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Skeletal muscle regeneration is delayed by reduction in Xin expression: consequence of impaired satellite cell activation?

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Nissar AA, Zemanek B, Labatia R, Atkinson DJ, van der Ven PF, Fürst DO, Hawke TJ. Skeletal muscle regeneration is delayed by reduction in Xin expression: consequence of impaired satellite cell activation?. *Am J Physiol Cell Physiol* 302: C220–C227, 2012. First published October 5, 2011; doi:10.1152/ajpcell.00298.2011.—Xin is a striated muscle-specific actin-binding protein whose mRNA expression has been observed in damaged skeletal muscle. Here we demonstrate increased Xin protein expression early postinjury (≤12 h) and localization primarily to the periphery of damaged myofibers. At 1 day postinjury, Xin is colocalized with MyoD, confirming expression in activated satellite cells (SCs). By 5 days postinjury, Xin is evident in newly regenerated myofibers, with a return to preinjury levels by 14 days of regeneration. To determine whether the increased Xin expression is functionally relevant, tibialis anterior muscles of wild-type mice were infected with Xin-short hairpin RNA (shRNA) adenovirus, whereas the contralateral tibialis anterior received control adenovirus (Control). Four days postinfection, muscles were harvested or injured with cardiotoxin and collected at 3, 5, or 14 days thereafter. When compared with Control, Xin-shRNA infection attenuated muscle regeneration as demonstrated by Myh3 expression and fiber areas. Given the colocalization of Xin and MyoD, we isolated single myofibers from infected muscles to investigate the effect of silencing Xin on SC function. Relative to Control, SC activation, but not proliferation, was significantly impaired in Xin-shRNA-infected muscles. To determine whether Xin affects the G0-G1 transition, cell cycle reentry was assessed on infected C2C12 myoblasts using a methylcellulose assay. No difference in reentry was noted between groups, suggesting that Xin contributes to SC activation by means other than affecting G0-G1 transition. Together these data demonstrate a critical role for Xin in SC activation and reduction in Xin expression results in attenuated skeletal muscle repair.

satellite cells; cardiotoxin

WHILE THE ABILITY for skeletal muscle to grow, adapt, and regenerate is well known, the numerous factors governing this ability are less well defined. Furthermore, satellite cells, the primary stem cell of skeletal muscle, are critical to these capacities of skeletal muscle and the factors governing their regulation are still being uncovered. In previous work, we identified Xin to be highly upregulated at the mRNA level within regenerating skeletal muscle, particularly during the early phases of regeneration (10).

Xin was initially observed as a critical protein for heart looping in developing chick embryos. To date, however, the role(s) within skeletal muscle for this striated muscle-specific, cytoskeletal protein has yet to be elucidated. In developing mouse skeletal muscle, Xin can be detected within somites at embryonic *day 10* (E10), and by E13.5 Xin is seen in some maturing skeletal muscle with expression expanding to include all skeletal muscles as the animal reaches maturity (10). That said, endogenous mRNA levels of Xin in healthy, uninjured, adult skeletal muscle are observed to be low (10), and protein expression appears to be primarily localized to the myotendinous junction (16). The mRNA expression within skeletal muscle changes dramatically in response to muscle injury, however, with increased Xin detected within 6 h of damage and a peak in expression noted at 12 h postinjury (10). With the use of in situ hybridization, the early expression of Xin mRNA was observed at the periphery of muscle fibers; an area consistent with the location of muscle satellite cells. Immunohistochemical staining of isolated single fibers confirmed the expression of Xin in satellite cells as it could be colocalized with Syndecan-4, a known satellite cell marker (6, 10).

Whereas the expression of Xin mRNA has been demonstrated to be increased within damaged skeletal muscle (1, 2, 10), it was unknown whether this translated to changes in protein expression and whether these changes were functionally significant to the muscle repair process. As well, it had yet to be elucidated if the expression of Xin within the satellite cell was critical for the regulation of this stem cell population. Thus the purpose of the present study was to define the protein expression of Xin within regenerating muscle and then to determine the functional contribution of Xin to skeletal muscle repair and satellite cell function through the use of Xin-short hairpin RNA (shRNA) adenoviruses. The in vivo results of this work demonstrate that Xin-shRNA adenoviruses injected into skeletal muscle lead to delayed regeneration following injury as demonstrated by fiber area quantification and Myh3 (myosin heavy chain-embryonic isoform) expression. Our in vitro results demonstrate that reduced expression of Xin leads to an impairment in the ability of satellite cells to become "activated" but does not affect their proliferative capacity. Taken together, the findings of the current study highlight Xin as a critical protein for satellite cell activation and reducing Xin expression during skeletal muscle repair attenuates the regenerative process.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice (3–4 mo of age) were obtained from Jackson Laboratories (Bar Harbor, ME). All mice had access to enrichment material (nesting material, cardboard tubing) and were provided with standard breeder chow and water ad libitum. Animals were housed in an environment maintained at 21°C, 50% humidity, and 12 h/12 h light-dark cycle. All experimental protocols were approved by the

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York University and McMaster University Committees on Animal Care and performed in accordance with the Canadian Council Animal Care guidelines.

Adenoviral Material

All adenoviral constructs were prepared by Vector Biolabs using adenoviral vector Type 5 (www.vectorbiolabs.com), purified by CsCl centrifiguation, and have been published previously (10). As previously noted, the Xin-shRNA adenovirus resulted in a greater than 50% decrease in Xin mRNA expression and an approximate 60% decrease in endogenous Xin, 4 days postinfection, compared with control (10). Briefly, to decrease endogenous Xin levels, the right tibialis anterior (TA) muscle of each mouse was infected with an adenovirus containing Xin-shRNA, which also expressed green fluorescent protein (GFP) used to determine adenoviral infection efficiency (Ad-CMV-Xin-shRNA-GFP; loop sequence: TTCAAGAGA; sense 5′-GATCCGGAAGAAAAGGGATATCAGTTCAAGAGACTGATATCCCTTTTCTTCCTTA-3′; antisense: 5′-AGCTTAAGGAAGAAAAGGGATATCAGTCTCTTGAACTGATATCCC TTTTCTTCCG-3′).

The contralateral leg of each animal was used as a control and infected with the same adenoviral vector containing GFP alone (Ad-CMV-GFP). Injection into the muscles was done using a Hamilton syringe with the needle inserted at the distal tendon in a distal to proximal direction, along the length of the muscle belly. Once the needle was inserted, the adenovirus [4.7×10^{10} plaque-forming units suspended in a 2:1 adenovirus to phophate-buffered saline (PBS) ratio by volume] was injected as the needle was slowly withdrawn (along the length of the muscle). In this way, ~75% of the muscle belly length was infected with similar degrees of infection observed between all mice regardless of vector. We estimated that ~35% of the muscle was infected by our injection strategy. This same procedure was undertaken to infect the extensor digitorum longus (EDL) (deep and lateral to TA) and the lateral compartment (peroneus) muscles for single muscle fiber experiments. Only areas/fibers expressing GFP were used in our measurements.

Adenoviral infection efficiency within myoblasts, regardless of construct, was routinely greater than 90% such that any difference in expression noted is the result of the degree of Xin repression rather than the degree of infection efficiency.

Skeletal Muscle Injury

Skeletal muscle injuries were induced using a 100-μl intramuscular injection of 10 μM cardiotoxin (CTX; Latoxan, Valence, France), as previously described (10). To establish a timeline for Xin expression in regenerating muscle, TA muscles were harvested before CTX injection (0 day) or at 0.5, 1, 3, 5, and 14 days postinjury ($n = 4$: 0d, 1d, 3d, 5d; $n = 3$: 0.5d, 14d). In the adenoviral experiments, CTX injury was induced 4 days following adenoviral infection in the left and right TA muscles. Muscles were harvested before injury (0d) or at 3, 5, and 14 days post-CTX injury.

Tissue Collection

Animals were euthanized by CO_2 inhalation followed by cervical dislocation. The TA muscles were excised and cut transversely in half. The proximal half was snap frozen in liquid nitrogen, whereas the distal half was coated in tissue-mounting medium and frozen in liquid nitrogen-cooled isopentane. Uninjured (0d) left and right EDL and peroneus longus muscles were harvested to isolate single muscle fibers.

Single Muscle Fiber Isolation

Single muscle fibers were obtained from left (control) and right (Xin-shRNA) EDL and peroneus muscles 1 day after adenoviral infection ($n = 5$), as previously described (11). Briefly, muscles were removed and digested in a collagenase solution. Resulting muscle bundles were triturated to isolate single fibers that were then collected using a sterile glass Pasteur pipette and placed in 24-well culture dishes containing plating media [10% normal horse serum, 0.5% chick embryo extract (MP Biomedicals) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen)].

Satellite Cell Activation and Proliferation Analysis

Satellite cell activation was assessed in floating culture by adding 10 μM 5-bromo-2-deoxyuridine (BrdU; Sigma 858811) to the plating media and incubating newly isolated single fibers for 24 h. After this duration, fibers were briefly fixed in 4% paraformaldehyde (PFA), incubated in 2 N HCl for 1 h at 37°C, followed by blocking buffer (1.5% normal goat serum, 1.5% normal horse serum) for 1 h. BrdU antibody (Sigma B2531) was diluted 1:25 in PBS and applied overnight at 4°C. A biotin-streptavidin (Vector Labs) detection system was used according to the manufacturer's protocol to visualize BrdU+ satellite cells. Briefly, streptavidin (20 min)-biotin (20 min) amplification was employed followed by a secondary anti-mouse biotin (2 μg/ml, 1:500) for 30 min. Fibers were rinsed and streptavidin anti-biotin fluorescein (1:100) with propidium iodide (200 μg/ml, 1:5; used to stain for all nuclei) was applied for 30 min. Satellite cells that became activated and entered the cell cycle-incorporated BrdU; therefore, activation was analyzed by the number of BrdU-positive cells per muscle fiber. The staining procedure for nonadherent single muscle fibers has been described in detail previously (20, 21).

Satellite cell activation was also assessed by infecting single fibers isolated from C57Bl/6 mice with 1 μl of 1×10^{10}/ml of either the Xin-shRNA Ad or GFP Ad constructs in Matrigel-coated plates to allow fibers to adhere. Fibers were left undisturbed for 24 h, after which the number of myoblasts that had migrated away from the fiber was determined using light microscopy. To assess proliferation, isolated muscle fibers were left undisturbed for 48 h on matrigel-coated dishes. After this time, the myofiber was removed, and the number of cells on the dish at this time (48 h) and 24 h later (72 h) were quantified to determine the fold increase in cell doublings. For satellite cell activation and proliferation assessments, a total of 50 fibers from an $n = 5$ were analyzed for all control and Xin-shRNA groups.

Western Blot Analysis

Proximal halves of TA muscles were ground in liquid nitrogen to a powder, which was then added to 2× SDS sample buffer, heated to 65°C for 15 min ensuring not to boil samples, and sonicated to shear DNA and homogenize (17). Proteins were separated using polyacrylamide gel electrophoresis and transferred to a PVDF membrane as previously described (13). Primary antibodies for Xin (1:50; 17) and MyoD (1:500; Dako) were used to detect proteins of interest. Protein expression was visualized by the binding of horseradish peroxidase-conjugated secondary antibodies (1:10,000) and the addition of Super Signal West Femto chemiluminescent reagent (Pierce Biotechnolog). The antibody used to detect Xin is directed toward the Xin repeats and thus detects both the A and B isoforms of Xin. As discussed later, Xin B was the primary isoform (~130 kDa) detected throughout these studies.

Images of blots were acquired with the Fusion Fx7 imager and accompanying software. Analysis of blots was performed using ImageJ freeware. It should be noted that significant changes in proteins normally used as loading controls (e.g., GAPDH) during skeletal muscle regeneration occur. Therefore, Ponceau-stained membranes were imaged, and an appropriate band was used to correct for protein loading. The 12- and 24-h time points were corrected with a band at 50 kDa, while the 0d, 3d, 5d, and 14d were corrected with a 37-kDa band. This allowed for the normalization of loading between the left and right TA samples of the same animal for each specific time point. Thus, in Fig. 3*C*, quantification is normalized for each time point but not between time points.

Cell Cycle Arrest and Reentry Assay

Control and Xin-shRNA-infected C2C12 cells were trypsinized and suspended in a final concentration of 3×10^5 into cell culture flasks containing 2% methylcellulose (Methocel A4M Premium; Dow Chemicals) in serum-containing DMEM and incubated for 72 h to ensure cell cycle arrest. Cells were recovered from the methylcellulose media by dilution in room temperature PBS, centrifugation, and PBS washes. Cells were further cleaned by passing through sterile 70-μm filters and a Ficoll gradient. Cells were then plated in proliferation medium (10% FBS, high-glucose DMEM) and allowed to reenter the cell cycle and proliferate for 12, 18, or 24 h. After the allotted time, cells were detached by trypsinization, washed in PBS, fixed with 70% cold ethanol while being vortexed, and then resuspended in a propidium iodide-RNAse A staining solution (1 mg/ml propidium iodide, 2 mg/ml RNAse A) overnight to label DNA and degrade RNA. With the use of a fluorescence-activated cell sorter flow cytometer (BD FACSCalibur; BD Biosciences), 10,000 live events were acquired for each sample using Cellquest software (BD Biosciences). The percentage of cells in each phase of the cell cycle was quantified using ModFit LT for Mac v3.0 software as previously described (8).

Histochemical and Immunofluorescent Analyses

Distal TA muscles were transversely cut into 8-μm sections and adhered to glass slides for subsequent histochemical or immunofluorescent staining.

Hematoxylin and eosin stain. Hematoxylin and eosin (H&E) stains were used for the determination of control and Xin-shRNA adenoviral-infected TA fiber area at 5 and 14 days of regeneration. Greater than 100 injured fibers (GFP-positive and centrally located nuclei) throughout each muscle section were analyzed.

Immunofluorescent staining. Embryonic myosin heavy chain (Myh3) expression was detected by immediately fixing cut sections in 2% PFA, incubating in block (10% normal goat serum and 1.5% bovine serum albumin) for 30 min, followed by mouse IgG block (Vector, BMK 2202) for 1 h at room temperature. Sections were then incubated with undiluted Myh3 antibody (Hybridoma Bank F1.652) at 4°C overnight. To visualize Myh3, sections were incubated in Alexa 488 anti-mouse antibody for 1 h (1:1,000) at room temperature. A drop of 1:10,000 4,6-diamidino-2-phenylindole (DAPI) was applied to the section for 5 min to identify nuclei. To validate our direct observations of adenoviral-mediated GFP expression using fluorescent microscopy, GFP expression within some infected muscle sections was also detected using a rabbit polyclonal antibody (1:400; Abcam ab290) and visualized with a Texas-Red conjugated secondary antibody (1:500).

Xin expression was detected by applying undiluted primary antibody (17) to the fixed section for 1 h at room temperature after the mouse IgG block. A biotin-streptavidin detection system was used as recommended (Vector Labs). The sections were then incubated in streptavidin antibody conjugated to Texas red diluted 1:100 for 30 min at room temperature. Blocking buffer was applied before costaining with laminin (Abcam; ab14055) or MyoD antibodies (Abcam; ab64159), which were diluted 1:250 and incubated overnight at 4°C. To detect laminin and MyoD, Alexa 488 anti-chick and anti-rabbit antibodies were applied respectively to the muscle section, followed by DAPI.

Image Analysis

High resolution images of stained muscle sections were captured with a Nikon 90i eclipse upright microscope ×20 objective. Using Nikon Elements software, fiber areas were determined by manually outlining fibers, and Myh3 stains were analyzed using threshold detection for positive fibers.

Statistical Analysis

Student's *t*-tests were performed to test for significant differences ($P < 0.05$) in data obtained from Xin-shRNA and control muscles. Data are presented as means ± SE.

RESULTS

Protein Expression of Xin is Observed Within Satellite Cells and Regenerating Muscle After Injury

While it was previously demonstrated that Xin expression at the mRNA levels was observed in the regenerating muscle (via RT-PCR and in situ hybridizations), it remained unknown if this increase in mRNA translated into an increase in protein expression. As can be seen in Fig. 1, the increase in Xin protein expression occurs within 12 h following cardiotoxin-induced

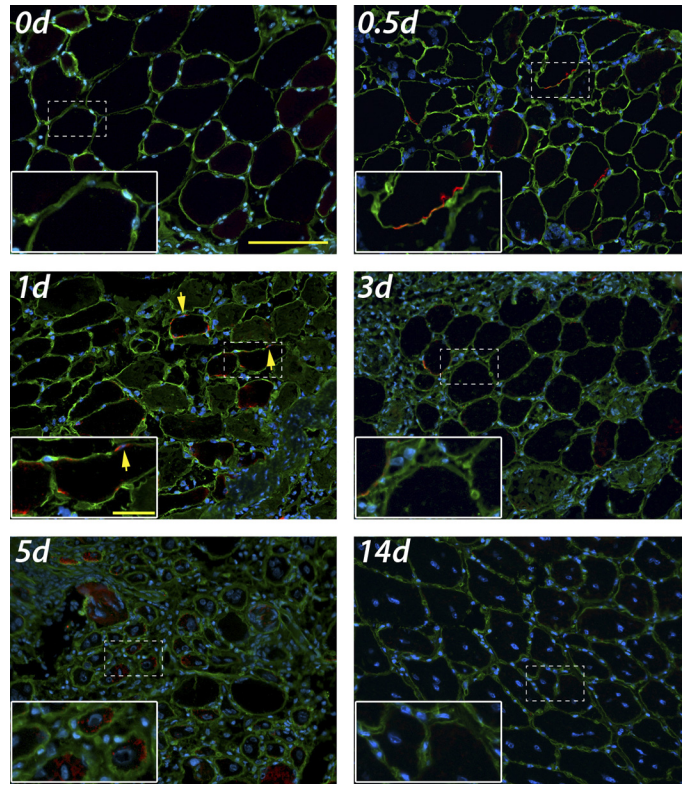

Fig. 1. Immunohistochemical expression of Xin during skeletal muscle regeneration. Protein expression of Xin (red) within the midbelly of the muscle is relatively low in the uninjured state (0 day; 0d). The expression increases within 12 h (0.5d) of cardiotoxin-induced muscle injury. The expression of Xin is largely localized to the periphery of the muscle fibers (arrowheads). By 1 day (1d) postinjury, the expression of Xin is generally sublaminar (laminin: green) and often at the periphery of the muscle fiber. There are also instances where this sublaminar Xin expression is colocalized with nuclei (4,6-diamidino-2-phenylindole, DAPI: blue) as noted by the yellow arrows. This expression pattern is characteristic of muscle satellite cells. By 3 days of regeneration (3d) there is a large inflammatory response evident as illustrated by the high level of interstitial nuclei. The expression of Xin is somewhat reduced compared with the 1 day time point though Xin expression is still observed at the periphery of muscle fibers. By 5 days postinjury (5d), the expression of Xin has increased and is observed primarily in newly regenerating muscle fibers (centrally located nuclei; arrowheads). At 14 days of regeneration, Xin expression has returned to levels consistent with the preinjury (0d) state. *Inset*, higher magnification highlight the expression/localization (or absence) of Xin at each time point. The dotted rectangle represents the region of interest that has been expanded upon in the bottom left corner of each panel (solid rectangle). Bar = 100 μm, which is consistent for all panels.

muscle damage, and the intensity increases by 24 h following injury, a finding consistent with an increase in mRNA at 6 and 12 h after damage (10). Though the mRNA expression appeared to decrease by 3 days postinjury (2), Xin protein was clearly evident at 5 days postinjury within newly regenerated muscle fibers (as illustrated by the centrally located nuclei). By 14 days of repair, the expression of Xin protein was very low, comparable to preinjury (0 day) levels.

At 24 h postinjury, the expression of Xin appeared in areas consistent with satellite cells (i.e., under the basal lamina and in association with nuclei; Fig. 1). While this association was not unexpected based on our previous work (10), we had not yet demonstrated that Xin was definitively expressed in activated satellite cells/myoblasts at the protein level within damaged skeletal muscle. To confirm this, we costained 24 h postinjury skeletal muscle samples with antibodies detecting Xin and the myogenic regulatory factor, MyoD (Fig. 2). Some examples of this colocalization are shown in Fig. 2, supporting the hypothesis that Xin is expressed in activated satellite cells, and its expression may be important in satellite cell function.

Repression of Xin In Vivo Leads to a Delay in Muscle Repair

To investigate the function of Xin within regenerating skeletal muscle, we undertook an adenoviral-mediated approach to downregulate Xin expression with one TA muscle receiving Xin-shRNA and the contralateral leg receiving the control adenoviral vector (Fig. 3). Western blot analysis for Xin expression in control and Xin-shRNA-infected myoblasts demonstrated a ~60% decrease in endogenous Xin expression at 4 days postinfection (Fig. 3*A*). This level of reduction is consistent with our in vivo repression level (0 day time point in Fig. 3*C*). As illustrated in Fig. 3*B*, both vectors contained GFP to allow for identification of infected areas. To verify the presence of GFP within the regenerating muscle (and thus confirm the presence of the adenovirus/expression of shRNA while ruling out an autofluorescent component), we stained regenerating muscle (14 day) with anti-GFP and detected this with a secondary antibody conjugated to Texas red secondary antibody. The infected area stained intensely for the presence of GFP protein even at 14 days of regeneration (18 days postinfection).

In Fig. 3*C*, the effect of Xin-shRNA can be observed in resting and regenerating skeletal muscle. In control infected muscles, Xin protein expression increased significantly in the first 12 h of regeneration with a peak in expression at 24 h. In contrast to our immunohistochemical observations, the expression of Xin (by immunoblotting) returned to the uninjured levels by 3 days of regeneration and remained at that level through the remaining 11 days of repair. The Xin-shRNA adenovirus infection was successful in blunting the rise in Xin expression at 12 and 24 h of regeneration. Interestingly, while the expression of Xin in control muscles returned to the preinjury state by 3 days of repair, the Xin-shRNA-infected muscles displayed a continued presence of elevated Xin expression (albeit blunted). By 5 days of regeneration in the Xin-shRNA muscle, the expression of Xin is similar to control levels and not until 14 days of repair does the expression return to levels significantly reduced compared with control infected muscles.

At all time points of regeneration, the expression of Xin B (~130 kDa) was the dominant isoform of expression with only faint or absent signal detected at the level of Xin A (~205 kDa). Therefore, only the expression of Xin B is presented here and we hereafter refer to the Xin B isoform as Xin.

The expression of MyoD, a known transcriptional regulator of Xin (2), increases in both groups within hours of muscle damage, but only in the Xin-shRNA-infected muscle does the expression remain significantly elevated at 3 days postinjury (Fig. 3*C*, *bottom*). Thereafter, MyoD expression in the Xin-shRNA muscle decreases to be modestly, but significantly, elevated at 5 days before returning to control levels at 14 days postinjury.

The robust expression of Xin during muscle regeneration suggests an important role for this striated muscle-specific protein in the repair process. The expression of myosin heavy

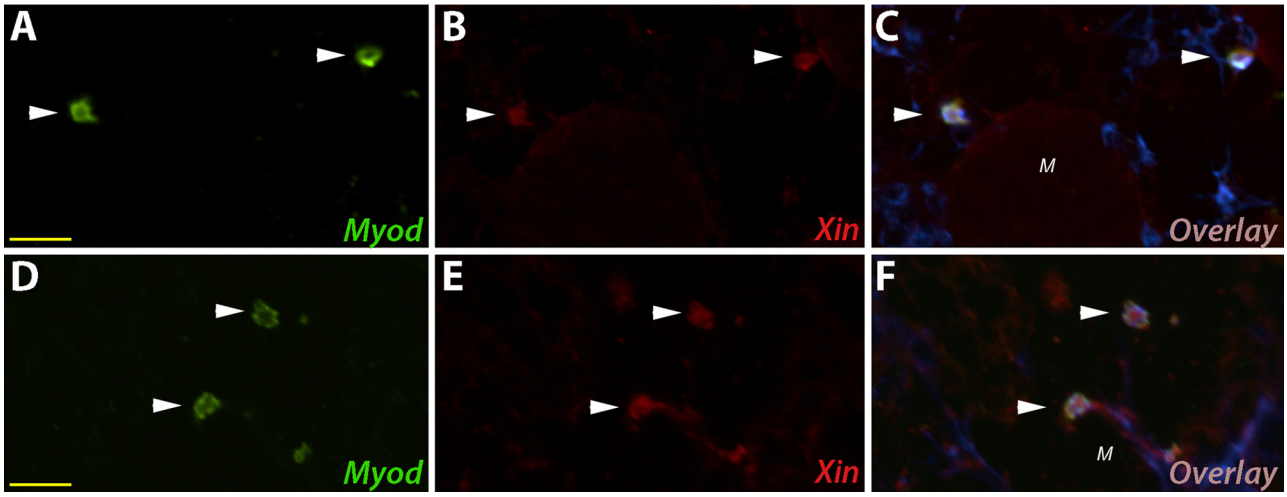

Fig. 2. Colocalization of Xin and MyoD at 24 h of skeletal muscle regeneration. Two examples of multiple MyoD-positive areas (*A* and *D*) that colocalize with Xin expression (*B* and *E*) as illustrated in the overlay (*C* and *F*), which also includes DAPI to label all nuclei. The background of the overlay images has been increased slightly to allow for an enhanced view of the surrounding structures. The *M* denotes the location of a muscle fiber. Bars in *A* and *D* = 15 μm, which are consistent for all panels.

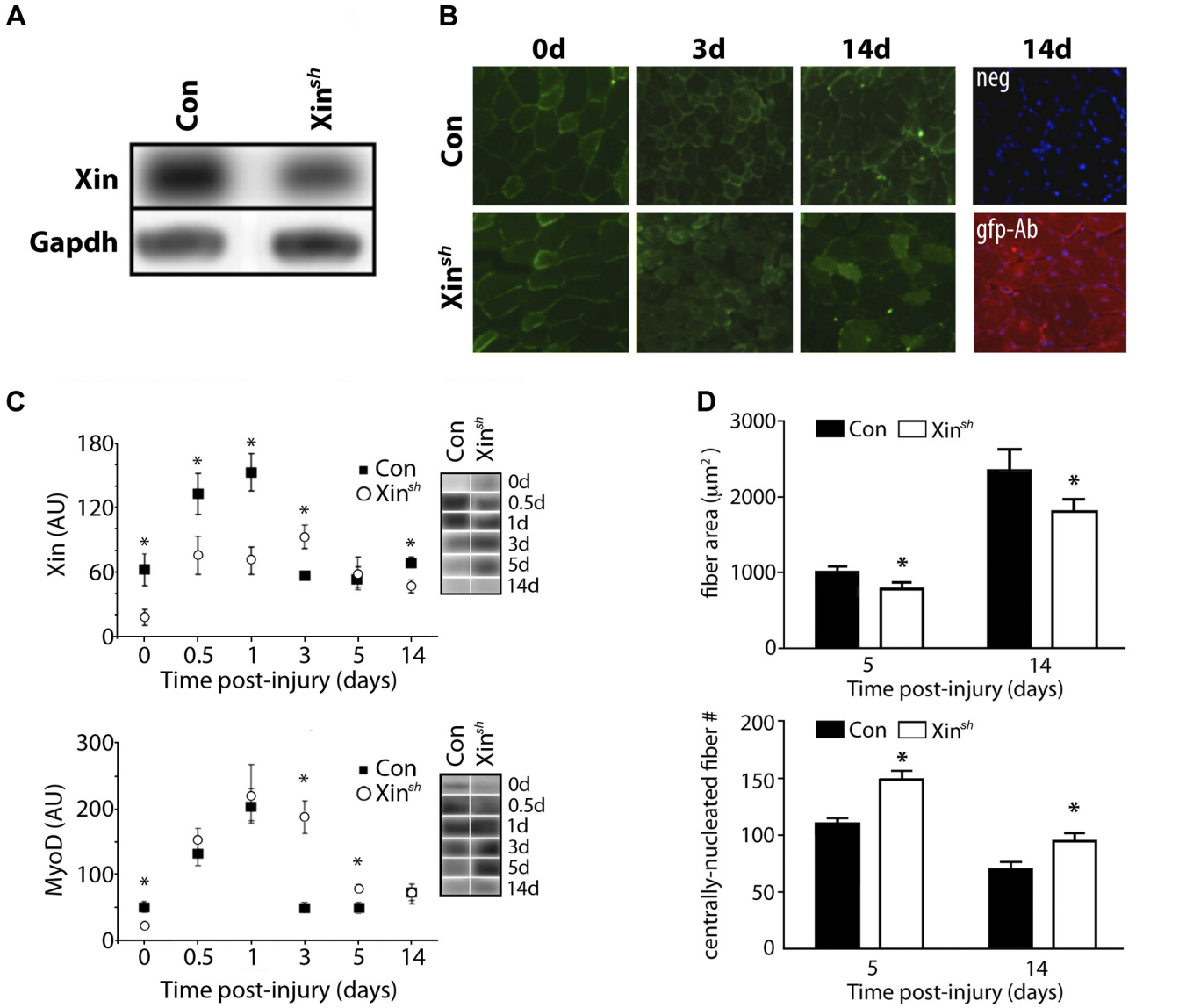

Fig. 3. Xin-short hairpin RNA (shRNA) infection impairs skeletal muscle repair. *A*: Xin protein expression in green fluorescent protein (GFP) (Con) and Xin-shRNA (Xin^{sh})-infected myoblasts demonstrated an ~60% decrease in endogenous Xin expression at 4 days postinfection. *B*: both Con and Xin^{sh} vectors contained GFP, thus regions of infection could be identified. The right tibialis anterior (TA) was infected with Xin^{sh} while the contralateral TA was infected with the Con adenovirus. Expression of GFP was evident throughout the experimental period for both vectors, and the expression was confirmed in Xin^{sh} muscle (14d) by using a GFP antibody and a Texas red secondary. Negative control (Neg; absence of primary antibody) showed no signal. *C*: Xin expression was detectable at ~130 kDa at all time points suggesting that the Xin B isoform is the predominant isoform in skeletal muscle regeneration. The expression of Xin A isoform (~205 kDa) was faint/absent at all time points throughout (not shown). Xin^{sh} infection significantly reduced in endogenous Xin (open circles) at *day 0* vs. control (black squares). At 3 days of regeneration, expression of Xin returned to 0d levels while in the Xin^{sh} muscles, expression remained elevated, suggesting a continued pressure to promote the expression of Xin during regeneration. MyoD expression was robustly elevated at 24 h and remained elevated at 3 days in Xin-shRNA, likely the result of delayed regeneration. Xin-shRNA and contralateral control muscle samples (n = 3–4/group) from each time point were run on the same gel to maintain temporal consistency. *Significant difference between control and Xin^{sh} samples at that given time point using a *t*-test. *D*: infection of skeletal muscle with Xin-shRNA resulted in a significant delay in muscle regeneration as noted by reduced regenerating myofiber areas at 5 and 14 days postinjury. The delayed regenerative capacity in Xin^{sh} muscles was further validated by quantifying the area of the muscle expressing embryonic myosin heavy chain (Myh3; *bottom*) at 5 days postinjury.

chain isoforms that are expressed only during defined periods of regeneration (e.g., Myh3) is routinely used to illustrate alterations in the rate of skeletal muscle repair. We observed a significant reduction in the expression of Myh3 at 5 days of regeneration in the Xin-shRNA-infected muscles (Fig. 3*D*; Xin-shRNA: 10.23 ± 0.25% vs. control-GFP: 15.01 ± 0.25%; n = 3/group;P = 0.0002). This result is consistent with our findings using muscle fiber area as a metric for myofiber maturation. When we quantified the areas of newly regenerating muscle fibers (GFP-positive muscle fibers with central nuclei) in Xin-shRNA and control-infected muscles at 5 and 14 days postcardiotoxin injury, we observed that the regenerating muscle fibers of Xin-shRNA-infected muscles are significantly smaller than the control-infected contralateral muscles at both 5 and 14 days of repair (Fig. 3*D*).Taken together, these results illustrate that, relative to control adenovirus-infected muscles,

there is a significant delay in the muscle repair process upon downregulation of Xin expression.

Decreasing Xin Expression in Primary Satellite Cells Impairs Activation But Does Not Directly Affect Proliferation

Having detected the expression of Xin in activated satellite cells both in vitro (10) and within regenerating skeletal muscle (present study), as well as our finding that reducing endogenous Xin expression impaired skeletal muscle repair, we hypothesized that reducing endogenous Xin expression may be affecting satellite cell activation. An impairment in satellite cell activation may therefore account, at least in part, for the delay in muscle repair. To test this hypothesis, we infected the skeletal muscle (and associated satellite cells) before the isolation of single fibers with our control or Xin-shRNA adenoviruses. The isolation procedure for single myofibers results in the activation of the resident satellite cells (20). By incubating the newly isolated fibers in a floating culture with the thymidine-analog BrdU and assessing its incorporation within 24 h of isolation, we obtain an estimate of the rate of satellite cell activation (20). Figure 4*A* illustrates a BrdU-positive satellite cell at the periphery of an isolated myofiber 24 h after isolation. When the number of BrdU-positive cells at the periphery of isolated myofibers are compared between control and Xin-shRNA-infected groups, we see a significant (~50%) reduc-

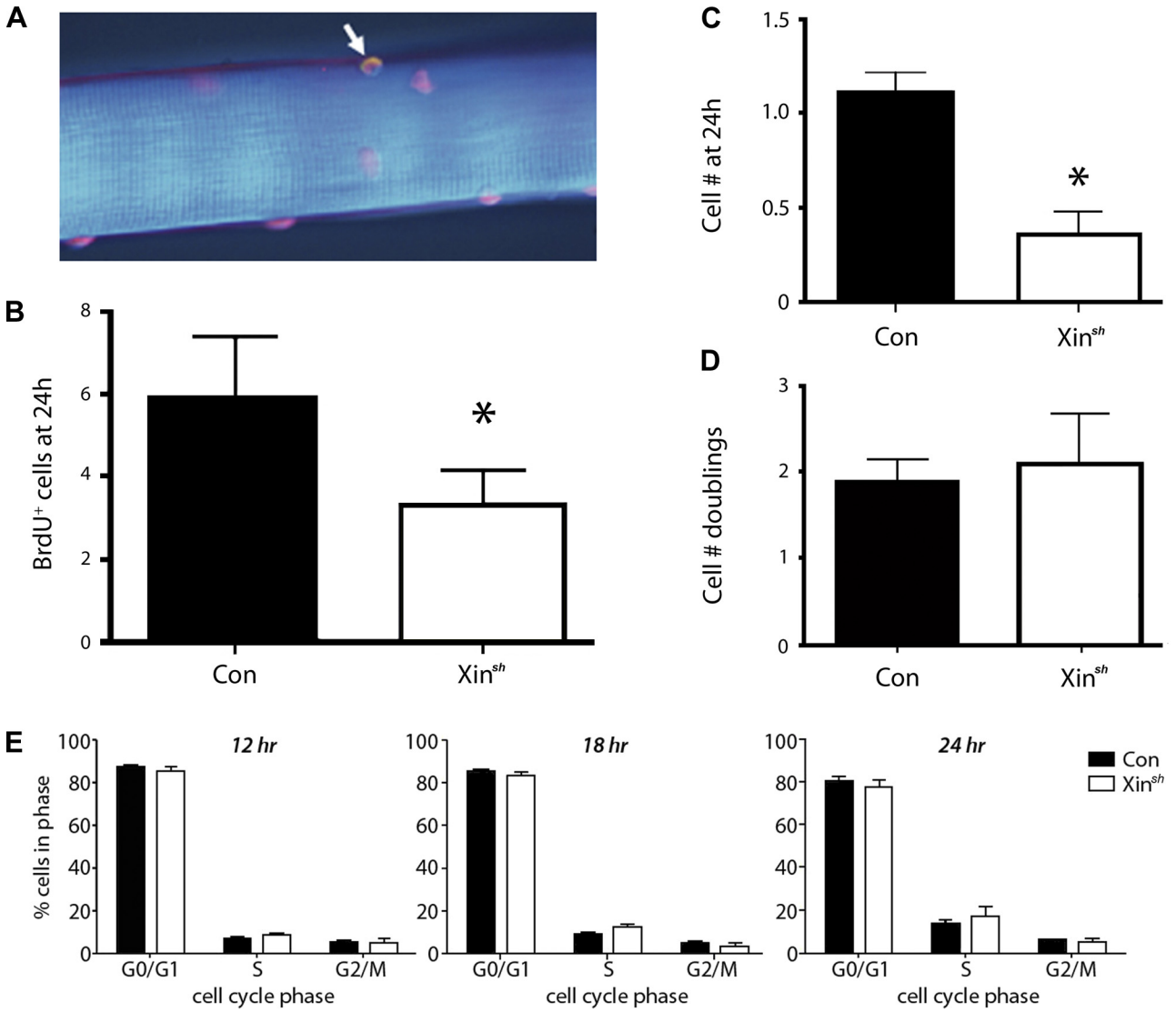

Fig. 4. Repression of Xin impairs satellite cell activation. Using floating single fibers infected with Xin-shRNA (Xin^{sh}) or control (Con) adenoviruses and incubated with 5-bromo-2-deoxyuridine (BrdU) for 24 h immediately following isolation, we were able to quantify the effect of Xin repression on the activation of satellite cells (20). *A*: BrdU-positive (arrow; fluorescein green) nucleus on the periphery of an isolated muscle fiber with all nuclei labelled with propidium iodide (red). *B*: significant reduction in the number of BrdU-positive satellite cells per fiber at 24 h postisolation was noted when Xin expression was repressed. *C*: an alternative approach to assess satellite cell activation is to quantify the number of satellite cells that migrate from the isolated, adherent muscle fiber onto the cell culture plate. There was a significant reduction in the number of satellite cells per fiber that had migrated to the cell culture plate within the first 24 h of isolation in the Xin^{sh}-infected muscle. *D*: there was no difference between Xin^{sh}- and Con infected myoblasts in proliferative capacity. *E*: to determine whether Xin plays a role in cell cycle entry, Xin^{sh}- and Con infected C2C12 myoblasts were arrested using a methylcellulose assay. Xin repression had no detectable effect on cell cycle reentry. *Significant ($P < 0.05$) difference between Con and Xin^{sh}.

tion in the number of BrdU-positive satellite cells in the presence of Xin-shRNA (Fig. 4*B*). Another means to assess satellite cell activation is to quantify the number of satellite cells that migrate off the isolated myofibers in an "adherent-fiber" preparation (11). Consistent with our BrdU-incorporation assay, the number of satellite cells that migrated off the myofiber onto the matrigel-coated plate was reduced by more than 60% upon infection of Xin-shRNA (Fig. 4*C*).

Though we had previously demonstrated a small, but significant, effect of Xin-shRNA on C2C12 myoblast proliferation (10), we did not observe any effect of Xin-shRNA on the proliferative capacity of primary myoblasts in culture (Fig. 4*D*). This effect may be the result of inherent differences between primary and cell line cultures. For example, given the greater proliferative capacity of C2C12 cells, small differences in proliferative capacity may be amplified within the short assay time compared with the longer cell cycle times associated with newly isolated myoblasts (11).

As Xin is an actin-binding protein, we hypothesized that Xin's role in satellite cell activation was to contribute to the large increase in cell size needed to accommodate the cellular hypertrophy immediately following stimulation. However, it was also conceivable that it was playing a role modulating cell cycle progression (e.g., G0-G1 transition). To investigate this, we infected C2C12 cells with Xin-shRNA or control adenoviruses and then rendered them quiescent through 72 h of culturing in a methylcellulose-supplemented medium (12, 15). We observed no significant difference between groups in their reentry into the cell cycle at 12, 18, or 24 h (Fig. 4*E*) lending support to our hypothesis that Xin is involved in cytoskeletal remodeling during satellite cell activation rather than directly affecting G0-G1 cell-cycle transition.

DISCUSSION

We had previously demonstrated a significant increase in the expression of Xin mRNA following skeletal muscle damage (10). At that point, however, it had remained unknown whether this expression pattern translated to significant changes in Xin protein levels and what the functional significance of Xin may be in muscle repair. In the present study, we substantiate a robust increase in Xin protein within hours of muscle damage, and many focal areas of Xin expression at these early stages of regeneration reveal colocalization with the myogenic regulatory factor MyoD. The colocalization of Xin and MyoD support the presence of Xin in activated satellite cells early after muscle injury. Interestingly, the expression of Xin protein is also readily detected in newly regenerating muscle fibers by 5 days of repair. By 14 days of regeneration, Xin expression is low, comparable to that observed in uninjured skeletal muscle. We further demonstrate that a reduction in Xin expression in vivo, through the use of adenovirally mediated Xin-shRNA, attenuates skeletal muscle regeneration as evidenced by reductions in the 5 days postinjury expression of Myh3 and reduced myofiber areas at 5 and 14 days postinjury. This effect appears to be mediated, at least in part, by an impairment in satellite cell activation. Taken together, these findings highlight a role for Xin within skeletal muscle repair, particularly within the resident muscle stem cell population.

An interesting observation from our immunohistochemical analysis in regenerating muscle is the apparent "biphasic" nature of Xin expression. While this is not readily apparent with PCR (10) or immunoblotting (present study), visualization of Xin expression both at the mRNA level by in situ hybridization (10) or of the protein by immunohistochemistry demonstrates an early expression phase (<24 h postinjury; particularly around the periphery of the muscle fibers) that is followed by a decrease in expression and subsequently by a second peak of expression (5–7 days) in small muscle fibers with centrally located nuclei. The heterogeneous expression pattern of Xin within these newly regenerated myofibers may reflect a temporal limitation of this study. That is, although Xin may be expressed in all regenerating fibers because of the asynchrony of regeneration (and the limitation of harvesting at 0.5, 1, 3, 5, and 14 days), we may be only observing fibers that are expressing Xin at that specific time. Whereas much of the early expression of Xin coincides with MyoD and represents the activated satellite cell population (5, 7), the purpose of the second phase of Xin expression is less clear. We hypothesize that this expression pattern may indicate a role for this cytoskeletal protein in myofibril assembly/formation, akin to that observed for other stress-fiber-related proteins in cultured chick cardiac myocytes (9). If, as we propose, Xin is involved in myofibrillar assembly, the expression of Xin in the early phase of regeneration that does not colocalize with MyoD may represent immediate remodeling/repair of myofibrils in response to damage in some muscle fibers. Further investigations are obviously needed to test this hypothesis and clarify the purpose of Xin in the terminal differentiation of regenerating muscle fibers.

To reveal the importance of Xin for skeletal muscle repair, we utilized an adenoviral Xin-shRNA-mediated knockdown approach. We have previously proven the efficacy of this adenovirus in cell culture but now also demonstrate an efficient knockdown of Xin expression levels in vivo. Although the injection of Xin-shRNA adenovirus did not result in a complete absence of Xin expression as would be observed in a knockout mouse model, this technique did offer some important advantages: *1*) injection of Xin-shRNA adenovirus into one muscle and the control adenovirus into the contralateral muscle allowed to obtain an internal control for each mouse; *2*) any systemic effect of adenoviral injection would affect both legs similarly; and *3*) the use of adenoviruses into wild-type muscle should reduce the contributions of compensatory proteins that would arise during development (14). The current study was limited, however, in that we did not define the amount of adenoviral infection within the skeletal muscle and supporting cells nor did we define the time course from infection of satellite cells and the subsequent expression of the Xin shRNA or GFP within infected muscles. Our findings do illustrate, however, that the expression of Xin is significantly decreased by 0.5 days of injury (in Xin-shRNA) resulting in a significant impairment in satellite cell activation (as assessed by BrdU incorporation). In this respect, and based on the present findings, it may be more prudent to suggest that Xin is an important protein in the process of satellite cell activation, rather than the actual exit from quiescence per se. Clearly future studies are needed to define the time lag between infection and expression of Xin-shRNA.

The repression of Xin expression resulted in significant delays in muscle regeneration as demonstrated both by smaller muscle fiber sizes at 5 and 14 days postinjury and a reduction

in the amount of embryonic myosin heavy chain at 5 days postinjury. The fact that Xin repression delays satellite cell activation could alone explain the delay in new myofiber growth. Given the expression of Xin protein in newly formed myofibers, we speculate that this second peak in the biphasic response of Xin protein supports the proposed role for Xin in myofibril assembly (and thus muscle fiber hypertrophy). Alternatively, it could be hypothesized that biphasic response by Xin protein may be indicative of satellite cells with differing states of activation capacity (more and less easily activated; reviewed by Ref. 20) with the continued expression of MyoD in Xin-shRNA regenerating muscle supporting this hypothesis. Future studies would investigate these ideas more thoroughly by examining regenerating muscles at later time points following injury; including the examination of myofiber number, fiber branching in Xin-shRNA muscles, satellite cell numbers in regenerated muscles, and the potential impact of the delay in activation/regeneration on the degree of fibrosis of Xin-shRNA and control muscles after regeneration.

Activation of the muscle satellite cell from a state of quiescence requires a number of coordinated events to allow the satellite cell to enter the proliferative phase (18, 20). Two major events occurring as the satellite cell becomes activated are *1*) an increase in cytoskeletal remodeling requisite for the increased cell size and organelle content, as well as *2*) the G0 to G1 cell cycle phase transition (3, 4). The methylcellulose assay has been used extensively to research factors responsible for activation/cell cycle reentry (8). The use of C2C12 cells in this assay allowed us to dissociate the potential role(s) of Xin in cell cycle reentry from a cytoskeletal remodeling role as these cells had been actively cycling before arrest. As we show in Fig. 4*E*, there is no significant effect of repressing Xin expression on cell cycle reentry as demonstrated using FACS analysis. While this supports the role for Xin in cytoskeletal remodeling, clearly future studies will be needed to investigate how Xin may be mediating this effect.

In conclusion, these findings contribute to our understanding of Xin within skeletal muscle particularly as an integral protein in skeletal muscle regeneration possibly through its role in activating satellite cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.A.N., B.Z., R.L., D.J.A., and T.J.H. performed experiments; A.A.N., B.Z., R.L., D.J.A., and T.J.H. analyzed data; A.A.N., B.Z., R.L., D.J.A., and T.J.H. interpreted results of experiments; A.A.N., B.Z., and T.J.H. prepared figures; A.A.N., B.Z., and T.J.H. drafted manuscript; A.A.N., P.F.v.d.V., D.O.F., and T.J.H. edited and revised manuscript; A.A.N., P.F.v.d.V., D.O.F., and T.J.H. approved final version of manuscript; P.F.v.d.V., D.O.F., and T.J.H. conception and design of research.

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