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Bex1 knock out mice show altered skeletal muscle regeneration

Jae Hyung Koo[a*], **Mark A. Smiley**[a], **Richard M. Lovering**[b], and **Frank L. Margolis**[a]

a*Department of Anatomy and Neurobiology, School of Medicine, University of Maryland Baltimore, Baltimore, MD 21201*

b*Department of Physiology, School of Medicine, University of Maryland Baltimore, Baltimore, MD 21201*

Abstract

Bex1 and Calmodulin (CaM) are upregulated during skeletal muscle regeneration. We confirm this finding and demonstrate the novel finding that they interact in a calcium-dependent manner. To study the role of Bex1 and its interaction with CaM in skeletal muscle regeneration, we generated Bex1 knock out (Bex1-KO) mice. These mice appeared to develop normally and are fertile, but displayed a functional deficit in exercise performance compared to wild type (WT) mice. After intramuscular injection of cardiotoxin, which causes extensive and reproducible myotrauma followed by recovery, regenerating muscles of Bex1-KO mice exhibited elevated and prolonged cell proliferation, as well as delayed cell differentiation, compared to WT mice. Thus, our results provide the first evidence that Bex1-KO mice show altered muscle regeneration, and allow us to propose that the interaction of Bex1 with Ca2+/CaM may be involved in skeletal muscle regeneration.

Keywords

Bex1; Bex1-KO mice; calmodulin; CaM-binding protein; muscle regeneration; Omnibank; calcium; myogenesis

Introduction

Skeletal muscle is a very adaptable tissue and can regenerate after injury. The process of skeletal muscle repair is a dramatic response to damage [1]. Regeneration is the result of many biological processes, such as inflammation, angiogenesis, arteriogenesis, and myogenic progenitor cell mediated myogenesis, all of which lead to the reconstitution of functional skeletal muscle tissue [2]. When this controlled repair system is impaired, continuous muscle degeneration and regeneration can occur [3]. Such is the case with Duchenne's muscular dystrophy (DMD), the most common form of inherited neuromuscular disorder [4]. The absence of dystrophin, a membrane-associated protein, is clearly the underlying cause [5]. Nonetheless, the molecular mechanisms of this disease and other myopathies are still under investigation [6]. It is, therefore, essential to identify molecules that contribute to mechanisms underlying muscular dystrophies in order to develop rational treatment strategies to target specific physiological processes [7].

*Corresponding Author: Jae Hyung Koo, Department of Anatomy and Neurobiology, University of Maryland Baltimore, Baltimore, MD 21201, USA, Phone: 410-706-8821, Fax: 410-706-2512, Email: jkoo001@umaryland.edu.

Recently, genome-wide discovery tools have been used to identify genes expressed in a temporal and spatial-specific fashion in skeletal muscle [8], and to identify their biological responses and how these relate to each other. Experiments performed using cDNA-based microarrays have identified highly coordinated molecular changes involved in skeletal muscle regeneration following cardiotoxin (CTX)-induced injury, a reproducible method to induce muscle regeneration [9,10]. *Mdx* mice [6] possess a mutation in the gene coding *dystrophin*, the protein that is absent in humans with DMD [4]. Histological findings, such as centrally nucleated fibers, inflammation, and heterogeneity of fiber size, are similar in the skeletal muscle of *mdx* mice and of patients with DMD [11]. In comparison to controls, many genes are differentially expressed in *mdx* mouse muscle [7]. Interestingly, the differential expression of Bex1 (Rex3) and Calmodulin (CaM) was reported in that study [7].

The Bex1 gene (Brain Expressed X-linked gene) is located on the X-chromosome and encodes a protein of the same name (Bex1 protein, ~15 kDa), which is expressed in several tissues [12,13]. There are several reasons why a Bex1 and CaM interaction during skeletal muscle regeneration would be important to elucidate. Expression of *Bex1* is transiently elevated at the end of the proliferation state of skeletal muscle regeneration [9,10] and several studies implicate the Bex1 protein in cell growth and differentiation [13,14]. On the other hand, CaM is a ubiquitous Ca2+ binding protein, but one that is poorly studied in muscle regeneration. It is reported that alteration of Ca2+ availability can modulate skeletal muscle differentiation [15], a phenomenon that could involve CaM. An understanding of the Bex1/CaM interaction in the process of skeletal muscle repair may contribute to the understanding of the regeneration process and provide further insight to myopathies such as DMD. Therefore, we tested the hypothesis that Bex1 is involved in skeletal muscle regeneration using Bex1-KO mice.

Here we report the generation of Bex1 knock out (Bex1-KO) mice, which may provide an important mouse model to study DMD, as the Bex1 gene family is located on the X-chromosome at XF1 (X57.5 cM) and the gene for human *dystrophin*-related protein (DRP2) maps to the X-chromosome [16] close to the Bex gene cluster. Interestingly, human Bex1 has been mapped to the Xq22 [13], while the *dystrophin* gene is located on the Xp21 region of the X-chromosome [17]. We report that Bex1-KO mice display a functional deficit in exercise performance. Furthermore, we demonstrate that muscle regeneration post CTX-induced injury is accompanied by enhanced and prolonged cell proliferation and delayed cell differentiation at distinct time points compared to wild type (WT) mice. Taken together, our investigations demonstrate that Bex1 interacts with CaM, and this interaction may modulate skeletal muscle regeneration via a Ca2+ involved pathway.

Materials and methods

Gel mobility shift assay

CaM (20μM, Sigma) was incubated with either recombinant mouse Bex1 protein (20μM) [12] or each of the synthetic Bex1 peptides (20μM, Biopolymer Lab, UMB) in 100mM Tris-HCl (pH 7.2) and 0.1mM $CaCl_2$ or 2mM EGTA for 1 h at the room temperature. The bound complexes were resolved by 15% non-denaturing PAGE and visualized by staining with Coomassie Brilliant Blue.

Calmodulin-agarose pull down assay

5 days post CTX-injury tibialis anterior (TA) muscle was extracted with 20 mM HEPES buffer containing protease inhibitors (Roche, Germant). After centrifugation (14,000 rpm, 30 min) the extract was incubated with calmodulin-agarose in the presence of 0.1mM calcium or 2mM EGTA at 4°C for overnight. The resin was washed with 20 mM HEPES buffer to remove

unbound proteins and then extracted with SDS sample buffer and analyzed by Western blot using rabbit antibody against Bex1 [12].

Genetic disruption of Bex1 in mice

To generate Bex1-KO mice, we used ES cells (Clone no. OST125186, OmniBank Library, Lexicon Genetics) derived by the gene trap method [18]. Using this clone, mice heterozygous for Bex1 were generated by blastocyst injection and backcrossed to 129/SvEv mice (Taconic Farms) that were also used as controls.

Exercise Performance Test

Uninjured, adult male Bex1-KO (*n*=7) and 129/SvEv control (*n*=8) mice were subjected to an exercise performance test, using a six lane rodent treadmill with an electric grid at the rear of the treadmill (Columbus Instruments, Columbus, OH). Mice were treadmill familiarized for 10 min at 10° incline for two days before exercise performance measurements: at a speed of 5 m/min on day one and 8 m/min on day two. For exercise performance measurements, the treadmill speed was started at 5 m/min for 5 min and then increased by 1m/min every minute until exhaustion, as indicated by an animal remaining on the electric grid for more than 20 seconds. The time to exhaustion was recorded.

Cardiotoxin Injection

Bex1-KO and littermate male mice (6 to 8 weeks old) were anesthetized (Ketamine, 165 mg per kg, and Xylazine, 10 mg per kg), and 100μl cardiotoxin (10μM in saline, Sigma) was injected into the right tibialis anterior (TA) muscle with a 30-gauge needle. PBS-injected left TA muscle was used as a control. The treated muscles were harvested at various times after CTX injection.

RT-PCR

PBS or CTX-treated muscle was dissected and tissues snap-frozen on dry ice. Total RNA preparation and RT-PCR were performed as described by Koo et al. [12] using the primers shown in Supplementary Material Table 1.

Western immunoblotting of tissue extracts

Muscle tissues were dissected onto dry ice and stored at −80°C. They were homogenized on ice in 10 volumes of 20 mM Hepes/NaOH buffer (pH 7.5) containing protease inhibitor cocktail (Roche, Germany). The preparation of tissue extracts and western blotting were performed as described previously [12] using various antibodies (Supplementary Material Table 2).

Results and Discussion

Bex1 interacts with Calmodulin

The primary sequence of Bex1 is highly conserved in mouse (GenBank accession no. Q9R224), rat (Q3MKQ2), pig (AY610481), dog (AC187985), cow (NM_001077034), water buffalo (DQ487028), chimp (XM_521190), monkey (Q2PG52), and human (Q9HBH7), and contains several conserved motifs [12,13]. To identify additional conserved sites on Bex1, we used the calmodulin target database (http://calcium.uhnres.utoronto.ca/ctdb) to analyze the Bex1 sequence, which resulted in the identification of a putative Ca^{2+}-dependent calmodulin (CaM) binding domain (CaMBD) in Bex1 (Supplementary Fig. 1). The Bex1 CaMBD matches the 1-8-14 (-FxxxxxxAxxxxxL-) motif, in which three basic residues (-RRR-) precede the first hydrophobic residue [19]. To confirm the interaction of Bex1 with CaM, the ability of recombinant Bex1 protein to bind CaM was analyzed by an electrophoretic mobility shift assay in the presence of Ca^{2+} (Fig. 1A) or +EGTA (data not shown). The interaction of Bex1 and

CaM was determined to be Ca^{2+} dependent. Recombinant Bex1 protein did not enter the native gel due to its basic isoelectric point (pI = 9.2). Therefore, it is not seen in the presence of Ca^{2+} and absence of CaM (Fig. 1A, lane 1). However, in the presence of both CaM and Ca^{2+}, a Bex1-CaM-Ca^{2+} complex was detected (Fig. 1A, lane 3). In addition, serial peptides of Bex1 were synthesized and purified and their CaM binding was evaluated by gel mobility shift assays under non-denaturing conditions (Fig. 1A). CaM only interacted with recombinant Bex1 protein as well as with a peptide containing Bex1-CaMBD in a Ca^{2+} dependent manner (Fig. 1A).

Bex1 expression is elevated following activation of skeletal muscle degeneration and regeneration in response to CTX-induced muscle injury [9,10]. This is consistent with our RT-PCR data, which demonstrates a dramatic induction of Bex1 mRNA (Fig. 4A) at 5 days after CTX treatment. In addition, Bex1 induced following muscle regeneration could be pulled down with CaM-agarose only in the presence of calcium (Fig. 1B). These experiments show that the Bex1 protein induced during skeletal muscle regeneration can bind CaM in a calcium dependent manner.

Generation of Bex1 knock out mice

To obtain insight into the physiological significance of Bex1 in skeletal muscle regeneration, we used gene trapping to generate Bex1-KO mice (Supplementary Fig. 2A) and confirmed this by genomic-DNA PCR (Supplementary Fig. 2B). The absence of Bex1 expression and Bex1-fused protein generated by the gene trap construct was confirmed using western blotting (Fig. 2). After the induction of muscle injury by CTX, expression of Bex1 was evaluated and detected by RT-PCR (Fig. 4A, Bex1) and western blotting (Fig. 4C, Bex1). Bex2 protein, which has 87% amino acid sequence identity to Bex1, showed no compensatory elevation of expression in Bex1-KO mice following saline or CTX-induced injury (data not shown). Bex1 expression was only induced in CTX-treated WT mice, further confirming the specificity of the Bex1-KO. Although we observed that mice lacking Bex1 are viable and fertile, their exercise performance measurements were 24% lower than WT mice (Fig. 3). This finding is consistent with the report that Bex1 protein is highly expressed in developing muscle, where it is localized in somatic mesenchyme [14]. We suggest that the lack of Bex1 in mice may alter normal development of muscle and lead to a reduction in exercise performance. It would be of interest in the future to elucidate the function of Bex1 in embryonic muscle development and determine if this is recapitulated in skeletal muscle regeneration [2].

Muscle regeneration characteristics of Bex1-KO and WT mice

To clarify the role of Bex1 expression in regenerating muscle, we used markers of myogenesis to characterize Bex1-KO and WT mice after CTX injection. Our RT-PCR data illustrate the virtual absence of Bex1 mRNA in controls and the presence of a small amount of Bex1 transcript 3 days after CTX injection, followed by a dramatic elevation after 5 days. Approximately 5 days after injury, when Bex1 is dramatically induced, myogenic cells withdraw from the cell cycle and either self-renew or form differentiated myotubes that contain a central nucleus [2]. At the onset of this differentiation, cell cycle dependent-kinase inhibitors (CDKI) such as p21CIP1, p27KIP1 and p57KIP2, together with myogenin, are induced to prevent cells from reentering the cell division cycle [20,21]. Increased expression of CDKIs and myogenin are hallmarks for the initiation of myogenic differentiation; the markers we used to approximate the kinetics of regenerating muscle differentiation in Bex1-KO and WT mice. Semi-quantitative RT-PCR was used to determine transcript levels of each gene at 5 days after CTX-injury. The transcript levels of p27, p57, and myogenin were reduced in regenerating muscle of Bex1-KO vs. WT mice by more than 27%, 40%, and 50%, respectively (Fig. 4A). By contrast, p21 transcript levels were similar between both groups. Even though the mRNA transcript levels of p27 and p57 were reduced in the regenerating muscle of Bex1-KO mice by

RT-PCR, it is essential to monitor the level of expressed functional protein. This is particularly important since these proteins were reported to be regulated by Ubiquitin-mediated degradation [22,23]. Therefore, levels of p21, p27, and p57 proteins, as well as myogenin, were measured by western blotting at different time points after CTX treatment. We did not observe expression of p21, p27, and p57 at 1- and 3-days post-injury. However, there was increased expression of these CDKIs at the initial stage of myogenic differentiation at 5-D post injury (Fig. 4B). Myogenin expression is induced with a similar time course to that seen for Bex1 expression after CTX-injury but showed no difference between Bex1-KO and WT mice (data not shown). The observed discordance between the mRNA and protein levels of myogenin at 5-D post-injury illustrated post-transcriptional regulation of its expression. In a recent report, up-regulation of the expression of myogenin and myogenic regulatory factors occurs concurrently through transcriptional and post-transcriptional mechanisms [24]. However, the levels of p27 and p57 proteins were reduced in regenerating muscle of Bex1-KO compared to that of WT mice at 7-D (Fig. 4B). These observations are consistent with our RT-PCR data at 5-D (Fig. 4A) while there was no expression of p27 protein and no significant difference of p57 protein between Bex1-KO and WT mice at 5-D (Fig. 4B). Our results indicate that different levels of mRNA expression at 5-D is reflected in reduced protein expression of p27 and p57 at 7-D post-injury. These data indicate that Bex1-KO mice showed a delay in muscle differentiation compared with WT mice.

To further evaluate muscle regeneration of Bex1-KO vs. WT mice, we analyzed the expression of proliferation marker proteins. Non-muscle β-cytoplasmic actin is expressed in essentially all dividing cells, but is inactive in myofibrils [25]. In primary myoblast cultures, β-actin mRNA increases sharply during the proliferative phase before fusion and steadily declines thereafter [26]. Genome wide gene expression profiling demonstrated the differential gene expression of cytoplasmic β-actin during muscle regeneration [9]. Therefore, β-actin represents a proliferative marker of regenerating muscle tissue. Immunoblotting of regenerating muscle tissue in Bex1-KO and WT mice using a β-actin specific monoclonal antibody showed that β-actin expression was transiently elevated at 5 days post-injury. Moreover, Bex1-KO regenerating muscle showed a dramatic increase in β-actin compared to WT mice at 5 days post injury that extends to 7 days (Fig. 4C). Similarly, our data using proliferating cell nuclear antigen (PCNA) antibodies (Fig. 4C) demonstrated that PCNA expression is prolonged to 7 days post-injury in Bex1-KO mice compared to WT mice. Together, these β-actin and PCNA results support an interpretation that in Bex1-KO mice muscle proliferation is enhanced and prolonged after CTX-induced injury in comparison to WT mice.

Western analyses for Bex1 and CaM at the same post-injury time-points showed that Bex1 and CaM proteins of regenerating skeletal muscle are maximally induced from 5 to 7 days post CTX-induced injury (Fig. 4C) in WT mice. However, there were no significant differences in CaM expression between Bex1-KO and WT mice. It has been previously shown that skeletal muscle differentiation can be altered by extracellular Ca^{2+} concentrations [15], suggesting that CaM, a ubiquitous Ca^{2+} binding protein induced 5–7 days post injury, interacts with Bex1 to modulate skeletal muscle regeneration.

In summary, our investigations show that Bex1 protein binds CaM in a calcium-dependent manner, Bex1-KO mice exhibit reduced treadmill exercise performance compared to WT mice, and the absence of Bex1 protein in mice results in increased and prolonged proliferation and delayed differentiation in skeletal muscle post CTX-induced injury. This suggests that Bex1-KO mice should show better muscle function compared to the WT mice. However, this is not observed attesting to the complexity of muscle development and regeneration and illustrating that further study is required. Additionally, the similar induction time points of Bex1 and CaM during skeletal muscle regeneration of the WT mice, as well as their ability to interact, provides the basis for us to propose that this interaction may influence the balance of proliferation and

differentiation through a Ca^{2+}-dependent pathway. Although CaM, which is ubiquitously expressed in most eukaryotic cells, has been extensively implicated in muscle contraction [27], we find only a few reports of CaM in muscle regeneration [28,29]. Thus, the data and hypothesis presented here draw attention to the roles of both Bex1 and CaM in skeletal muscle regeneration. Furthermore, an understanding of the factors regulating skeletal muscle regeneration may lead to development of strategies for the treatment of myopathies such as Duchenne's muscular dystrophy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

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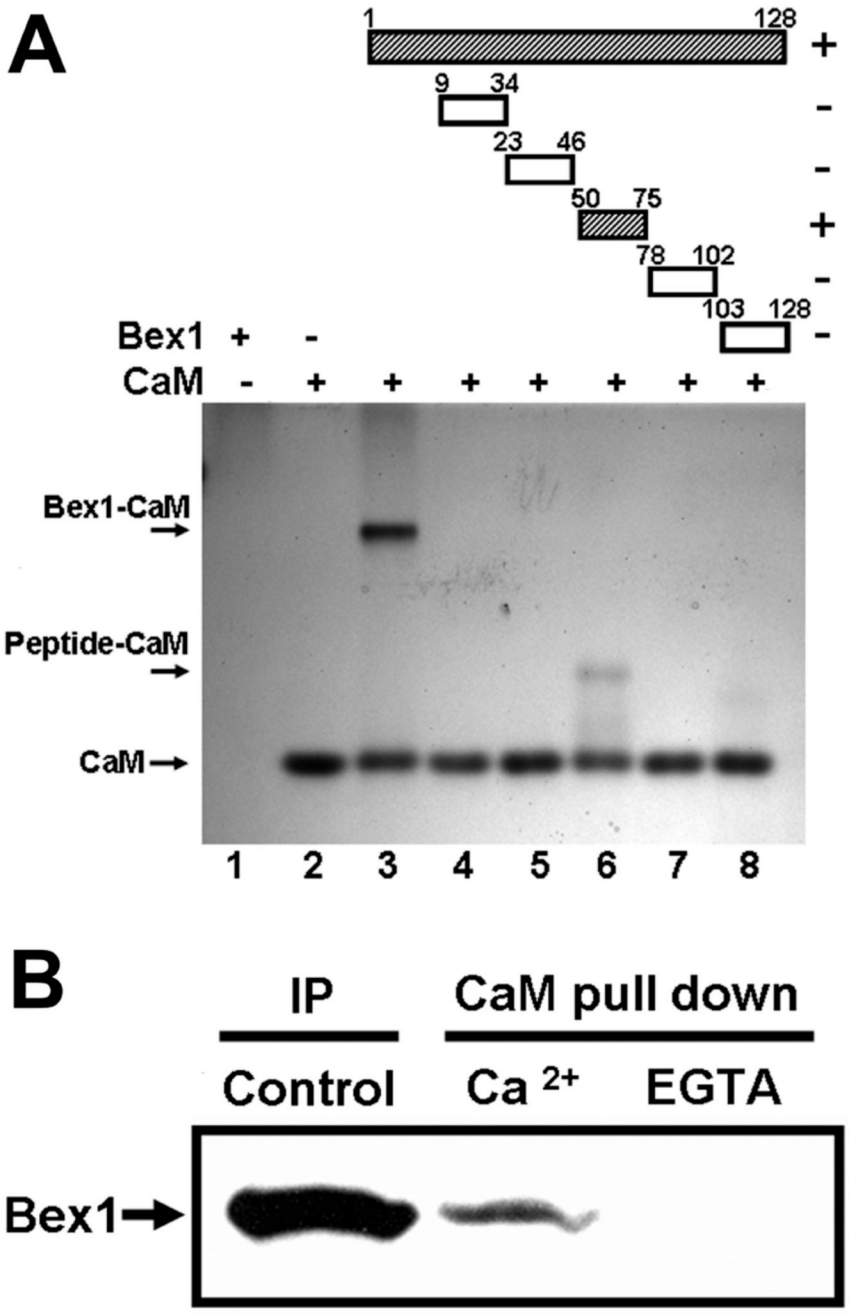

Fig. 1. Bex1 interacts with Calmodulin (CaM) in a Ca^{2+} dependent manner

(A) CaM-binding activity of recombinant Bex1 protein and of synthetic serial peptide fragments of the mouse Bex1 sequence. The CaM binding ability is shown in the right-hand column (+, binds CaM; -, does not bind CaM). Bex1 protein and a 25-mer peptide (Bex1 50-75) containing the 1-8-14 motif exhibit CaM binding activities on the Coomassie Blue stained native gel. (B) Endogenous Bex1 from regenerating muscle extract pulled down by calmodulin-agarose in the presence of calcium. Bex1 proteins bound to calmodulin-agarose preferentially in the presence of 0.1mM calcium as compared with 2mM EGTA. Bex1 was confirmed by subsequent immunoprecipitation of column eluates with Bex1 antibody and immunoblotting.

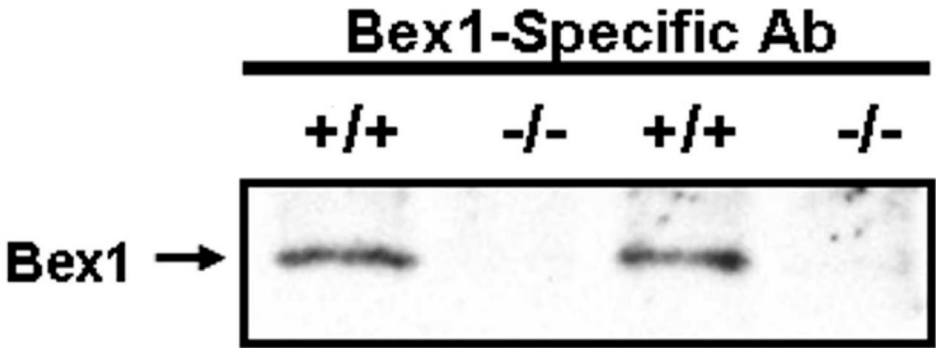

Fig. 2. Genetic characterization of Bex1 knock out (Bex1-KO) mice

Expression of Bex1 in *Bex1*$^{+/+}$ and *Bex1*$^{-/-}$ mice, determined by immunoprecipitation (IP) with polyclonal anti-Bex1 antibody (Ab) followed by western blotting with Bex1-specific antibody confirms that the mice are Bex1 (−/−).

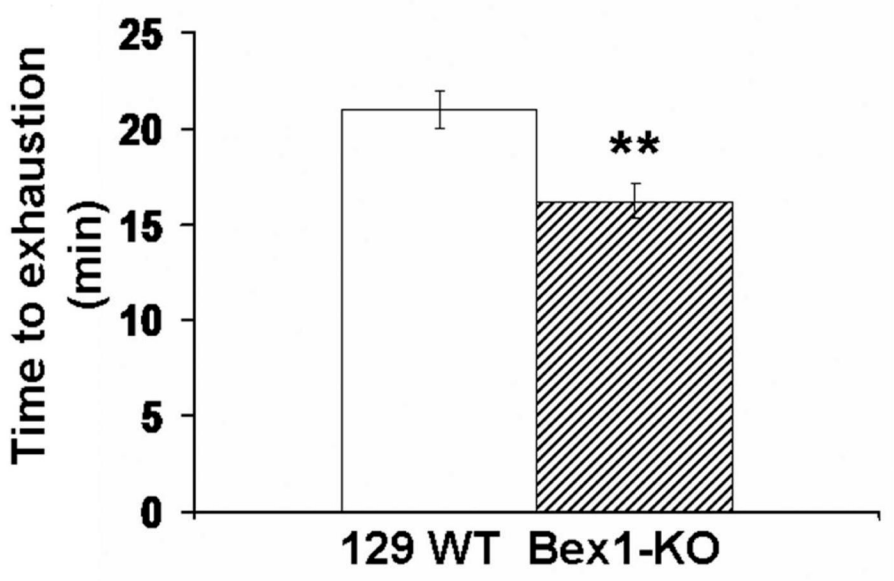

Fig. 3. Treadmill-running tolerance test

Bex1-KO mice (n=7) displayed reduced performance and earlier exhaustion compared to control mice (n=8) (Student's t-Test, $^{**}p < 0.004$). Data are means ± SEM. Experiments were performed as described in Materials and Methods.

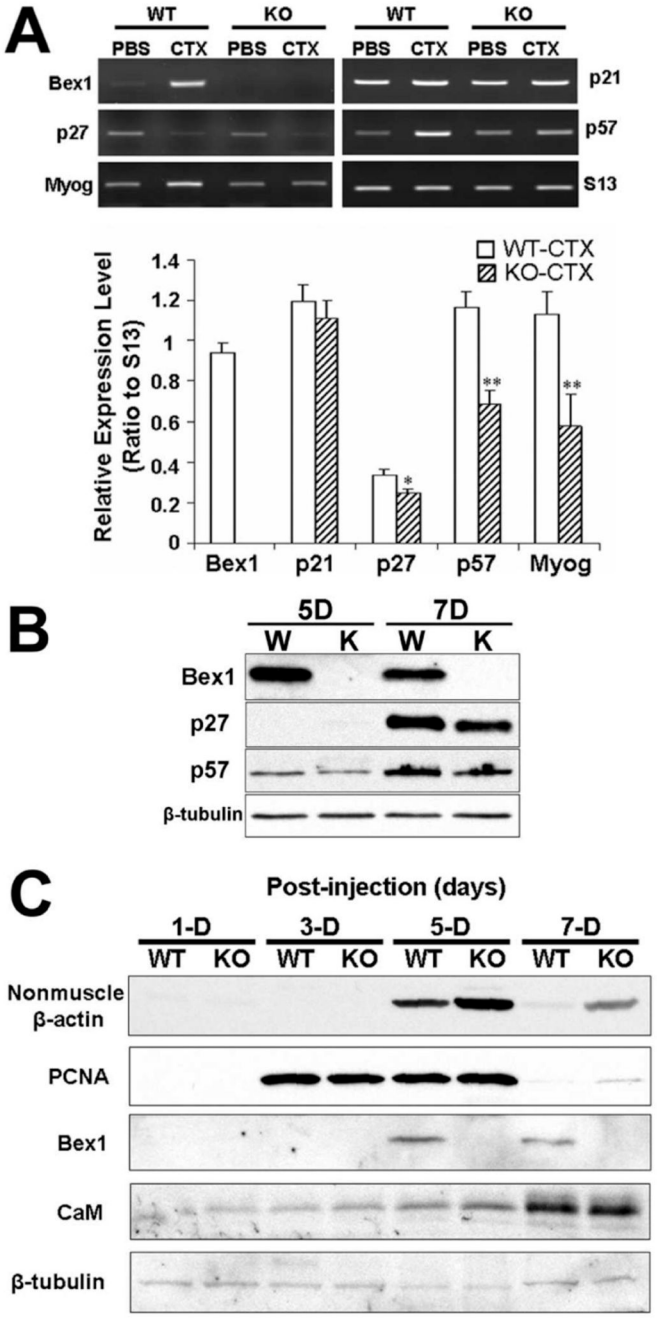

Fig. 4. Regenerating muscle from Bex1-KO mice exhibits increased and extended cell proliferation and delayed differentiation

(A) The appearance of differentiated muscle protein transcripts as measured by semi-quantitative RT-PCR analysis for Bex1, cyclin-dependent kinase inhibitors (CDKIs e.g. p21, p27, and p57), myogenin (Myog), and ribosomal protein (S13) as an internal control in *Bex1*$^{-/-}$ (KO) and *Bex1*$^{+/+}$ (WT) regenerating muscles (Panel A, top). PBS indicates saline-treated control. Regenerating muscles from Bex1-KO mice have reduced expression of differentiation markers 5d after cardiotoxin (CTX) treatment. The data are for KO and WT mice and were quantified by densitometry (Panel A, bottom). This experiment was independently replicated three times and plotted as means + SEM (*n*=3) in the bottom panel.

**, $P < 0.01$. *, $P < 0.05$. The reduced expression levels of p27 and p57 CDKIs and myogenin demonstrate that regenerating muscle of Bex1-KO mice shows delayed differentiation. (B) Levels of p27 and p57 CDKIs proteins are reduced in Bex1-KO at 7 d post-injury, confirming delayed differentiation of regenerating muscle of Bex1-KO mice. (C) Differential cell proliferation at 5 and 7 days after CTX treatment was evaluated by western analysis for non-muscle cytoplasmic β-actin and proliferating cell nuclear antigen (PCNA) in regenerating muscles of WT and KO mice. Seven days after CTX treatment, PCNA is still expressed in KO, whereas it is undetectable in WT demonstrating that regenerating muscle in the KO has prolonged cell proliferation. Bex1 protein is maximally induced at 5 and 7 days in the WT regenerating muscle and calmodulin (CaM) is also induced at the same time interval. Similar results were obtained in four independent experiments. β-tubulin is loading control.