Fas-Associated Protein with Death Domain Regulates Notch Signaling during Muscle Regeneration

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Key Words

Fas-associated death domain · Mouse · Muscle regeneration · Notch · Protein kinase C · Wnt

Abstract

Notch signaling plays critical roles during myogenesis by promoting the proliferation and inhibiting the differentiation of myogenic progenitors. However, the mechanism of the temporal regulation of Notch signaling during the myogenic lineage progression remains elusive. In the present study, we show that a constitutively phosphoryl-mimicking mutation of Fas-associated death domain (FADD-D) enhances Notch-1 signaling and compromises Wnt signaling in both cultured myoblasts and regenerating muscles, which results in inhibited myogenic differentiation and muscle regeneration. Inhibition of Notch signaling recovers the regeneration ability in injured FADD-D muscles through rescuing Wnt signaling. Furthermore, we found that protein kinase C α mediates FADD-D-induced Notch-1 signaling by stabilizing Notch-1. Collectively, these data identify a novel mechanism for the temporal regulation of Notch signaling during myogenic lineage progression and muscle regeneration.

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Introduction

Notch signaling is activated immediately in the early phase of muscle regeneration. Studies have shown that the inhibition of Notch signaling impairs the proliferative expansion and myogenic progression of myoblasts [Conboy et al., 2003]. In addition to Notch, previous studies have indicated that fibroblast growth factors also promote myoblast activation and proliferation, which in turn promotes muscle regeneration [Anderson et al., 1995; Husmann et al., 1996; Chakkalakal et al., 2012; Yin et al.,

Abbreviations used in this paper

CHX	cycloheximide
cPKC	conventional protein kinase C
FADD	Fas-associated death domain
FADD-D	constitutively phosphoryl-mimicking mutation of FADD
NICD	intracellular domain of Notch-1
PKC	protein kinase C
TA	tibialis anterior
WT	wild-type

2013]. Given that Notch signaling represses the differentiation of myoblasts, it should be compromised at a certain time to assure myogenic progression. A signaling switch from Notch to Wnt has been revealed as critical for myogenic lineage progression [Brack et al., 2008]. Wnt signaling is activated in the later phases of myogenic lineage progression to facilitate differentiation events [Otto et al., 2008]. However, the regulatory mechanisms involved in this switch have yet to be elucidated.

Protein kinase C (PKC) controls diverse biological processes, such as proliferation and differentiation, and is activated by secondary messengers generated by growth factors [Newton, 2010]. PKC conveys extracellular signals to initiate a cascade of cellular events, and evidence from diverse cell types has demonstrated that PKC regulates cell cycle progression to control proliferation and differentiation [Black, 2000]. We have shown that Fas-associated death domain (FADD) phosphorylation regulates conventional PKC (cPKC) signaling termination and cell migration [Cheng et al., 2012]. Moreover, we have also established the role of FADD phosphorylation in muscle stem cell self-renewal and commitment, which occurs mainly through cPKC-mediated Notch signaling [Cheng et al., 2014]. How cPKC mediates FADD-induced Notch signaling remains unknown.

Here, we show that FADD is also involved in myoblast differentiation and skeletal muscle regeneration. A constitutively phosphoryl-mimicking mutation of FADD (FADD-D) promotes the phosphorylation of PKC α , which stabilizes Notch-1, leading to the inhibition of β -catenin accumulation and compromised regeneration of muscles. Inhibition of Notch or restoration of Wnt signaling rescues regeneration efficiency in FADD-D muscles. Furthermore, PKC α interacts with full-length Notch-1 as determined by the phosphorylation state of PKC α to suppress ligand-independent Notch-1 degradation. These data collectively suggest a novel mechanism for Notch signaling regulation during muscle regeneration.

Materials and Methods

Cell Culture, Plasmids and Reagents

C2C12 and 293T (ATCC, Manassas, Va., USA) cells were cultured in DMEM with 10% fetal bovine serum, penicillin and streptomycin. The full-length cDNA clone of Notch-1 was generously provided by Dr. Raphael Kopan [Schroeter et al., 1998]. Full-length wild-type (WT) PKC α was kindly provided by Dr. Christer Larsson and cloned into the pRK5 vector as previously described [Cheng et al., 2012]. Go6976, cycloheximide (CHX), MG132, L685, 458 and BIO were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Anti-PKC α , anti-myogenin and anti-total GSK3 β were

purchased from BD Biosciences (Franklin Lakes, N.J., USA). Anti-FLAG and anti-HA were purchased from Sigma-Aldrich. Anti-myc and anti-active β -catenin were purchased from Millipore (Billerica, Mass., USA). Anti-Notch-1 and anti-GSK3 β (Y216) were purchased from Epitomics.

Animal Care

The FADD phosphorylation mutant knockin mice (FADD-D in FADD^{-/-} alleles) were crossed and genotyped by PCR as described previously [Hua et al., 2003]. Adult (3–6 months old) FADD-D mice and their littermates were then used for the experiments. All the mice were housed in a specific pathogen animal facility in Nanjing Drum Tower Hospital, which is authorized by the Association for the Assessment and Accreditation of Laboratory Animal Care in Jiangsu Province. All animal care and experimental procedures were approved by the Animal Care and Use Committee of the School of Life Sciences of Nanjing University.

Myoblast Isolation and Culture

Primary myoblasts were isolated as described previously with minor modifications [Cheng et al., 2014]. Briefly, the mice were sacrificed by cervical dislocation. Skeletal muscles were cut from the hind limb and cut into pieces. Muscles were then digested with 0.2% collagenase II (Worthington, N.J., USA) in a cell culture incubator for 45 min. The digested mixture was then filtered twice through 40- μ m cell strainers (BD Biosciences) and centrifuged. Myoblasts were then enriched by differential sedimentation. Primary myoblasts were counted and then cultured in 8-well chamber slides (BD Biosciences) that were coated with Matrigel (BD Biosciences) in the presence of growth medium (Ham's F-10 nutrient mixture containing 20% FBS and 1% penicillin/streptomycin with 5 ng/ml of basic fibroblast growth factor). For myoblast differentiation, the growth medium was switched to low-serum medium (2% horse serum in DMEM). BIO (500 nM) or Go6976 (5 μ M) was added to the differentiation medium 24 h after cell plating. To determine myoblast proliferation, Go6976 (5 μ M) and EdU (10 μ M) were added to the C2C12 myoblasts and cultured for 12 h.

Muscle Injury

The mice were anesthetized with isoflurane before the injury procedures. The skin was opened to expose the tibialis anterior (TA) muscles. The muscle injury was performed as described [Cheng et al., 2012]. Briefly, TA muscles were frozen by a 15-second application of a metallic rod precooled in liquid nitrogen. The skin was closed and the mice were kept on a warm plate until they recovered. For myoblast isolation, gastrocnemius muscles were injected with 20 μ l of cardiotoxin (20 μ M; Sigma-Aldrich) at 20 different sites. γ -Secretase inhibitor L685,458 (1 μ M, 10 μ l) and PKC inhibitor Go6976 (2 μ M, 10 μ l) were injected at the times indicated. The contralateral leg was injected with an equal volume of vehicles as the control. To determine the cross-section area of newborn myotubes, L685,458 or Go6976 was injected at 5 days postinjury and muscles were collected 7 days postinjury except where otherwise indicated.

Immunoprecipitation

Plasmids were transiently transfected into 293T cells with Lipofectamine 2000 for 24 or 48 h following the manufacturer's instructions. Cells were then lysed in lysis buffer as previously described [Cheng et al., 2012]. Cell lysis was then immunoprecipi-

Fig. 1. FADD-D inhibits muscle regeneration. **a** Hematoxylin and eosin sections of injured TA muscles ($n = 4$). Scale bar = 20 μm . **b** Normalized quantification of myofiber cross-section area (CSA) from **a**. The CSA from uninjured WT or FADD-D mice was set as 1 (mean $\pm$ SD, $n = 4$). **c** Quantitative RT-PCR analysis of primary myoblasts isolated from injured muscles at 4 days postinjury (mean $\pm$ SD, $n = 3$).

tated with the appropriate antibody as indicated overnight. The antibody was then incubated with protein A/G-agarose (Millipore) for 2 h and washed 3 times with lysis buffer.

Histology

Frozen sections (10 μ m thick) or cells were fixed with a freshly prepared solution of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 10 min. All of the images were captured by an AxioCam HR camera on an Axioplan 2 fluorescence microscope (Carl Zeiss). The fusion index was calculated as the ratio of nuclei inside the myotubes to total nuclei multiplied by 100, and was calculated at 4 days after the start of differentiation.

Quantitative RT-PCR

Total RNA was isolated (TRIzol, Invitrogen, Carlsbad, Calif., USA) and then reverse transcribed using the SuperScript III Cells-Direct cDNA Synthesis Kit (Invitrogen) to synthesize the first-strand cDNAs. Real-time RT-PCR was performed with a StepOne/StepOnePlus™ Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master mix according to the manufacturer's instructions (Roche) with specific primers: Pax7_F: aggacgacgaggaaggagaca; Pax7_R: tcattcagacgggtccctt; Hes1_F: gacaaaccaagacggcctct; Hes1_R: ccatgataggcttgatgacttct; Myogenin_F: accaggagccccacttctatgatg; Myogenin_R: gcccgcggcctctgtga; β -catenin_F: tcctgcgtccctcagat; β -catenin_R: aatggcggcgtgtgtg; Axin2_F: gcccgccaccaagactacatacg; Axin2_R: cgctgggtggctgtgcaagacat; Aurora A_F: tgctgcaaacggataggaa; Aurora A_R: acctgctcaagtttctggg; Aurora B_F: tcgctgtgtttccctctct; Aurora B_R: gatcttgagtgcacgatga; Survivin_F: catcgccacttcaagaact; Survivin_R: agctgtcaattgactgacg; Ki67_F: agagctaactgcgtgact; Ki67_R: actccttccaaacaggcagg.

Statistical Analysis

A minimum of 3 and a maximum of 6 replicates were prepared for all of the experiments. The data are presented as the mean and standard error of the mean (SD). Comparisons within groups were conducted using a t test with repeated measures; the p values indicated in the figures are: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Results

FADD-D Impairs Muscle Regeneration

Our previous studies indicated that skeletal muscles containing FADD-D exhibited a reduced myofiber size [Cheng et al., 2014]. Here, we wanted to establish whether FADD-D affected the regeneration capacity of muscles by monitoring freeze-injured TA muscle regeneration. As shown in figure 1a, muscle degeneration seemed comparable between WT and FADD-D animals at 4 days postinjury. In contrast, the size of newly formed myofibers (denoted by nuclei-centralized myofibers) in WT injured animals reached half the size of the uninjured myofibers in WT mice, whereas the newly formed myofibers in injured FADD-D animals were only ~20% the size of uninjured myofibers in FADD-D animals at 9 days postinjury (fig. 1a, b). Furthermore, the size of newly

formed myofibers in WT injured animals was nearly 80% that of uninjured myofibers in WT mice at 15 days after injury. However, the size of the myofibers in injured FADD-D animals only reached ~40% of that of uninjured myofibers in FADD-D mice (fig. 1a, central right and right panels, b). At 30 days postinjury, both WT and FADD-D mouse myofibers regenerated to reach their uninjured sizes (fig. 1a, b). Consistent with the delayed regeneration in FADD-D mice, myogenin, an indicator of myoblast differentiation, was significantly downregulated in regenerating myoblasts from FADD-D animals, whereas the expression of Pax7, a marker of undifferentiated myoblasts, was increased in FADD-D (fig. 1c). These data indicate that the progression of muscle regeneration is largely compromised in FADD-D animals.

FADD-D Inhibits Wnt Signaling

It has been shown that Wnt signaling promotes myofiber differentiation in physiological and aged contexts [Brack et al., 2007, 2008]. Here, we examined Wnt signaling in FADD-D muscles. Interestingly, GSK3 β phosphorylation at Y216, which corresponds to GSK3 β activation, was increased ~2-fold in FADD-D muscle compared with WT (fig. 2a, b). We then examined β -catenin levels in FADD-D muscle. Consistent with the increased GSK3 β phosphorylation, protein levels of β -catenin were reduced more than 70% in FADD-D myoblasts, whereas the mRNA levels were comparable (fig. 2c). Accordingly, the transcriptional levels of Axin2, a direct transcriptional target of Wnt signaling, were also suppressed ~35% in FADD-D myoblasts from uninjured muscles (fig. 2d). Moreover, ~55% of β -catenin were repressed in regenerating muscles compared with WT regenerating muscle (fig. 2e), whereas its mRNA levels remained comparable (fig. 2f). These data indicate that Wnt signaling is suppressed in FADD-D muscle.

Notch Inhibition Restores β -Catenin Accumulation in FADD-D Myoblasts

Previous studies have indicated that Notch inhibits Wnt through activation of GSK3 β during muscle regeneration [Brack et al., 2008]. Interestingly, myoblasts from regenerating FADD-D muscle indeed contained higher GSK3 β activity (fig. 3a). We then determined whether Notch mediated GSK3 β activation in FADD-D myoblasts. As expected, Notch inhibition by L658,458, a selective inhibitor of γ -secretase inhibitor, largely compromised the induced GSK3 β activation in FADD-D myoblasts (fig. 3a). Consistently, β -catenin was largely restored by Notch inhibition in FADD-D cells (fig. 3a). The efficiency of Notch

Fig. 2. FADD-D inhibits β -catenin accumulation. **a** Protein levels of β -catenin and phospho-GSK3 β in primary myoblasts from uninjured muscles. Representative results were shown ($n = 3$). **b** Normalized quantification of β -catenin and phospho-GSK3 β protein

levels. **c, d** Quantitative RT-PCR of β -catenin and Axin2 in primary myoblasts from uninjured muscles (mean $\pm$ SD, $n = 4$). **e, f** Quantitative RT-PCR of Axin2 and β -catenin in primary myoblasts from regenerating muscles (4 days postinjury; mean $\pm$ SD, $n = 3$).

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inhibition was confirmed by Hes1 expression, which is a direct transcriptional target of Notch signaling (fig. 3b). Consistent with the increased Notch signaling, Hes1 was increased ~2-fold in FADD-D myoblasts compared with WT cells, while ~71% of Hes1 was repressed in myoblasts from FADD-D muscles injected with L685,458 (fig. 3b). Furthermore, a selective inhibitor of GSK3 β (BIO) promoted the differentiation of FADD-D myoblasts in culture conditions promoting differentiation, indicating that GSK3 β inhibits myoblast differentiation in FADD-D (fig. 3c). To examine the effects of Notch signaling on muscle regeneration in FADD-D muscle, we then determined whether Notch inhibition restored regeneration efficiency in FADD-D muscle. Indeed, Notch inhibition increased the size of newly formed myofibers in FADD-D ~2.5-fold compared with that of control FADD-D muscles injected with vehicles (fig. 3d, e). Furthermore, consistent with the restored regeneration, Pax7, the repression of which favors myoblast differentiation, was reduced ~56% in FADD-D myoblasts from muscles injected with L685,458 (fig. 3f). In contrast, myogenin was increased in FADD-D myoblasts by L685,458 injection (fig. 3f). These data indicate that enhanced Notch signaling compromised Wnt signaling in FADD-D myoblasts.

PKC Promotes Notch Signaling in FADD-D Myoblasts

We have shown that FADD phosphorylation promotes PKC α activation, the inhibition of which represses the increased Notch-1 protein levels in FADD-D myogenic satellite cells [Cheng et al., 2014]. We then investigated whether PKC α also modulated Notch-1 expression in myoblasts. Compared to myoblasts isolated from the regenerating contralateral leg of FADD-D mice treated with vehicles, myoblasts exposed to a selective inhibitor of PKC α (Go6976) exhibited reduced Notch-1 levels in FADD-D (fig. 4a). The inhibition efficiency was validated by ERK phosphorylation, which is a direct effector of PKC signaling (fig. 4a). Consistently, more than 60% of

Hes1 mRNA was inhibited in FADD-D myoblasts from Go6976-treated muscles (fig. 4b). We then analyzed whether or not PKC α affected β -catenin accumulation in regenerating muscles from FADD-D mice. Notably, PKC α inhibition restored β -catenin expression in FADD-D regenerating myoblasts (fig. 4c). In addition, PKC α inhibition also resulted in compromised Pax7 expression in FADD-D myoblasts, while myogenin expression was restored (fig. 4d). To determine whether PKC α regulates myoblast differentiation in FADD-D cells, we monitored the differentiation of myoblasts from FADD-D muscle. As expected, inhibition of PKC α promoted the differentiation of myoblasts in FADD-D (fig. 4e). Thereafter, we were interested in whether the activated PKC α regulated muscle regeneration in FADD-D mice. Compared to the contralateral leg, Go6976 restored the size of regenerating myofibers efficiently in FADD-D mice (fig. 4f). The newborn myofiber size was increased ~4-fold by Go6976 treatment in regenerating FADD-D muscles compared to that of vehicle-injected muscle (fig. 4g). These data indicate that enhanced Notch-1 signaling in FADD-D myoblasts is mediated by PKC.

PKC α Regulates Notch-1 Stability

Because Notch-1 has a rapid turnover rate, we investigated whether PKC α could regulate the stability of Notch-1. To test this, we used CHX, which blocks mRNA translation, to monitor Notch-1 degradation. Forced expression of PKC α in myoblasts largely compromised Notch-1 loss induced by CHX treatment (fig. 5a). Moreover, Notch-1 protein loss induced by CHX was also compromised in FADD-D myoblasts compared with WT myoblasts (fig. 5b). Given the fact that Notch-1 is a transmembrane receptor and PKC α also translocates to the inner cellular membrane after activation, we then went on to investigate if these two proteins interacted with each other in vivo. Interestingly, PKC α coimmunoprecipitated with full-length Notch-1 (fig. 5c), indicating that PKC α was able to interact with Notch-1 in vivo. Previous studies have indicated that phosphorylation events in the hydrophobic and turn motif regions of PKC α are critical for its activation and membrane translocation [Newton, 2010]. We then examined whether the phosphorylation of PKC α regulated its interaction with Notch-1. As shown in figure 5d and e, constitutively phosphorylated mutations in the hydrophobic and turn motif regions of PKC α promoted its interaction with Notch-1, suggesting that the interaction between PKC α and Notch-1 is regulated by the phosphorylation status of PKC α . Furthermore, no coimmunoprecipitation was observed between the released intra-

Fig. 3. Notch signaling mediates Wnt signaling in FADD-D myoblasts. **a** Immunoblotting of β -catenin and phospho-GSK3 β in primary myoblasts from regenerating muscles (5 days postinjury) injected with L685,458 as indicated at 3.5 days postinjury. **b** Quantitative RT-PCR of Hes1 in samples as in **a**. **c** Quantification of cultured fused myoblasts (mean $\pm$ SD, $n = 3$). **d** Hematoxylin and eosin sections of injured TA muscles injected with L685,458; representative images are shown ($n = 4$). **e** Normalized quantification of the myofiber cross-section area (CSA) from **d** (mean $\pm$ SD, $n = 3$). **f** Quantitative RT-PCR of Pax7 and myogenin in samples in **d** (mean $\pm$ SD, $n = 3$). Scale bar = 20 μ m.

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cellular domain of Notch-1 (NICD) and PKC α , suggesting that PKC α only interacts with full-length Notch-1 rather than its activated form (fig. 5f). These data suggested that Notch-1 was stabilized by PKC α .

PKC α Regulates Muscle Regeneration

Given that PKC α and FADD phosphorylation were induced in regenerating muscles and previous studies have indicated a critical role of PKC α in cell proliferation [Black, 2000], we hypothesized that increased PKC α phosphorylation promotes myoblast expansion. Indeed, PKC α inhibition by Go6976 largely reduced nuclei-centralized myoblasts in regenerating areas, suggesting a role of PKC α in myoblast formation (fig. 6a, b). Consistently, cell cycle regulators including Ki67, Aurora A, survivin and Aurora B were downregulated significantly in myoblasts from Go6976-treated regenerating muscles (fig. 6c). To verify whether PKC α regulates myoblast proliferation, we examined DNA synthesis in myoblasts. PKC α inhibition suppressed DNA synthesis (denoted by EdU incorporation) and proliferation in C2C12 cells and cultured primary myoblasts (fig. 6d, e), indicating that PKC α modulates myoblast proliferation. We then went on to examine whether PKC α affects differentiation. Forced PKC α expression inhibited MHC expression in myoblasts cultured in differentiation medium, suggesting that myoblast differentiation was compromised by PKC α (fig. 6f). These data suggest dual roles of PKC α in myogenic progression.

Discussion

The roles of Notch signaling in myogenic expansion have been well defined: when activated by its ligands, Notch undergoes proteolysis to release the intracellular domain, which translocates to the nucleus to activate transcriptional programs [Kopan and Ilagan, 2009]. At

the early phase of mouse muscle regeneration, one Notch ligand, Delta, is upregulated and promotes Notch-1 cleavage and Notch signaling [Conboy and Rando, 2002]. This ligand-dependent activation model has been widely accepted as the principal mechanism for the regulation of Notch signaling. In addition to this, previous studies have indicated that compromised endocytic trafficking of Notch receptor from plasma membrane leads to an increase in Notch signaling [Vaccari and Bilder, 2005; Chastagner et al., 2008]. These observations imply that the availability of Notch receptor for signaling is also involved in the regulation of Notch signaling. In the present study, our data indicate that ligand-independent Notch-1 degradation in myoblasts is controlled by PKC α . The protein level of Notch-1 has been observed to be increased in regenerating muscles compared to uninjured counterparts [Conboy et al., 2003]. Given that injury-induced muscle regeneration requires urgent Notch signaling to support immediate myoblast proliferation, it is reasonable to conclude that the availability of Notch-1 affects Notch signaling. PKC α phosphorylation is induced at the early phase of myogenic progression and also promotes increased interaction of PKC α with Notch-1. These observations suggest a mechanism for the sustainability of Notch signaling by modulating Notch-1 abundance on the plasma membrane in proliferating myoblasts. Our data show that PKC only interacts with full-length Notch-1 rather than cleaved NICD, suggesting the existence of a competence between TACE (tumor necrosis factor α -converting enzyme) and PKC. The recruitment of TACE might lead to disassociation between PKC and Notch-1. Further explorations are required to verify this hypothesis.

Here we also identified that PKC α inhibition resulted in myoblast proliferation repression. It has been shown that growth factors play critical roles in myoblast proliferation in regenerating muscles [Husmann et al., 1996]. As a classic intracellular effector of growth factors, PKC α may play a role in conveying these proliferative signals *in vivo* to facilitate myoblast proliferation. The present data showed that newborn myofiber size in FADD-D was largely restored by PKC α inhibition, which reached 80% of WT myofibers (fig. 4f, g), indicating that PKC α is a critical mediator involved in FADD-mediated muscle regeneration.

It has been shown that overexpression of PKC α induces Notch-4, instead of Notch-1, in breast cancer cells [Yun et al., 2013], which is inconsistent with our observations in myoblasts. Given that PKC α is highly expressed in breast cancer cells [Lahn et al., 2004], it is possible that the forced expressed PKC α is unable to interact with endogenous

Fig. 4. PKC regulates Notch in FADD-D muscles. **a** Regenerating gastrocnemius muscles were injected with Go6976 at 3 days postinjury for an additional 2 days. Representative results are shown ($n = 3$). **b** Quantitative RT-PCR of Hes1 in samples from **a** (mean $\pm$ SD, $n = 3$). **c** Protein levels of β -catenin in primary myoblasts isolated from regenerating muscles 7 days postinjury. **d** Quantitative RT-PCR of Pax7 and myogenin in primary myoblasts from regenerating muscle as in **c** (mean $\pm$ SD, $n = 3$). **e** Quantification of fused myoblasts (mean $\pm$ SD, $n = 3$). **f** Hematoxylin and eosin staining of sections from regenerating TA muscles injected with Go6976. Representative images are shown ($n = 4$). Scale bar = 20 μ m. **g** Normalized quantification of myofiber cross-section area (CSA) from **f** (mean $\pm$ SD, $n = 3$).

Fig. 5. PKC α regulates Notch-1 stability. **a** C2C12 cells expressing PKC α or vector were treated with CHX (top panel). The bands were then quantified (bottom panels). **b** Primary myoblasts from uninjured muscles were treated with CHX and samples were collected at the times indicated (top panel); the bands were then quantified (bottom panels). All the experiments were performed at least three times and representative results are shown. **c** Coimmunopre-

cipitation of PKC α with Notch-1 in 293T cells. **d** Coimmunoprecipitation of WT PKC α and its phosphorylation mutations in Thr638 with full-length Notch-1 in 293T cells. **e** Coimmunoprecipitation of WT PKC α and its phosphorylation mutations in Ser657 with Notch-1 in 293T cells. **f** Coimmunoprecipitation of Notch-1 and NICD with PKC α in 293T cells. The 120-kDa portion of Notch-1 and NICD are indicated (arrowheads).

Fig. 6. PKC α regulates myogenic progression. **a** Regenerating muscles were injected with Go6976 at 2 days postinjury. Representative images are shown ($n = 4$). Newborn myofibers were denoted by central localized nuclei (arrows). Scale bar = 20 μ m.

b Quantification of **a**. **c** Quantitative RT-PCR of cell cycle proteins in samples from **a** (mean $\pm$ SD, $n = 3$). **d** C2C12 myoblast proliferation in the presence of Go6976. **e** Primary myoblast cell proliferation in the presence of Go6976 (mean $\pm$ SD, $n = 3$).

Fig. 6. PKC α regulates myogenic progression. **f** Protein levels of MHC in PKC α overexpressed C2C12 cultured in differentiation medium.

Notch-1, which already binds enough PKC α in breast cancer cells. Alternatively, this discrepancy also suggests the effects of PKC α on Notch-1 depend on the cell context.

It remains unknown how Notch promotes GSK3 β activation. Notch has been shown to inhibit AKT

phosphorylation by facilitating AKT dephosphorylation [Hales et al., 2013]. AKT plays classical inhibitory roles in GSK3 β activation [Cohen and Frame, 2001]. More importantly, AKT is required for the initiation of myoblast differentiation [Gardner et al., 2012]. These observations suggest AKT as a candidate for mediating Notch-induced GSK3 β , which needs to be determined by further studies.

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Disclosure Statement

The authors declare no conflict of interests.

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