mL 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 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 Trizolol Kit (Life Technologies) according to the manufacturer's instructions. Muscle tissue was homogenized with TissueLyser (Qiagen), and RNA was extracted with Trizolol 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 myoblasts were prepared from the extensor digitorum longus (EDL) muscles from 6-wk-old C57/B6 or Myf5nLacZ/+ mice (3, 4) as described (5). Briefly, muscles were dissected and digested in 2% (wt/vol) collagenase type 1 (Sigma–Alrich) in DMEM (Gibco) for 1.5 h at a 35 °C water bath. Myoblasts were isolated by gentle trituration of the muscle using a customized heat-polished Pasteur pipette. Individual intact myoblasts were washed by serially transferring them through three dishes of warmed DMEM supplemented with 4 mM t-glutamine (Sigma–Alrich) and a 1% penicillin and streptomycin solution (Sigma–Alrich). 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-Cripto) 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 × 10^3 transducing unit (TU)/μL.

Immunofluorescence staining on C57/B6 fibres 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/+ myoblasts 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.

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.)
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 × 10⁸–6 × 10⁹ pfu/mL). Adenovirus encoding empty vector has been used as a control (Ad-Control) at the highest concentration (6 × 10⁹ pfu/mL). Ad-Control 0 vs. Ad-sCripto: 0.23 ± 0.06 ng/mg at 6 × 10⁸ pfu/mL, 0.83 ± 0.04 ng/mL at 3 × 10⁹ pfu/mg, and 2.12 ± 0.5 ng/mg at 6 × 10⁹ 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 ± 2% after Ad-sCripto vs. 4.6 ± 1% after Ad-Control on day 4; 9.1 ± 4% after Ad-sCripto vs. 5.2 ± 3% after Ad-Control on day 8; 5.7 ± 2% after Ad-sCripto vs. 6.9 ± 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::CriptoloxP/− and CriptoloxP/− 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

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<th>Primer (forward) 5′-3′</th>
<th>Primer (reverse) 5′-3′</th>
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<td>1.GCCAAGAGCCATGACAGAGATGG</td>
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<td>Cripto-del</td>
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<td>raldehyde</td>
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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

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