

# Necdin mediates skeletal muscle regeneration by promoting myoblast survival and differentiation

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**R**egeneration of muscle fibers that are lost during pathological muscle degeneration or after injuries is sustained by the production of new myofibers. An important cell type involved in muscle regeneration is the satellite cell. Necdin is a protein expressed in satellite cell-derived myogenic precursors during perinatal growth. However, its function in myogenesis is not known. We compare transgenic mice that overexpress necdin in skeletal muscle with both wild-type and necdin null mice.

After muscle injury the necdin null mice show a considerable defect in muscle healing, whereas mice that overexpress necdin show a substantial increase in myofiber regeneration. We also find that in muscle, necdin increases myogenin expression, accelerates differentiation, and counteracts myoblast apoptosis. Collectively, these data clarify the function and mechanism of necdin in skeletal muscle and show the importance of necdin in muscle regeneration.

## Introduction

Skeletal muscle tissue is characterized by a very slow turnover. Turnover increases, however, upon certain physiological stimuli or in pathological conditions, such as primary myopathies, leading to an extensive repair process aimed at preventing the loss of muscle mass.

The initial phase of muscle repair is characterized by necrosis of the damaged tissue and activation of an inflammatory response (Charge and Rudnicki, 2004). Local cues, produced by growth factors and inflammatory cytokines released by infiltrating cells, lead to the activation of quiescent myogenic cells, the satellite cells located beneath the basal lamina of muscle fibers that start to proliferate, differentiate, and fuse, leading to new myofiber formation and reconstitution of a functional contractile apparatus.

Muscle satellite cell activation resembles embryonic myogenesis in several ways, including the *de novo* induction of the myogenic regulatory factors (MRF). Genetic studies have defined a temporal and functional hierarchy among the myogenic basic helix-loop-helix transcription factors during somitic myogenesis, the first of which are Myf5 and MyoD (Cossu and Biressi, 2005; Buckingham et al., 2006). Quiescent satellite cells express another important transcription factor, Pax7, which is involved in myogenic specification (Relaix et al., 2006; Kuang et al., 2007). Upon injury, satellite cells are activated and start proliferating and expressing Myf5 and MyoD, whereas Pax7 expression is progressively reduced. At this stage they are often referred to as myogenic precursor cells. Subsequently, expression of myogenin and MRF4 (MRF members) is up-regulated in cells beginning their terminal differentiation program, which is followed by permanent exit from the cell cycle, activation of muscle-specific proteins such as sarcomeric myosin, and fusion with damaged muscle fibers or with themselves, to produce new fibers that replace the dead ones. A fraction of activated cells down-regulates expression of Myf5/MyoD and returns to a quiescent state to maintain a more or less constant pool of satellite cells. A delicate balance between cell proliferation and exit from

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Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; CTX, cardiotoxin; GAPDH, antiglyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin; MyLC, myosin light chain; MRF, myogenic regulatory factor; MyHC, myosin heavy chain; P, postnatal day; PCNA, proliferating cell nuclear antigen; TA, tibialis anterior; wt, wild type; XSA, cross-sectional area.

The online version of this article contains supplemental material.



cell cycle, differentiation, and fusion is required for the correct muscle regeneration to occur, and many proteins have been found to play a crucial role in these processes, including insulin-like growth factor, myostatin and follistatin (Musaro and Rosenthal, 1999; Lee and McPherron, 2001; Iezzi et al., 2004; Pisconti et al., 2006), intracellular mediators like p66 (Zaccagnini et al., 2004), and transcription factors other than MRF, such as E2F1 and myocyte nuclear factor (Garry et al., 2000; Li et al., 2000; Yan et al., 2003).

Necdin is a member of the melanoma antigen-encoding gene family (Barker and Salehi, 2002), a large family of proteins initially isolated from melanomas. The predominant feature of these proteins is a large central region termed the melanoma antigen-encoding homology domain. Deficiency of necdin in transgenic mice causes neonatal respiratory distress that is usually fatal, and surviving mice exhibit behavioral and cognitive disruptions that bear some resemblance to the phenotype observed in humans affected by the Prader Willi syndrome (Gerard et al., 1999; Muscatelli et al., 2000). Studies in neuroblastoma cell lines and dorsal root ganglia cultures have suggested that necdin may act as a growth suppressor, facilitating cell cycle exit and differentiation while inhibiting apoptosis in neurons (Muscatelli et al., 2000; Kobayashi et al., 2002; Takazaki et al., 2002; Kuwako et al., 2005). The precise cellular mechanism leading to cell cycle arrest is still unclear, although evidence points to interaction with proteins involved in cell cycle progression such as p53 and E2F (Taniura et al., 1999; Kuwako et al., 2004; Kurita et al., 2006; Kuwajima et al., 2006). We have also shown that necdin cooperates with the homeobox transcription factor *Mx2* during smooth muscle differentiation of mesoangioblast cells (Brunelli et al., 2004). Whether necdin acts as a transcriptional cofactor or as a direct transcriptional repressor or activator in these different scenarios is still unknown.

Necdin is also expressed in developing skeletal muscle, which suggests that it plays a relevant role in this tissue (Brunelli et al., 2004; Kuwajima et al., 2004; Watrin et al., 2005). Its function in skeletal muscle and myogenesis, however, has not been investigated in detail.

Here, we provide the first evidence that necdin plays an important role in skeletal muscle growth and repair *in vivo*, as shown by using loss- and gain-of-function experiments in necdin knockout mice and mice overexpressing necdin specifically in skeletal muscle. We also demonstrate that necdin activity is mediated through stimulation of myoblast differentiation and protection from apoptotic cell death.

## Results

### Necdin expression in adult skeletal muscle is restricted to myogenic precursor cells

To study the expression of necdin in postnatal skeletal muscle we performed immunofluorescence on cryostat sections of tibialis anterior (TA) muscle from postnatal day (P) 5 and P10 pups (Fig. 1, A–H) and 2-mo-old mice (not depicted). In P5 (Fig. 1, A–D) and P10 (Fig. 1, E and F) muscle sections, we observed colocalization of necdin with Myf5-positive cells, indicating that these cells are myogenic precursor cells. No other cells in the muscle appeared to express necdin. We did not detect staining for necdin in 2-mo-old muscle (not depicted). Using Western blot, we also analyzed the profile of necdin expression in TA muscle from mice at different ages. As indicated in Fig. 1 I, necdin expression was high in muscle from 5–10-d-old pups, it decreased in pups from 15 d to 1 mo old, and it was undetectable at 2–3 mo, in agreement with the immunofluorescence data. This suggests that necdin is restricted to active myogenic progenitor cells.

We isolated myoblasts from newborn mice and studied the temporal expression profile during differentiation. Proliferating myoblasts expressed high levels of necdin, which decreased as differentiation proceeded, along with increased expression of sarcomeric myosin and decreased expression of the proliferation marker proliferating cell nuclear antigen (PCNA; Fig. 1 J).

We also isolated single muscle fibers from 2-mo-old mice and analyzed the expression of necdin, together with that of Pax7, a marker of quiescent satellite cells and early myogenic progenitors, and of MyoD, a marker of activated satellite cells and myogenic precursor cells (Cooper et al., 1999; Tajbakhsh, 2005; Relaix et al., 2006; Kuang et al., 2007). Expression of necdin and these two markers was analyzed from cells on a single fiber or from cells that migrated away from the fiber, which were subsequently maintained in proliferating or differentiating medium. We found that necdin was coexpressed with Pax7 in very few cells (Fig. 1, K–L; and Fig. S1, A–D; available at <http://www.jcb.org/cgi/content/full/jcb.200701027/DC1>). In most cases it was coexpressed with MyoD (Fig. 1, M and N, Q–S', and T–U'). Necdin localization was in the cytoplasm but staining was also observed in some nuclei (Fig. 1, Q–S' and T–U') and in both cells on the fibers and cells leaving the fibers. In differentiated myotubes, the level of necdin expression was lower and predominantly in the cytoplasm (Fig. 1, O and P).

marker of myogenic differentiation, PCNA as a proliferation marker, and GAPDH as a loading control. (K–U') Single muscle fibers were isolated from 2-mo-old mice and then either maintained in proliferation medium or switched to differentiation medium. (K–L) Immunofluorescence on myoblasts derived from single fibers maintained in proliferation medium, using antibodies specific for necdin (green; K) and Pax7 (red; L). Nuclei in K are identified by DAPI staining (blue). (M and N) Immunofluorescence on myoblasts derived from single fibers maintained in proliferation medium using antibodies specific for necdin (green; M) and MyoD (red; N). Nuclei in M are identified by DAPI staining (blue). (O and P) Immunofluorescence on myoblasts derived from single fibers maintained in differentiation medium, using antibodies specific for necdin (green; O and P) and sarcomeric MyHC (red; P). Nuclei in O are identified by DAPI staining (blue). (Q–S') Immunofluorescence on the single muscle fiber using antibodies specific for necdin (green; Q, Q', and S') and MyoD (R and R'). Nuclei in S, R', and S' are identified by DAPI staining (blue). In S, Q', R', and S', the fluorescence image is merged with the phase-contrast image. (T–U') Immunofluorescence on myoblasts derived from single fibers maintained in proliferation medium, using antibodies specific for necdin (green; T–U') and MyoD (red; U and U'). Nuclei in T' are identified by DAPI staining (blue). Bars: (A–D) 75  $\mu$ m; (E–H) 150  $\mu$ m; (K–P) 100  $\mu$ m; (Q–U') 30  $\mu$ m.



## Necdin is required for normal muscle growth

To investigate the role of necdin in myogenesis *in vivo*, we analyzed gain and loss of necdin function in transgenic mice.

We first generated a transgenic mouse overexpressing necdin in skeletal muscle. We cloned the ORF of *necdin*, downstream of the myosin light chain (MyLC) 1F promoter and upstream of the MyLC enhancer (Jiang et al., 2002; Fig. 2 A). The linearized vector was used to generate transgenic lines by pronuclear injection. Two founders, MlcNec2 and MlcNec9, screened by PCR and selected on the basis of transgene transmission to the progeny, were maintained as two independent lines on a CBA/C57BL6 background (Fig. 2 B). We tested the expression of the transgene on tissues and myoblasts by RT-PCR using specific primers (Fig. 2, C and U) and the overexpression of the protein by immunofluorescence and Western blot (Fig. 2, D–T). The transgenic animals showed increased expression of necdin in isolated adult myogenic progenitors, and the protein was also found in different adult muscles (diaphragm, soleus, and TA) where levels of expression in the wild type (wt) were undetectable (Fig. 2, C and D). Of notice, the MyLC1F promoter, derived from the locus of a fast myosin-type gene, was also able to drive necdin expression in the soleus, a muscle with an abundance of slow fibers. The transgene was expressed in both proliferating myoblasts and differentiating myotubes, indicating that the promoter also allows expression of this transgene in cells not terminally differentiated (Fig. 2 U). In addition, it was possible to observe expression of necdin in nuclei and also faintly in the fiber cytoplasm on sections of adult muscle (Fig. 2, E–H; and Fig. S2 E, available at <http://www.jcb.org/cgi/content/full/jcb.200701027/DC1>). In some cases, transgenic necdin colocalized with rare Myf5-positive activated satellite cells (Fig. 2, E–H). As in the case of wt, expression of necdin in myoblasts was both in the nucleus and cytoplasm (Fig. 2, I–L), and we could detect overexpression of necdin in the cytoplasm and nucleus of cells on single muscle fiber coexpressing MyoD (Fig. 2, M–T). The tissue specificity of the promoter and the transgene expression was verified by testing the expression of necdin in other tissues and organs where necdin is expressed at very low, barely detectable levels (lung, kidney, and heart; Fig. 2, C and D). The transgenic mice from both lines showed no overt differences in terms of weight, fertility, behavior, and life span with respect to the littermate controls (not depicted).

We decided to compare the results in these mice to the results of necdin loss-of-function transgenic mice, i.e., the *Ndn*<sup>-/-</sup> mice (Muscatelli et al., 2000) obtained from *Ndn*<sup>-/+</sup> intercrosses. Necdin has been described as an imprinted gene, paternally expressed only, but the lack of expression from the maternal chromosome in myogenic cells has never been investigated. We performed analysis of necdin RNA and protein expression in brain and myoblasts from *Ndn*<sup>-/-</sup>, *Ndn*<sup>-m/+p</sup>, *Ndn*<sup>+m/-p</sup>, and wt. We observed that heterozygous mice carrying the mutated gene on the paternal chromosome showed expression of necdin neither in brain nor in myoblasts, indicating that necdin also undergoes complete maternal silencing in the myogenic lineage (Fig. 2, V and W).

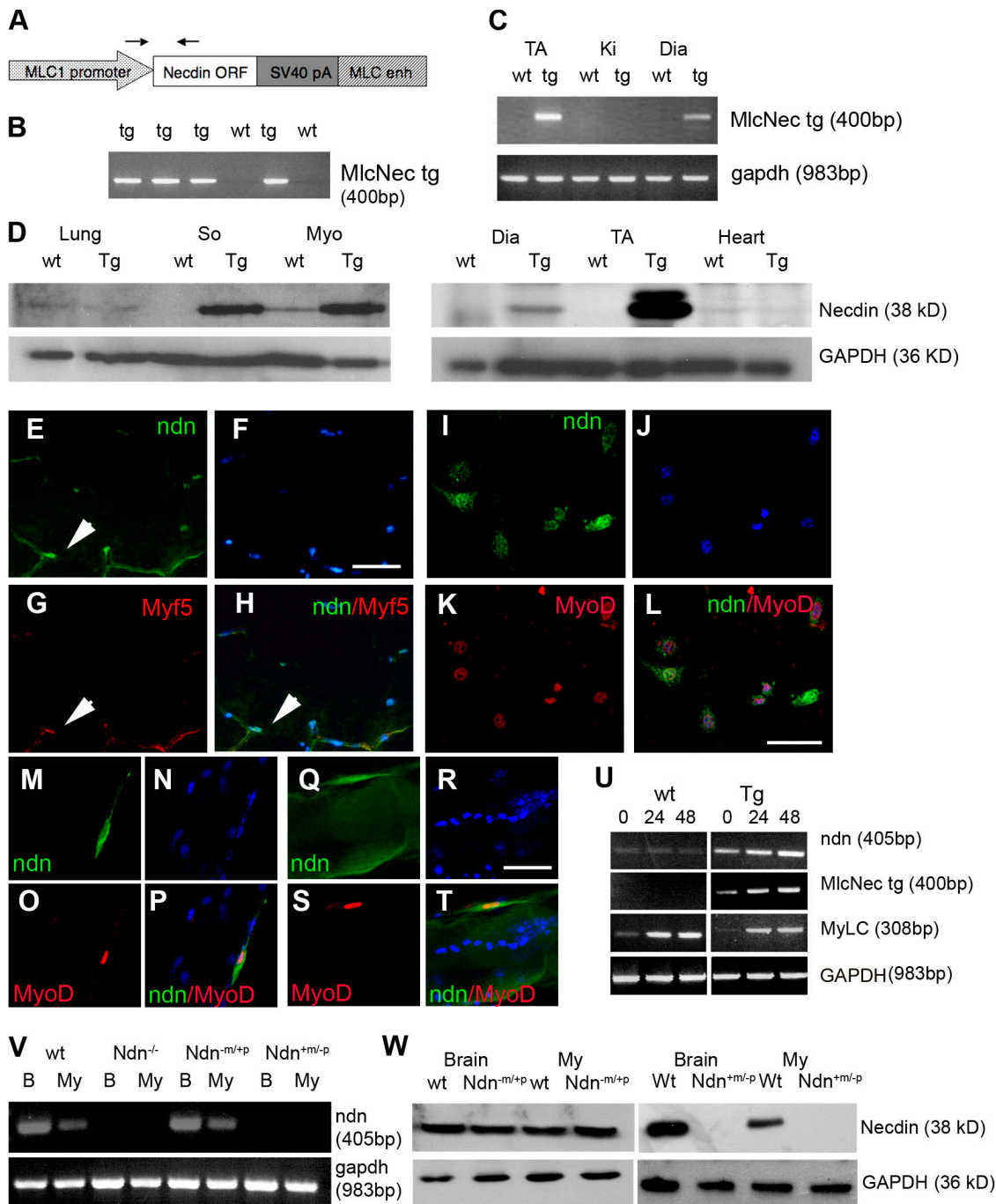
To determine whether necdin plays a functional role in skeletal muscle *in vivo*, we examined myofiber size in wt, *Ndn*<sup>-/-</sup>, and MlcNec2 muscles from P10 pups (Fig. 3, A–C, G, and I) and 2-mo-old mice (Fig. 3, D–F, H, and J–O). The TA muscles were collected from adult wt, *Ndn*<sup>-/-</sup>, and MlcNec2 mice. In the adult we also collected the soleus. Sections were stained with hematoxylin and eosin (H&E; Fig. 3, A–F). As confirmed by cross-sectional area (XSA) analyses (Fig. 3, I–K), *Ndn*<sup>-/-</sup> myofibers were significantly smaller than wt myofibers in both P10 and adult mice, whereas MlcNec2 myofibers were consistently larger, particularly in the adult (Fig. 3, G and H). We also determined the mean number of myofibers in TA and soleus muscle in the adult. We observed that MlcNec2 muscle contained a similar number of myofibers, whereas *Ndn*<sup>-/-</sup> showed a higher number of fibers (Fig. 3, L and M). Finally, we measured the maximum XSA of the TA and soleus muscle; the results indicate that MlcNec2 mice have an increased XSA (Fig. 3, N and O). The effects of loss and gain of function of necdin on morphological parameters of muscle was greater in the TA with respect to the soleus, reflecting the relative amount of necdin expression in the two muscles. All these data suggest that necdin is involved in myofiber growth and maintenance.

## Necdin is required for efficient muscle regeneration *in vivo*

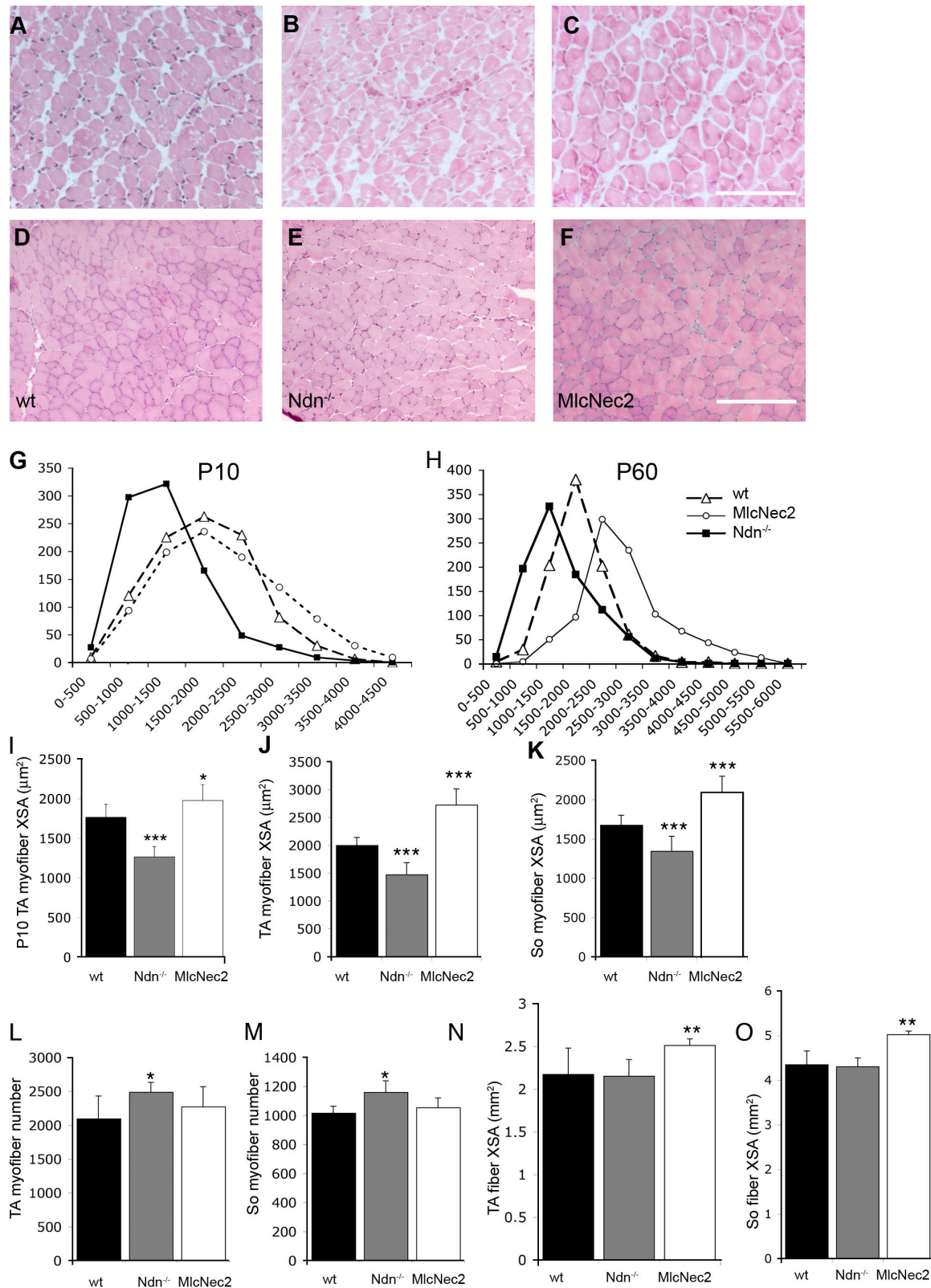
To analyze the overall regeneration capacity of these mice, injury and regeneration were induced in the TA of 3-mo-old transgenic and wt mice by injecting 25  $\mu$ l of 10  $\mu$ M cardiotoxin (CTX). Mice were killed 3, 7, and 14 d after the CTX injection; the injured TA was cryostat sectioned and stained with H&E or Azan-Mallory.

In wt mice, new myotube formation in TA muscle was observed after 7 d (Fig. 4 B), even if inflammatory infiltrates were still present. In contrast, MlcNec2 mice displayed a more advanced rate of regeneration and formation of newly regenerated fibers by 3 d after the CTX injection (Fig. 4, E and F). The accelerated regeneration in MlcNec2 mice was accompanied by high expression of neonatal myosin, a marker of muscle regeneration in the adult (Fig. 4, K, L, and S). Conversely, *Ndn*<sup>-/-</sup> mice showed a marked delay in the regeneration process with increased inflammatory infiltrates, decreased numbers of new regenerating, centronucleated fibers (Fig. 4, C and D), and no expression of neonatal myosin (Fig. 4, I and J) with respect to the wt muscle (Fig. 4, G and H). 14 d after the crush, the overall architecture was completely restored in the wt, MlcNec2, and *Ndn*<sup>-/-</sup> muscle, and they were undistinguishable from uninjured muscle except for centrally located nuclei (Fig. 4, M, O, and Q).

To further investigate whether delayed regeneration in *Ndn*<sup>-/-</sup> mice may indicate a major defect in this process, we caused more severe damage by injecting 100  $\mu$ M CTX, a higher concentration than before. We analyzed the mice after 14 d and observed that in wt animals, regeneration was occurring normally, even if inflammatory infiltrates were still present (Fig. 4 N). In contrast, MlcNec2 mice showed a complete regeneration of the muscle (Fig. 4 R). A dramatic effect was seen on the *Ndn*<sup>-/-</sup> animal. At dissection, the injured TA already appeared atrophic (not depicted) and no regenerating fibers were seen (Fig. 4 P).



**Figure 2. Analysis of the expression of necdin in the gain- and loss-of-function transgenic mice.** (A) Map of the gain-of-function transgene; arrows indicate the primers for the genotyping. (B) PCR analysis of the MlcNec2 transgenic mice using primers specific for the transgene (MlcNec tg). (C) Expression of MlcNec tg and GAPDH by RT-PCR analysis, using specific primers on total RNA from kidney (Ki), diaphragm (Dia), and TA from 2-mo-old MlcNec2 transgenic mice or wt littermates. (D) Expression of necdin and GAPDH by Western blot analysis using specific antibodies on protein extract from lung, heart, diaphragm, soleus (So), and TA from 2-mo-old MlcNec2 transgenic mice or wt littermates, or primary myoblasts (Myo) from newborn transgenic or wt mice. (E–G) Immunofluorescence on TA sections from MlcNec2 2-mo-old mice using antibodies specific for necdin (green; E) or Myf5 (red; G). Nuclei are stained with DAPI (F). (H) The merged image of E, F, and G. Arrowheads point to a double Myf5/necdin-positive myogenic precursor. (I–K) Immunofluorescence on myoblasts cells from MlcNec2 newborn mice, using antibodies specific for necdin (green; I) and MyoD (red; K). Nuclei are stained with DAPI (J). (L) The merged image of I, J, and K. (M–T) Immunofluorescence on a single muscle fiber from 2-mo-old MlcNec2 mice, using an antibody specific for necdin (green; M and Q) and MyoD (red; O and S), showing single activated satellite cells. Nuclei are stained with DAPI (N and R). P and T show the merged images of M, N, and O and Q, R, and S, respectively. (U) Expression of necdin (ndn), MlcNec tg, MyLC, and GAPDH by RT-PCR analysis, using specific primers on total RNA from myoblasts from newborn wt or MlcNec2 mice, kept in proliferation medium for 24 h (O) or differentiated for 24 and 48 h. For the detection of necdin, PCR reaction was stopped at 28 cycles. A low level of MyLC transcript can be observed in both wt and transgenic myoblasts kept in proliferation medium. (V) Expression of ndn analyzed by RT-PCR, using specific primers on RNA from brain or primary myoblasts (My) from wt, Ndn<sup>m/+p</sup>, Ndn<sup>m/-p</sup>, or Ndn<sup>-/-</sup> newborn mice. (W) Expression of necdin analyzed by Western blot, using a specific antibody on protein extract from brain or primary myoblasts from wt, Ndn<sup>m/+p</sup>, or Ndn<sup>m/-p</sup> newborn mice. Bars: (E–H) 75  $\mu$ M; (I–T) 50  $\mu$ M.



**Figure 3. Myofiber XSAs are decreased in *Ndn*<sup>-/-</sup> muscle and increased in *MlcNec2* muscle.** (A–C) Representative sections of wt (A), *Ndn*<sup>-/-</sup> (B), and *MlcNec2* (C) TA muscles from P10 mice stained with H&E. (D–F) Representative sections of wt (D), *Ndn*<sup>-/-</sup> (E), and *MlcNec2* (F) TA muscles from 2-mo-old (p60) mice stained with H&E. (G) Frequency histogram showing the distribution of myofiber XSA in wt (*n* = 867 myofibers), *Ndn*<sup>-/-</sup> (*n* = 954 myofibers), and *MlcNec2* (*n* = 890 myofibers) TA muscles from P10 mice. (H) Frequency histogram showing the distribution of myofiber XSA in wt (*n* = 987 myofibers), *Ndn*<sup>-/-</sup> (*n* = 1,023 myofibers), and *MlcNec2* (*n* = 997 myofibers) TA muscles from 2-mo-old mice. (I) Mean myofiber XSA was calculated for wt, *Ndn*<sup>-/-</sup>, and *MlcNec2* TA myofiber XSA from P10 mice. The mean XSA of *Ndn*<sup>-/-</sup> TA myofibers was reduced by 28.4% compared with wt, whereas the mean XSA of *MlcNec2* TA myofiber was increased by 11.0%. *n* = 4 per genotype. (J) Mean myofiber XSA was calculated for wt, *Ndn*<sup>-/-</sup>, and *MlcNec2* TA muscles from 2-mo-old mice. The mean XSA of *Ndn*<sup>-/-</sup> TA myofibers was reduced by 26.2% compared with wt, whereas the mean XSA of *MlcNec2* TA myofiber



The muscle was mostly composed of necrotic fibers in the presence of massive inflammatory infiltrates. As expected, no expression of neonatal myosin could be observed even by RT-PCR (Fig. 4 T). We also extended the analysis at 30 d after severe damage and observed similar results (Fig. S2 and not depicted).

These results indicate that necdin enhances the regeneration of muscle after damage and that it is necessary to the regeneration process when the damage is severe. This observation strongly suggests that necdin may increase proliferation and/or differentiation and/or protect the myogenic progenitor cells from apoptosis.

### Necdin accelerates myogenic progenitor cell differentiation and fusion

We performed several experiments *in vitro* to investigate in more detail the mechanism through which necdin exerts its effects on myogenesis.

Myoblasts from newborn transgenic MlcNec2 mice, Ndn<sup>-/-</sup>, and wt littermates were isolated as described in Sabourin et al. (1999) and seeded at clonal density. Cells were either maintained in proliferation medium or switched to differentiation medium. At different time points we either obtained cell lysates for Western analysis or we fixed the cells and performed immunofluorescence using the MF20 antibody that recognizes the sarcomeric myosin heavy chain (MyHC). Myoblasts cultured from MlcNec2 mice initiated differentiation more rapidly and formed myotubes with an increased fusion index with respect to wt controls (Fig. 5, A and B). Myoblasts cultured from Ndn<sup>-/-</sup> mice consistently showed a reduced differentiation and fusion index (Fig. 5, A and B).

We also investigated the effect of necdin overexpression in myoblasts by infecting wt newborn myoblasts with a lentiviral vector expressing necdin under the cytomegalovirus constitutive promoter (pLentiNecdinIRESGFP) or the empty vector (pLentiIRESGFP). Cells were infected 24 h after plating at clonal density, and then maintained in proliferation medium for 72 h. We did not observe any effect on myoblast proliferation or number and size of the clones generated (not depicted), but necdin-overexpressing myoblasts underwent spontaneous differentiation, forming myotubes with an increased fusion index with respect to controls (Fig. S3, A–F, available at <http://www.jcb.org/cgi/content/full/jcb.200701027/DC1>). A similar effect on differentiation was also seen in C2C12 cells after transfection with a necdin-expressing vector (Fig. S3, G–L).

Increased or reduced differentiation of myoblasts may depend on changed expression of MRF and/or decreased cell death. We decided to investigate which of these parameters was altered by first analyzing the protein levels of MyoD, myogenin, and sarcomeric MyHC (Fig. 5, C and D). The increased and decreased differentiation levels we observed in the myoblast cultures from MlcNec2 and Ndn<sup>-/-</sup> mice, respectively, were accompanied by increased and decreased expression of MyHC, respectively. Interestingly, MyoD was more highly expressed in Ndn<sup>-/-</sup> satellite cells, possibly reflecting an accumulation of myoblasts, whereas myogenin expression at the beginning of differentiation in the MlcNec2 cultures was significantly increased in comparison with wt and even more so in comparison with Ndn<sup>-/-</sup>.

Necdin has been described as acting both as a coactivator and a corepressor of transcription mediated by other transcription factors (Kuwako et al., 2004; Kurita et al., 2006; Kuwajima et al., 2006). To determine whether the modified expression of myogenic markers was caused by a direct transcriptional activation of these genes by necdin, we tested the ability of this protein to transactivate the myogenin promoter–driving expression of a chloramphenicol acetyltransferase (CAT) reporter gene by cotransfecting a necdin expression construct (pIRES-GFP-necdin; Brunelli et al., 2004) together with the reporter construct pMyo-1565CAT (Edmondson et al., 1992) in 10T1/2 or C2C12 cells. We observed that in C2C12, where the myogenin promoter already had a high basal activity caused by the presence of endogenous myogenic factors such as MyoD, necdin alone was able to activate four- to fivefold the expression of the CAT reporter (Fig. 5 E). In contrast, necdin was unable to activate the reporter gene when transfected alone in 10T1/2, but when necdin was cotransfected with a MyoD-expressing construct (Edmondson et al., 1992), it increased CAT activity fivefold in comparison with MyoD alone. These results indicate that necdin is involved in the transcriptional regulation of myogenic genes by cooperating with other MRFs.

We decided to investigate the mechanism of action of necdin by analyzing its ability to bind and/or interact with the *myogenin* promoter. C2C12 cells were transfected with the necdin overexpressing construct pCMV3B-Myc-necdin, and chromatin immunoprecipitation was performed using anti-necdin, anti-Myc, and anti-MyoD–specific antibodies. As expected, we found that MyoD was able to bind to the *myogenin* promoter (Fig. 5 F). Interestingly, necdin was also equally able to bind this DNA sequence, which was precipitated both by anti-necdin or anti-Myc antibodies (Fig. 5 F).

was increased by 36.3%.  $n = 6$  per genotype. (K) Mean myofiber XSA was calculated for wt, Ndn<sup>-/-</sup>, and MlcNec2 soleus muscles from 2-mo-old mice. The mean XSA of Ndn<sup>-/-</sup> soleus myofibers was reduced by 19.8% compared with wt, whereas the mean XSA of MlcNec2 soleus myofiber was increased by 25.0%.  $n = 6$  per genotype. (L) Mean number of myofibers per muscle was calculated for wt, Ndn<sup>-/-</sup>, and MlcNec2 TA muscles from 2-mo-old mice. No significant difference was observed in the mean number of myofibers between MlcNec2 and wt TA muscles, whereas there was a slight but significant increase in the mean number of myofibers (14.1%) in the Ndn<sup>-/-</sup> TA muscle with respect to the wt.  $n = 6$  per genotype. (M) Mean number of myofibers per muscle was calculated for wt, Ndn<sup>-/-</sup>, and MlcNec2 soleus muscles from 2-mo-old mice. No significant difference was observed in the mean number of myofibers between MlcNec2 and wt soleus muscles, whereas there was a slight but significant increase in the mean number of myofibers (12.2%) in the Ndn<sup>-/-</sup> soleus muscle with respect to the wt.  $n = 6$  per genotype. (N) Mean fiber XSA was calculated for wt, Ndn<sup>-/-</sup>, and MlcNec2 TA muscles from 2-mo-old mice at the level of maximum XSA. No significant difference was observed in the mean fiber XSA between Ndn<sup>-/-</sup> and wt TA muscles, whereas there was a significant increase in the mean XSA (15.5%) in the MlcNec2 TA with respect to the wt.  $n = 6$  per genotype. (O) Mean fiber XSA was calculated for wt, Ndn<sup>-/-</sup>, and MlcNec2 soleus muscles from 2-mo-old mice at the level of maximum XSA. No significant difference was observed in the mean fiber XSA between Ndn<sup>-/-</sup> and wt soleus muscles, although there was a significant increase in the mean XSA (11.5%) in the MlcNec2 soleus with respect to the wt.  $n = 6$  per genotype. Error bars represent SEM. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  versus wt. Bars: (A–C) 250  $\mu\text{m}$ ; (D–F) 500  $\mu\text{m}$ .

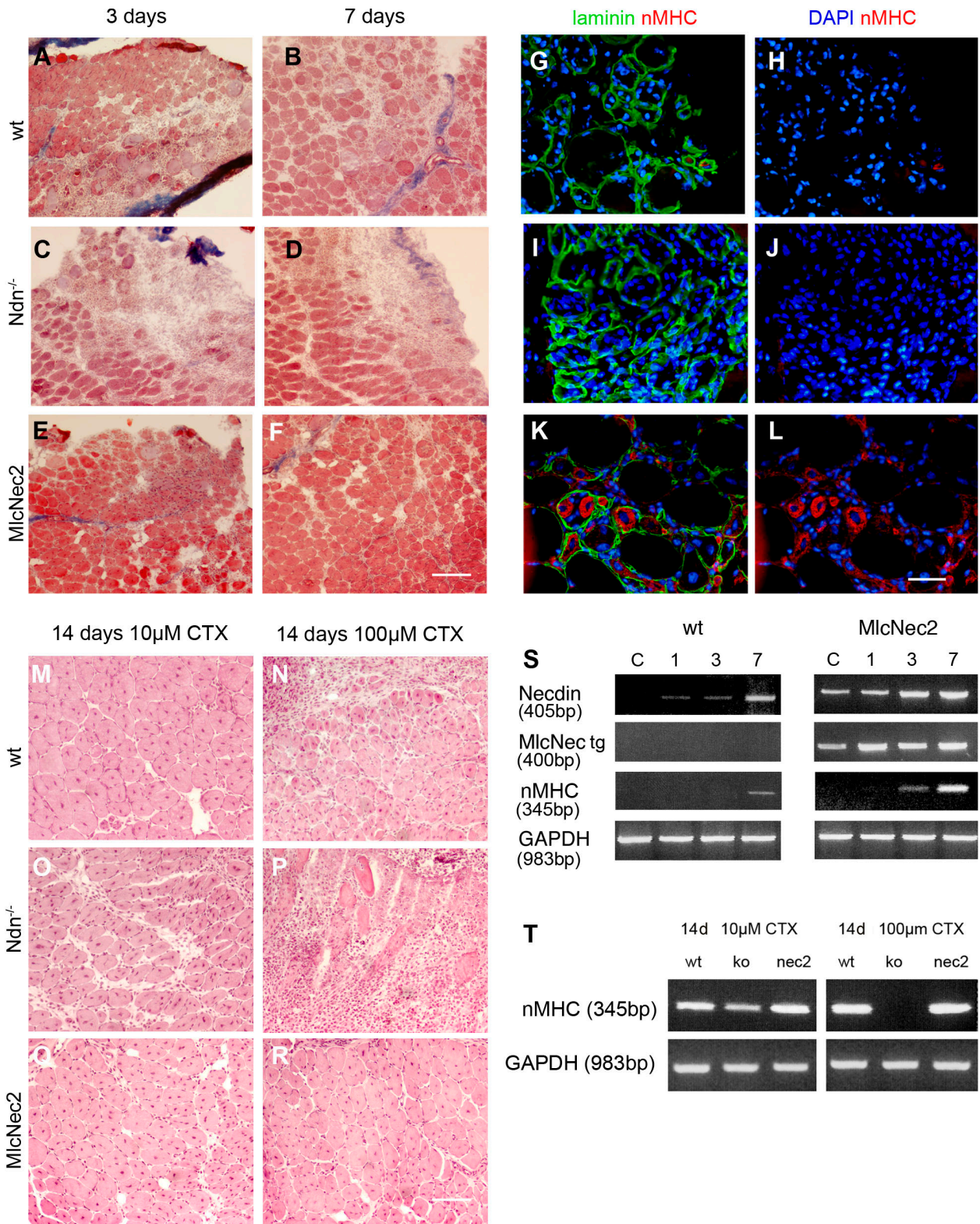
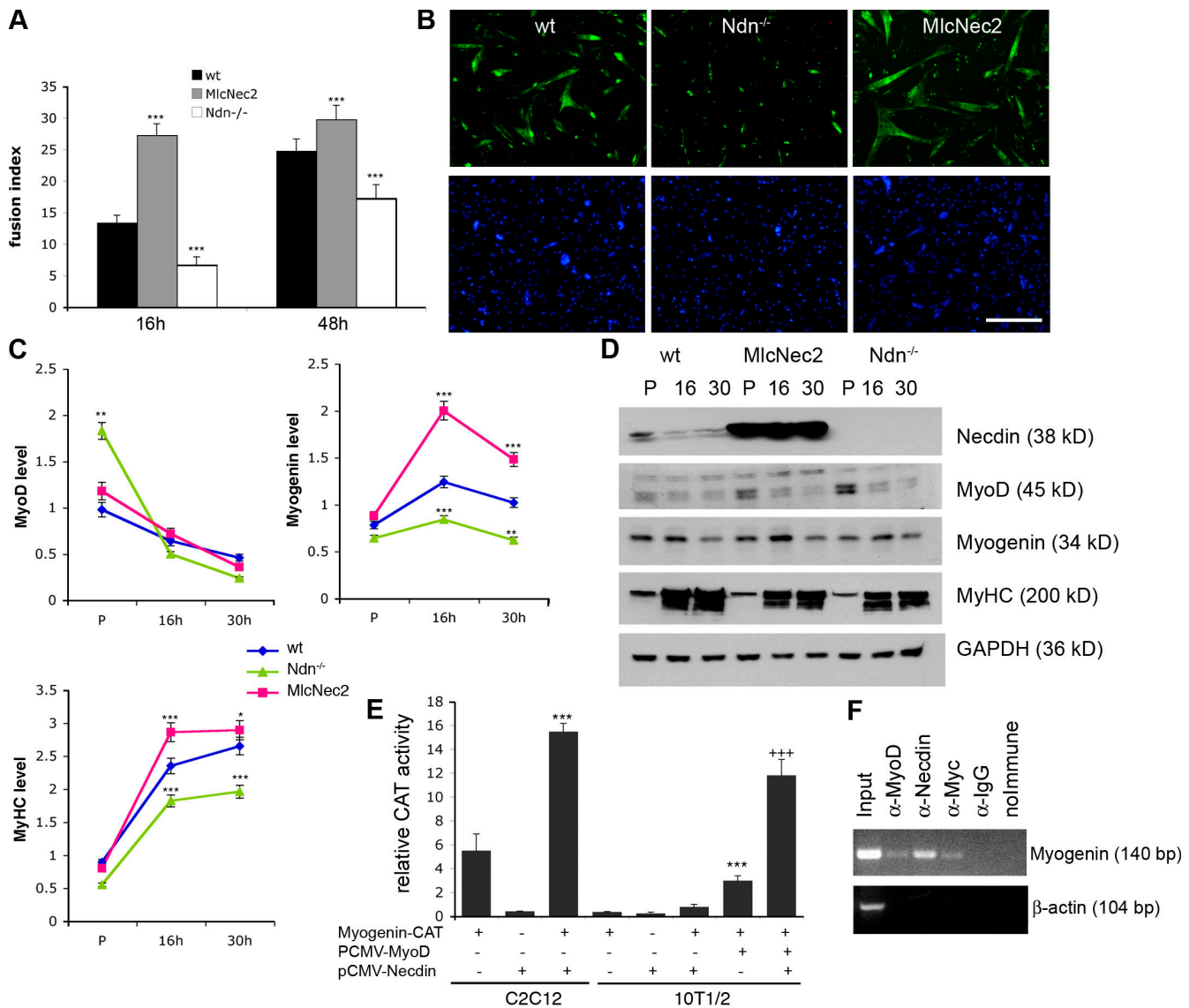


Figure 4. **Necdin is required for normal muscle regeneration after injury.** (A–F) 3 (A, C, and E) and 7 d (B, D, and F) after CTX injury, wt (A and B), *Ndn*<sup>-/-</sup> (C and D), and *MlcNec2* (E and F) TA sections were stained with Azan Mallory. Representative sections are shown. (G–L) 3 d after CTX injury, neonatal myosin (nMHC) expression was analyzed by immunofluorescence (red) using a specific antibody on wt (G and H), *Ndn*<sup>-/-</sup> (I and J), and *MlcNec2* (K and L) TA sections. Laminin expression (green) was also analyzed (G, I, and K). Nuclei are identified with DAPI staining. (M–R) 14 d after injection with 10 (M, O, and Q) or 100 μM CTX (N, P, and R), wt (M and N), *Ndn*<sup>-/-</sup> (O and P), and *MlcNec2* (Q and R) TA sections were stained with H&E. Representative sections are shown. (S) Expression of necdin, *MlcNec2* tg, nMHC, and GAPDH by RT-PCR on RNA from TA muscle from wt and *MlcNec2* mice, before (written as C) and 1, 3, and 7 d after CTX injection. (T) Expression of nMHC and GAPDH by RT-PCR on RNA from TA muscle from wt, *Ndn*<sup>-/-</sup>, and *MlcNec2* mice 14 d after injection with 10 or 100 μM CTX. Bars: (A–F) 400 μm; (G–L) 200 μm; (M–R) 300 μm.





**Figure 5. Necdin accelerates differentiation and cooperates with MyoD in myogenin transcriptional activation.** (A and B) Primary myoblasts from wt, Ndn<sup>-/-</sup>, and MlcNec2 newborn mice were maintained for 24 h in growth medium and differentiated for 16 or 48 h, and then immunofluorescence was performed using a MyHC-specific antibody. (A) Fusion index (percentage of nuclei within MyHC-positive myotubes with more than two nuclei) was calculated after 16 and 48 h in differentiation medium. Error bars represent SEM. \*\*\*,  $P < 0.001$  versus wt. (B) Representative images of immunofluorescence on wt, Ndn<sup>-/-</sup>, and MlcNec2 myoblast culture after 16 h in differentiation medium, using an antibody specific for sarcomeric MyHC (top). Nuclei in the bottom panels are visualized with DAPI. Bar, 100  $\mu$ m. (C and D) Expression of MyoD, myogenin, and MyHC by Western blot analysis from myoblasts maintained in proliferation medium (P) or differentiated for 16 or 30 h. (C) Densitometric analysis of Western blot with the bands normalized for GAPDH expression ( $n = 4$ ). Error bars represent SEM. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  versus wt. (D) Representative image of a Western blot. (E) C2C12 and 10T1/2 cell were transfected with or without myogenin-CAT (pMyo-1565CAT), pRESNecdin, and pEMSV-MyoD1. CAT activity was measured after 48 h (in proliferation medium for 10T1/2 and in differentiation medium for C2C12) by ELISA assay. ( $n = 6$ ). \*\*\*,  $P < 0.01$  myogenin-CAT only; +,  $P > 0.01$  versus myogenin-CAT + PCMV MyoD. (F) Soluble chromatin was prepared from cultures of C2C12 myoblasts transfected with pCMVNecdin-Myc, differentiated for 48 h in differentiating medium, and immunoprecipitated with antibodies specific for MyoD, necdin, or Myc. Parallel extracts were exposed to rabbit IgG to control for nonspecific precipitation of chromatin. Precipitated genomic DNA was analyzed by PCR, using primers designed to amplify sequences in the *myogenin* promoter. Input refers to DNA input control in which PCR amplification was performed before immunoprecipitation. A sample where no antibody was used for immunoprecipitation was run in parallel (noImmune).

We then wanted to investigate whether necdin was able to directly interact with other proteins known to bind the *myogenin* promoter, e.g., MyoD, myogenin, and Mef2A. We performed co-immunoprecipitation experiments on C2C12 cells transfected with the same necdin-Myc-overexpressing construct. As shown in Fig. S4 A (available at <http://www.jcb.org/cgi/content/full/jcb.200701027/DC1>), we did not detect any direct interaction between necdin and MyoD, myogenin, or Mef2A in these conditions.

### Necdin protects myoblasts from apoptosis

In neural cells, necdin protects against apoptosis, even if the mechanism is not completely clear (Muscatelli et al., 2000; Takazaki et al., 2002; Kuwako et al., 2005; Andrieu et al., 2006). Based on this observation in neural cells, we studied whether necdin protected myoblasts from apoptosis, and whether such protection contributed to accelerated regeneration in vivo in the MlcNec2 mice and delayed regeneration in the Ndn<sup>-/-</sup> animals.

We used TUNEL assay to measure the level of cell death in CTX-injured muscles (Fig. 6, A and B). We observed an increased TUNEL staining in *Ndn*<sup>-/-</sup> TA muscle with respect to wt (Fig. 6 A), whereas *MlcNec2* showed a decreased number of TUNEL-positive cells (Fig. 6 A). We confirmed that satellite cell-derived myoblasts were among the TUNEL-positive cells as demonstrated by expression of nLacZ in a *Myf5*<sup>nLacZ/+</sup> background (Fig. S4, B–E; and not depicted).

We then investigated whether overexpression of necdin had prosurvival effects on myoblasts in vitro. To this end, wt, *Ndn*<sup>-/-</sup>, and *MlcNec2* myoblasts were exposed for a further 24 h to 20  $\mu$ M of the cytotoxic stimuli staurosporine or 100  $\mu$ M of the reactive oxygen species generating agent H<sub>2</sub>O<sub>2</sub>, or exposed for 1 min to UV radiation. Cell death was determined 24 h later by measuring both Annexin V staining of phosphatidylserine exposed on the outer leaflet of the plasma membrane and propidium iodide incorporation. Results obtained are summarized in Fig. 6 C, and representative dot plot analyses showing the results using H<sub>2</sub>O<sub>2</sub> as the death-inducing stimulus are shown in Fig. 6 D. Cell death induction by all stimuli was decreased in *MlcNec2* cells with respect to wt. On the contrary, cell death was increased in all conditions in the *Ndn*<sup>-/-</sup> cells, including basal cell death. To investigate which cell death pathway necdin was involved in, we measured the levels of expression of the activated effector caspase 3 and the initiating activated caspase 9, mainly involved in mitochondrial-mediated cell death (Perrotta et al., 2004). We observed that *Ndn*<sup>-/-</sup> myoblasts challenged with either staurosporine or H<sub>2</sub>O<sub>2</sub> display a higher level of activated caspase 9 and 3, with respect to wt cells, whereas activated caspase 3 and 9 were almost undetectable in *MlcNec2* myoblasts (Fig. 6 E). We observed a similar effect when C2C12 myoblasts transfected with necdin were treated with the same apoptotic stimuli (Fig. S4 F).

Thus, necdin appears to act as a prosurvival factor involved in the protection of cell death through inhibition of the mitochondrial cell death pathway.

## Discussion

Stem cells are capable of both self-renewal and generating progenitors that finally undergo terminal differentiation to ensure the regeneration of tissues whose differentiated cells cannot divide. In tissues with a high cell turnover such as blood and skin, a constant flow of precursors for terminal differentiation is needed. In contrast, adult skeletal muscle needs only new myofibers for repair. In accordance, satellite cells are normally quiescent in adult muscle and activated only in response to specific signals provoked by physiological or pathological stimuli. The activation of satellite cells and their subsequent progression through cell cycle, differentiation, and fusion need to be finely regulated and tuned to ensure correct growth and maintenance of the fibers; therefore, understanding the molecular pathways involved in myoblast differentiation and survival is crucial to the development of treatments for impaired muscle growth associated with age, disease, and atrophy.

Here, we demonstrate that necdin is an important player in skeletal muscle differentiation and maintenance. We found

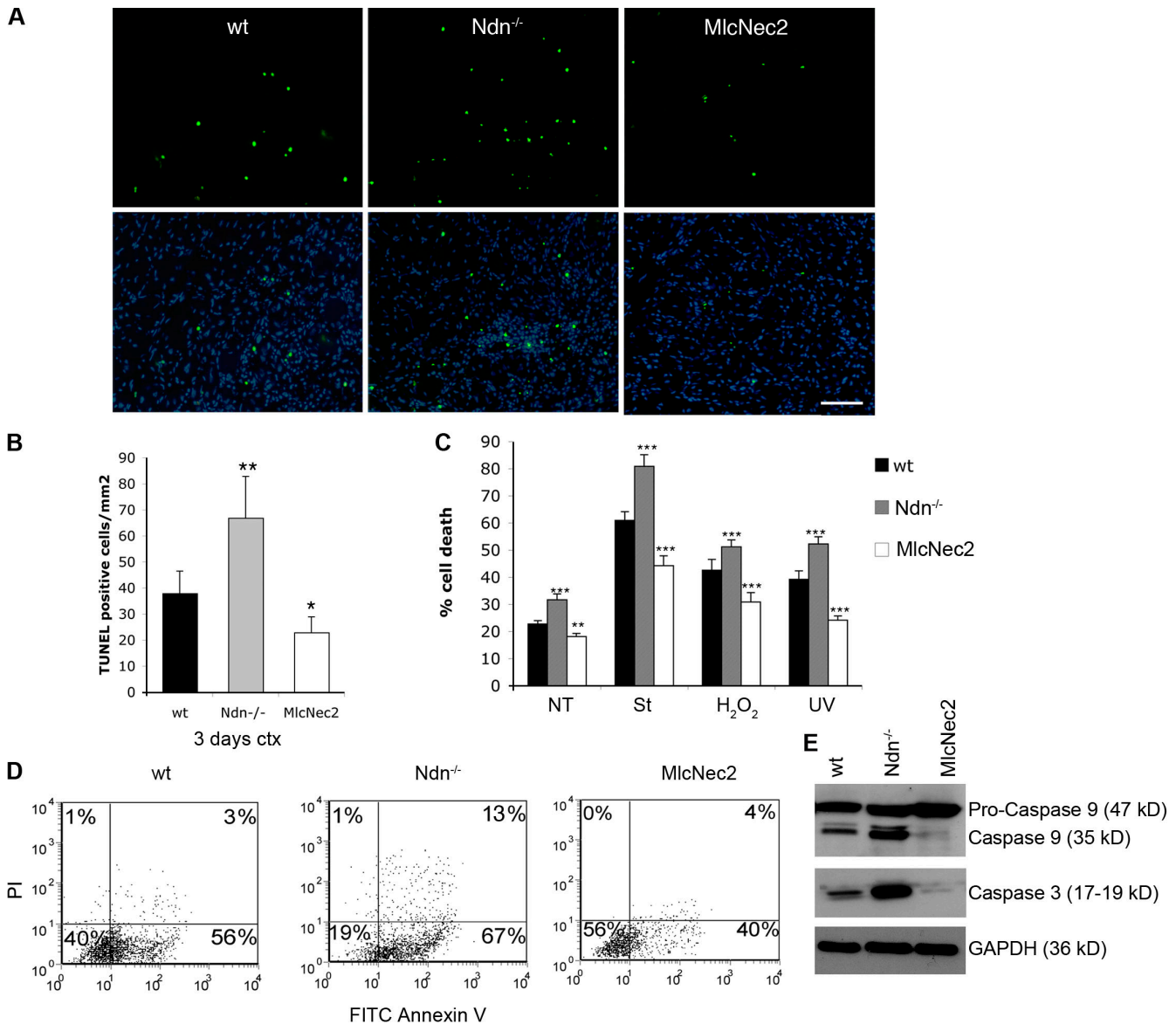
that necdin acts on two different pathways: (1) it acts on myoblast differentiation through direct transcriptional regulation of myogenin, in cooperation with MyoD; and (2) it protects myoblasts from cell death.

### Necdin and terminal differentiation

Necdin was initially identified as a nuclear protein expressed solely in neuronal tissue (Maruyama et al., 1991); subsequent studies demonstrated abundant necdin expression in the cytoplasm of differentiated neurons (Niinobe et al., 2000), in smooth muscle (Brunelli et al., 2004), and in brown adipocyte tissue (Boeuf et al., 2001). Necdin was initially identified by subtraction cloning in embryonic carcinoma cells induced to differentiate into neurons by retinoic acid, where necdin expression increased markedly during early differentiation (Maruyama et al., 1991), and by overexpression of necdin in neuroblastoma cells, which both suppresses cell growth through inhibition of S phase entry (Taniura et al., 1998) and induces differentiation (Kobayashi et al., 2002; Takazaki et al., 2002).

The effect of necdin on differentiation may be cell type specific. In contrast to neuronal cells, where growth arrest is required before final differentiation, adipocyte differentiation occurs with initial growth arrest, cell cycle reentry, and clonal expansion, followed by a second growth arrest and by terminal differentiation (Rangwala and Lazar, 2000). In these cells, increased expression of necdin correlates with impaired differentiation, and reduction of necdin expression restores the differentiation capacity, indicating an inhibitory effect of necdin on adipocyte differentiation (Tseng et al., 2005). This inhibition has also been associated with a down-regulation of adipocyte-specific prodifferentiation regulators, CEBP- $\alpha$  and PPAR- $\gamma$  (Goldfine et al., 2006). This suggests that necdin may act either by interfering with the cell cycle progression or by direct transcriptional regulation of downstream target genes.

As in the case of adipocytes, satellite cells during the regeneration process undergo cell cycle reentry, expansion, and then terminal differentiation. Our results show that necdin is expressed in these cells upon activation and during the expansion phase, as it is coexpressed with Pax7 and MyoD. When myoblasts differentiate and start to fuse, necdin expression decreases. Continuous overexpression of necdin in differentiating myoblasts and in muscle fibers leads to significant hypertrophy of myotubes, myofibers, and muscle (Fig. 3, I–O; and Fig. 5 A). In addition, when muscle regeneration is induced in adult muscle after crush injury the reconstitution of new fibers is accelerated, indicating that myoblasts are actively differentiating. The gain-of-function effect in vitro in myoblast cultures correlates with an increased fusion index and with changes in the expression of different myogenic markers, including myogenin and sarcomeric myosin. Conversely, necdin loss of function leads to the presence of smaller fibers, and when the muscle is damaged the regeneration process is severely impaired. When damage is severe, repair does not occur in the absence of necdin, indicating the obligatory role of this protein. The critical role of necdin appears not to be restricted to muscle because lack of necdin leads to altered motoneuron development (Pagliardini et al., 2005; unpublished data). The relative contribution of



**Figure 6. Necdin is required for myoblast survival in vivo and in vitro.** (A and B) 3 d after CTX injury, TUNEL analysis was performed on TA sections of wt, Ndn<sup>-/-</sup>, and MlcNec2 mice. (A) Representative sections are shown; in the top panels TUNEL staining is shown; in the bottom panels a merge with DAPI staining reveals nuclei. Bar, 400  $\mu$ m. (B) Histogram representing the number of TUNEL-positive nuclei measured on serial TA sections. Values shown are the results of experiments on six animals per group,  $\pm$  SEM. \*\*,  $P < 0.01$ ; \*  $P < 0.05$  versus wt. (C and D) Primary myoblasts from wt, Ndn<sup>-/-</sup>, and MlcNec2 newborn mice were maintained for 24 h in growth medium and incubated for 24 h in the absence (NT) or presence of 5  $\mu$ M Staurosporine (St) or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> or exposed to UV radiation for 1 min. (C) Cell death via apoptosis was determined by flow cytometry measuring Annexin V staining of phosphatidylserine exposed on the outer leaflet of the plasma membrane in propidium iodide–excluding cells ( $n = 4$ ). Error bars represent SEM. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$  versus wt. (D) Representative dot plot analyses showing the results obtained in one of four similar experiments using H<sub>2</sub>O<sub>2</sub> as the cell death–triggering stimulus. Percentage indicates the proportion of cells that are propidium iodide and Annexin V double negative (bottom left section of the plot), propidium iodide and Annexin V double positive (top right), propidium iodide positive (top left), and Annexin V positive (bottom right). (E) Expression of procaspase 9, activated caspase 9, activated caspase 3, and GAPDH as shown by Western blot analysis on myoblasts from wt, Ndn<sup>-/-</sup>, and MlcNec2 newborn mice maintained for 24 h in growth medium and incubated for 3 h in the presence of 5  $\mu$ M staurosporine as the cell death–triggering stimulus.

necdin in motoneurons and skeletal muscle needs to be further investigated.

Myoblasts from these mice show decreased levels of myogenin and myosin and a decreased fusion index when they differentiate. This suggests that necdin may be mainly required during the first phases of differentiation as well as in the growth and repair of the muscle fiber. Our results are consistent with this role, showing that necdin is highly expressed in the

embryonic and fetal myoblasts (Biressi et al., 2007) and during the perinatal stages, whereas it decreases as postnatal development proceeds.

In vitro data in 10T1/2 embryonic fibroblast cells and C2C12 myoblast cells, using a reporter gene driven by myogenin promoter, indicated an enhancement of the transcriptional activation achieved by MyoD or other MRFs. Necdin may therefore act by recruiting or stabilizing transcriptional



complexes on the promoter. Necdin has been described to behave as a coactivator of transcription mediated by other transcription factors such as Dlx2 (Kuwajima et al., 2006), as a corepressor, as in the case of E2F-1 (Kuwako et al., 2004; Kurita et al., 2006), or as a repressor of Msx2 (Kuwajima et al., 2004). Other genes have been described that act as activators or repressors depending on the molecular partner, as observed in the case of Tcf–Groucho versus Tcf– $\beta$ -catenin molecular complexes (Tutter et al., 2001). Here we show that necdin is able to directly interact with sequences on the myogenin promoter. It has been previously shown that necdin may recognize guanosine-rich sequences, similar to the canonical GC box recognized by Sp family members (Matsumoto et al., 2001). Several of these sequences are found in the myogenin promoter, in particular around position  $-170$  to  $150$  from the start site and near the binding sites for Mef2 and E boxes (de la Serna et al., 2005). We did not detect any direct association between necdin and MyoD, Mef2A, or myogenin by coimmunoprecipitation; this is of relevance and suggests that necdin acts directly on these sequences, perhaps with other interacting proteins on the promoter to enhance myogenin transcription.

As previously shown in different cell types, we also provide evidence that necdin interacts with p53 in differentiating myoblasts (Taniura et al., 1999; unpublished data). Many studies support the hypothesis that p53 is activated during myogenic differentiation and participates with MyoD to promote myogenesis (Tamir and Bengal, 1998; Porrello et al., 2000). In addition to acting directly on myogenin transcription, necdin may also cooperate with p53. Nevertheless, because p53 expression is not essential for muscle development and regeneration (Donehower et al., 1992) we hypothesize the role of necdin to be complex.

More studies will be required to get definitive insight into the ability of necdin to bind the myogenin promoter, to characterize necdin molecular partners in myogenic precursor cells and during skeletal muscle differentiation, and to clarify its role in the stabilization of other transcriptional complexes.

### Necdin and cell death

Upon muscle injury, disruption of the myofiber sarcolemma, resulting in increased myofiber permeability, leads to generation of several proapoptotic cues, including the release of free radicals or other oxidants by activated macrophages (Fielding et al., 1993; Tidball, 2005) that can promote additional muscle damage (Hampton et al., 1998; Tidball, 2005) by stimulating apoptosis of myogenic cells. Two major forms of apoptotic cell death have been identified. The extrinsic pathway is initiated by extracellular insult, which triggers receptor (e.g., Fas)-mediated caspase 8 cleavage and activation, which, in turn, catalyses cleavage of the downstream effector caspase 3 that acts on a series of protein targets, initiating the cellular changes leading to irreversible apoptotic cell death (Ashe and Berry, 2003). The intrinsic death pathway is activated by intracellular events, factors such as DNA damage, resulting in mitochondrial-mediated cell death (Saelens et al., 2004). Proapoptotic members of the Bcl-2 family, such as Bax and Bid, undergo conformational change, oligomerization, and translocation to the mitochondria, resulting in cytochrome *c* release that ultimately induces caspase 9

cleavage and activation; activated caspase 9 in turn activates caspase 3 (Twiddy et al., 2004).

Our studies revealed that necdin acts as a prosurvival factor in muscle cells. In particular, myogenic precursor cells from both necdin-overexpressing MlcNec2 mice and C2C12 cells were protected from apoptosis induced by three different stimuli (radiation, hydrogen peroxide, and staurosporine), whereas cell death of  $Ndn^{-/-}$  myoblasts was greatly increased, indicating that necdin acts as an antiapoptotic agent. This observation *in vitro* correlates with the fact that in the crushed muscle of  $Ndn^{-/-}$  mice, delayed regeneration is accompanied by an increased number of TUNEL-positive cells, some of which corresponded to myogenic precursors. Conversely, MlcNec2 mice show a decreased number of TUNEL-positive cells in the crushed muscle. A prosurvival role of necdin consistent with the one we show here has also been described in neurons (Muscatelli et al., 2000; Andrieu et al., 2006; Kurita et al., 2006) where caspase 3 was found to be activated; however, the molecular mechanisms that regulate this action of necdin and the relationship to differentiation were unclear. Here we show that in addition to caspase 3 activation, caspase 9 was greatly activated in the  $Ndn^{-/-}$  cells, whereas when necdin was overexpressed in both primary myoblasts or in C2C12, there was a dramatic reduction in caspase 9 activation and a subsequent reduction in activated caspase 3. This observation establishes for the first time a link between necdin and the extrinsic, caspase 9–dependent apoptosis pathway. In addition, our observation that necdin actively protects from a variety of different proapoptotic stimuli suggests that it switches off the entire apoptotic program triggered in muscle rather than acting as a simple negative modulator of selected apoptotic signals; thus it presumably acts by limiting events in the common pathway to apoptosis converging on caspase 9 activation.

More studies will be needed to clarify whether the antiapoptotic role of necdin is mainly related to its transcriptional or cotranscriptional activity, mediated, e.g., by interaction with p53, or through a direct interaction with the apoptotic pathway in the cytosol.

In conclusion, we identify necdin as a novel, important factor required for proper myoblast differentiation *in vitro* and *in vivo*, for the first phase of muscle fiber growth, and for efficient repair upon muscle injury. We also show that necdin acts at different levels: it cooperates to promote the transcriptional activation of myogenin by direct binding to the promoter, and possibly by contributing to p53 action, and by accelerating myoblast differentiation. In addition, it exerts a prosurvival, antiapoptotic action *in vitro* and *in vivo*, counteracting the cytotoxic effect of several apoptotic agents, including the formation of oxidant species. This necdin prosurvival action can explain: (a) the protection of myogenic precursor cells from apoptosis *in vitro*; (b) the accelerated regeneration *in vivo* in necdin gain-of-function mice; and (c) the dramatic loss of the muscle regeneration capacity of the necdin null mice. This may derive either from the loss of myogenic precursors through cell death and/or from secondary muscle damage caused by the inflammatory cells.

## Materials and methods

### Materials

The following reagents were purchased as indicated: anti-necdin pAb from Upstate Biotechnology; anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb from Biogenesis; anti-MyoD mAb from DakoCytomation; anti-PCNA mAb, anti-MyoD, anti-Mef2A, and anti-Myf5 pAbs from Santa Cruz Biotechnology, Inc.; antisarcomeric myosin MF20, antimyogenin, and anti-Pax7 mAbs from Developmental Studies Hybridoma Bank; anti-developmental MyHC (NCL-MyHCd) from Novocastra; antilaminin and anti-Myc from Sigma-Aldrich; anti-GFP from Invitrogen; and antiactivated caspase 3 pAb and anti-caspase 9 mAb (recognizing activated and non-activated forms) from Cell Signaling. In immunofluorescence analysis, primary antibodies were detected by appropriate Alexa-conjugated (Alexa 489 or Alexa 594) secondary antibodies (Invitrogen). In immunoblot analysis, primary antibodies were detected by chemiluminescence with appropriate horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories). Cell culture media and sera were purchased from Cambrex. Bicinchoninic acid-based assay was purchased from Perbio. The Hoechst dye, CTX, and other chemicals were purchased from Sigma-Aldrich.

### Animals

Transgenic mice were generated by injecting the purified linearized construct represented in Fig. 2 A (as in Gordon [1993]) into the pronuclei of fertilized oocytes (CBA/C57BL6 F1).

Transgenic mice were identified by PCR analysis of tail DNA using the following primers: TgMex2 forward: 5'-GTGTC AAGGTTCTATTAGG-CACTA-3'; and TgNec reverse: 5'-GGTCAACATCTTCTATCCGTC-3'.

Expression of the transgene in the various tissues was evaluated by Western blot using the necdin antibody or by RT-PCR (see Protein extraction and immunoblot analysis and RT-PCR sections).

Lines were established from two founders, MlcNec2 and Nec9, that exhibited correct overexpression of necdin only in the skeletal myoblasts and skeletal muscle and were maintained on a CBA/C57BL6 background or backcrossed on a C57BL6 background. MlcNec2 and MlcNec9 on both backgrounds show the same phenotype in terms of muscle morphology and myoblast behavior; therefore, in this work we only considered results obtained using the MlcNec2 line.

Ndn<sup>-/-</sup> mice (provided by F. Muscatelli) were genotyped by PCR analysis of tail DNA using the following primers: Ndn-KO-S: 5'-TCTCA-TGCTTGAAGTCA-3'; Ndn-KO-AS: 5'-CAGGTAATTCTGCTGGAC-3'; NECF1: 5'-GTCCTGCTGATCCGAAGG-3'; and TgNec reverse: 5'-GGT-CAACATCTTCTATCCGTC-3'.

Mice were also crossed with CBA mice and maintained on either a pure C57BL6 or a CBA/C57BL6 background. In some experiments, mice were crossed with Myf5<sup>nec2/+</sup> mice (provided by S. Tajbakhsh, Pasteur Institute, Paris, France).

### Primary myoblast cell culture and infection

**Isolation of primary myoblasts.** Primary myoblasts from newborn mice of the different strains were isolated as described in Cossu et al. (1980) and plated at clonal density. Cells were grown either in proliferation medium (DME supplemented with 20% FBS, 3% chick embryo, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamycin) or in differentiation medium (DME supplemented with 2% horse serum, 100 U/ml penicillin, and 100 µg/ml streptomycin). We observed that already after 24 h in proliferation medium some cells started to express MyLC and MyHC.

**Single fiber preparation.** Single myofibers were prepared from the extensor digitorum longus and TA muscles of 6–12-wk-old mice as described by Rosenblatt et al. (1995; two animals of each strain for experiments). Individual myofibers were plated on matrigel-coated wells of a 24-well multiwell in proliferating medium (20% FBS, 10% horse serum, and 1% chick embryo extract in DME) or switched to differentiation medium (2% FCS, 10% horse serum, and 0.5% chick embryo extract in DME).

Fibers and myoblasts that exit the fibers were fixed for immunofluorescence experiments at different stages of activation and differentiation.

The necdin lentiviral vector (in pRRLsin.PPT.CMV.NTRiresGFPpre) was generated and prepared as described in Brunelli et al. (2004). The final MOI was 10<sup>7</sup> TU/ml. After 24 h in culture, myoblast cells were infected with an MOI of 100 in proliferation medium overnight. The next day, the medium was changed and cells were maintained in proliferation medium. Immunofluorescence was performed after 72 h, using antibodies specific for sarcomeric myosin and GFP.

### Histology and immunohistochemistry

TA, diaphragm, or soleus muscles were dissected from adult mice (2–3 mo) and frozen in liquid N<sub>2</sub>-cooled isopentane. 8-µm serial muscle sections were either stained with H&E or Azan Mallory or immunostained as described in Sciorati et al. (2006).

### Regeneration assay

To evaluate the ability of the satellite cell to participate in the regeneration process *in vivo*, injury was performed on the TA of 3-mo-old MlcNec2, Ndn<sup>-/-</sup>, and wt mice by injecting 25 µl of 10 µM or 100 µM CTX (four animals per group). Mice were killed at 1, 3, 7, 14, or 30 d after the CTX injection, and the muscle was collected and either sectioned for histological analysis or subject to protein or RNA extraction.

### Morphometry

Morphometric analyses were performed on sections collected from similar regions of each TA muscle and from the belly of each soleus muscle. Two images were captured from each section and Image 1.63 (Scion Corporation) was used to determine the XSA of 700–1,000 myofibers per section.

### Cell lines, plasmids, and transfections

C2C12 and 10T1/2 cell lines were obtained from American Type Culture Collection. C2C12 and 10T1/2 cells were cultured in DME supplemented with 15% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (proliferation medium). C2C12 cells were differentiated in DME supplemented with 2% horse serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (differentiation medium) as described in Pisconti et al. (2006).

10T1/2 and C2C12 cells were transiently transfected with Lipofectamine Plus reagent (Invitrogen). In brief, 10<sup>5</sup> cells were seeded in triplicate on 35-mm dishes, and the next day transfected with 0.5 µg of the reporter construct pMyo-1565CAT (Edmondson et al., 1992) and 0.5 µg pCMV-βGal (CLONTECH Laboratories, Inc.), alone or together with various combinations of plasmids mixed as follows: 1 µg pIRESNecdin; 1 µg pEMSV-Myf3 (MyoD1); and 1 µg pIRESNecdin + 1 µg pEMSV-Myf3 (MyoD1). Cells were grown for 2 d in DME, 10% FCS (or in the case of C2C12 cells, in differentiation medium, DME, and 2% horse serum); proteins were then extracted by repeated freezing–thawing. The levels of CAT protein were determined using an enzymatic immunoassay (CAT-ELISA; Roche Diagnostics). Cell extracts were normalized for protein concentration and CAT expression was further normalized to β-galactosidase activity.

C2C12 cells were also transfected with pIRESNecdin to assess effect on differentiation. Immunofluorescence was performed after 5 d in differentiating medium, using antibodies specific for sarcomeric myosin and GFP. Alternatively, C2C12 cells were transfected with pCMV3B-Myc-necdin, and coimmunoprecipitation or chromatin immunoprecipitation was performed after 48 h in differentiating medium.

### Immunofluorescence

Immunofluorescence on the cell cultures and cryosections was performed according to Brunelli et al. (2004), using antibodies specific for necdin, sarcomeric myosin MF20, MyoD, MyHCd, laminin, Pax7, Myf5, and GFP. For fluorescent detection, we used appropriate secondary antibodies conjugated with either Alexa 488 (green) or Alexa 594 (red; Invitrogen).

Apoptotic cells on muscle cryostat sections were detected using the ApopTag fluorescein direct *in situ* apoptosis detection kit (Intergen).

The fusion index of differentiating myoblasts and C2C12 cells was measured as the number of nuclei in sarcomeric myosin-expressing cells with more than two nuclei versus the total number of nuclei.

### Cell resistance to apoptosis

Satellite cells from the different genotypes were incubated with or without 100 µM H<sub>2</sub>O<sub>2</sub> or 5 µM staurosporine for 24 h, or exposed to UV light for 1 min. Cells were detached and stained with fluorescein isothiocyanate–Annexin V and propidium iodide, according to the manufacturer's instructions (Bender MedSystems), and analyzed by flow cytometry as described in Sciorati et al. (1997). Cells that showed single staining for Annexin V or double staining for Annexin V and propidium iodide were considered dead cells. Alternatively, cells were lysed 3 h after treatments and analyzed for expression of activated caspases by Western blot.

### Protein extraction and immunoblot analysis

Cells were homogenized in 50 mM Tris/HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Complete; Roche Diagnostics) and centrifuged at 1,000 g for 20 min at 20°C to discard cellular debris.

Muscle tissues were dissected and homogenized in 100 mM NaHCO<sub>3</sub>, 1 mM EDTA, 2% sodium dodecyl sulfate, and protease inhibitor cocktail and centrifuged at 1,000 g for 10 min at 4°C to discard cellular debris. Sample preparation and Western blot analyses were performed as described in Pisconti et al. (2006).

After electrophoresis, polypeptides were electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell), and antigens were revealed by the respective primary antibodies and the appropriate secondary antibodies.

#### Chromatin immunoprecipitation

6 × 10<sup>6</sup> of differentiated C2C12 cells, previously transfected with Myc-tagged necdin, were treated with 1% formaldehyde and sonicated in 600 μl of protease inhibitor-containing buffer. The chromatin was then immunoprecipitated with, respectively, mouse anti-MyoD (Santa Cruz Biotechnology, Inc.), mouse anti-Myc (Sigma-Aldrich), and rabbit anti-necdin (Upstate Biotechnology), as in Bodega et al. (2007). The input DNA was an aliquot of the supernatant from centrifuged sonicate (DNA size range: ~200–1,000 bp), and the preimmune chromatin was immunoprecipitated with normal rabbit and mouse IgG (Santa Cruz Biotechnology, Inc.). After an overnight incubation with the antibodies, 10 μl of 20 mg/ml rRNA, 20 μl of 10 mg/ml salmon sperm DNA, and 20 μl of protein G-agarose beads were added to the 1-ml samples and incubated for 3 h at 4°C with constant agitation. The purified immunoprecipitated DNA was dissolved in 60 μl of 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. PCRs were performed with GoTaq (Promega) for 40 cycles. The primers used were myogenin forward (5'-GAATCACATGTAATCCACTGGA-3'), myogenin reverse (5'-ACGCCA-ACTGCTGGTGCCA-3'), β-actin forward (5'-GCTTCTTGACGCTCCTC-GTTG-3'), and β-actin reverse (5'-TTTGACATGCCGGAGCCGTTGT-3').

#### Coimmunoprecipitation

For detection of necdin interactors, C2C12 cells were transfected with pCMV3B-Myc-necdin. Cells were harvested after 48 h in differentiating medium and lysed in RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate sodium, 1% NP-40, and 1× protease inhibitor mixture (Roche). The 2 mg of lysates were incubated overnight at 4°C with 5 μg of polyclonal antibody against necdin (Upstate Biotechnology) or rabbit anti-IgG. The complexes were pelleted with protein G-Sepharose (Invitrogen), separated by 10% SDS-PAGE, and transferred to nitrocellulose membrane by electroblotting. The membranes were incubated with antibodies against MyoD, Mef2A, myogenin, or Myc, and washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgGs (Bio-Rad Laboratories). Proteins were visualized by the chemiluminescence method (ECL Western blot detection reagent; GE Healthcare).

#### RT-PCR

1 μg RNA, collected from cells or tissues using RNeasy mini (or micro) kit (QIAGEN), was converted into double stranded cDNA using the cDNA synthesis kit ThermoScript RT-PCR system (Invitrogen), according to the manufacturer's instructions. The primers used were the following: GAPDH forward: TGAAGGTCGGTGTGAACGGATTTG; GAPDH reverse: CATGTGGGCCATG-AGGTCCACCAC; dMyHC forward: ACTGAGGAAGACC GCAAGAATG; dMyHC reverse: AAGTAAACCCAGAGAGGCCAAGTGACC; MyLC1 forward: GATCACCTTAAGTCAAGG; MyLC1 reverse: gcaacgctctacctt; necdin NEC F2: 5'-CACTGATAGTTTCTGACCCATAC-3'; NEC R1: 5'-GCCAGTGAAGTC-ATATGGAG-3'; necdin transgene TgNec reverse: 5'-GGTCAACATCTTCTATC-CGTTC-3'; and TgMex2 forward: 5'-GTGTCAAGGTTCTATTAGGCACTA-3'.

#### Image acquisition and manipulation

Fluorescent and phase-contrast images were taken on microscopes (Eclipse E600 [Nikon]; Plan Fluor lenses: 10×/0.33, 20×/0.50, and 40×/0.75; [Leica AF6000]; lenses: HCX PL FLUOTAR L40×/0.60). Images were acquired using digital cameras (DXM1200 [Nikon]; DFC 350 FX [Leica]) and acquisition software (ACT-1 [Nikon]; AF6000 [Leica]). The imaging medium was PBS buffer; images were taken at room temperature. Images were assembled in panels using Photoshop 7.0 (Adobe). Images showing double or triple fluorescence were separately acquired using the appropriate filters, and the different layers were merged with Photoshop 7.0.

#### Statistical analysis

The results are expressed as means ± SEM; *n* represents the number of individual experiments. Statistical analysis was performed using the analysis of variance test. Asterisks in the figures refer to statistical probabilities versus wt controls, respectively. Statistical probability values of <0.05 were considered significant.

#### Online supplemental material

Fig. S1 shows coexpression of necdin with Pax7 on single fiber, expression of necdin on a TA section of adult MlcNec2 mice, and expression of necdin and Myf5 on a TA section of Ndn<sup>-/-</sup> P5 mice. Fig. S2 shows histology of TA of wt, MlcNec2, and Ndn<sup>-/-</sup>, 30 d after injection of 100 μM CTX. Fig. S3 shows the differentiation of primary myoblasts or C2C12 cells after overexpression of necdin. Fig. S4 shows absence of coimmunoprecipitation of necdin with MyoD, myogenin, and Mef2A; costaining of TUNEL-positive cells and β-galactosidase-positive cells in Myf5<sup>nlacZ/+</sup>, Ndn<sup>-/-</sup> mice after CTX injection; and expression of activated caspase 3 and 9 in C2C12 cells overexpressing necdin. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200701027/DC1>.

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