

Lipin1 is required for skeletal muscle development by regulating MEF2c and MyoD expression

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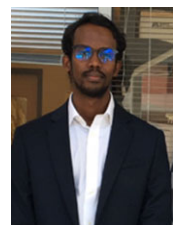
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Key points

- Lipin1 is critical for skeletal muscle development.
- Lipin1 regulates MyoD and myocyte-specific enhancer factor 2C (MEF2c) expression via the protein kinase C (PKC)/histone deacetylase 5-mediated pathway.
- Inhibition of PKC μ activity suppresses myoblast differentiation by inhibiting MyoD and MEF2c expression.

Abstract Our previous characterization of global lipin1-deficient (*fld*) mice demonstrated that lipin1 played a novel role in skeletal muscle (SM) regeneration. The present study using cell type-specific Myf5-cre;Lipin1^{fl/fl} conditional knockout mice (Lipin1^{Myf5cKO}) shows that lipin1 is a major determinant of SM development. Lipin1 deficiency induced reduced muscle mass and myopathy. Our results from lipin1-deficient myoblasts suggested that lipin1 regulates myoblast differentiation via the protein kinase C μ (PKC μ)/histone deacetylase 5 (HDAC5)/myocyte-specific enhancer factor 2C (MEF2c):MyoD-mediated pathway. Lipin1 deficiency leads to the suppression of PKC isoform activities, as well as inhibition of the downstream target of PKC μ , class II deacetylase HDAC5 nuclear export, and, consequently, inhibition of MEF2c and MyoD expression in the SM of lipin1^{Myf5cKO} mice. Restoration of diacylglycerol-mediated signalling in lipin1 deficient myoblasts by phorbol 12-myristate 13-acetate transiently activated PKC and HDAC5, and upregulated MEF2c expression. Our findings provide insights into the signalling circuitry that regulates SM development, and have important implications for developing intervention aimed at treating muscular dystrophy.

Abdulrahman Jama is a first-year PhD student in the Department of Biochemistry & Molecular Biology at Wright State University. He has been working on identifying the molecular mechanisms underlying myopathy induced by lipin1 deficiency.



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Introduction

Lipin1 catalyses the penultimate step in triglyceride synthesis and acts as a nuclear transcriptional coactivator (Finck *et al.* 2006; Han *et al.* 2006; Kim *et al.* 2013). Studies of lipin1 have revealed multiple roles: its lipid-storing function in adipose tissue, its role in Schwann cell and macrophage biology and its role in hepatic lipid metabolism (Rehmark *et al.* 1998; Finck *et al.* 2006; Nadra *et al.* 2008; Reue & Dwyer, 2009; Ren *et al.* 2010; Nadra *et al.* 2012; Hu *et al.* 2013; Kim *et al.* 2013; Mitra *et al.* 2013; Schweitzer *et al.* 2015; Wang *et al.* 2016; Vozenilek *et al.* 2018), as well as new roles in autophagy and mitophagy in other recent studies (Zhang *et al.* 2014; Alshudukhi *et al.* 2018; Schweitzer *et al.* 2018). There are three mammalian lipin genes, lipin1, lipin2 and lipin3, with distinct but partially overlapping expression pattern (Donkor *et al.* 2007), although lipin1 is the predominant lipin isoform in skeletal muscle (SM), accounting for almost all phosphatidic acid phosphatase activity in SM (Han *et al.* 2006; Reue & Dwyer, 2009). Clinical studies have identified a population carrying a homozygous or compound heterozygous *LPIN1* gene mutation, which could be the result of a premature stop codon insertion, a large intragenic deletion, frameshift mutation and single amino acid replacements, comprising the major cause of rhabdomyolysis (Zeharia *et al.* 2008; Michot *et al.* 2010; Michot *et al.* 2012). Our earlier work in global lipin1-deficient (*fld*) mice demonstrated that lipin1 plays a major role in SM regeneration (Jiang *et al.* 2015). In particular, *fld* mice exhibited smaller regenerated muscle fibre cross-sectional areas in response to injury, accompanied by reduced MyoD expression compared to wild-type (WT) mice. A key challenge in these studies, however, is that lipin1-deficient *fld* mice have systemic metabolic abnormalities and develop severe neuropathy and locomotor defects that complicate or preclude a detailed analysis of SM function (Rehmark *et al.* 1998). Thus, for the present study, we generated Lipin1^{Myf5cKO} mice to specifically deplete lipin1 expression in Myf5-expressing progenitors to further investigate the role of lipin1 in myogenic determination and differentiation.

SM development involves a highly ordered cascade of events comprising myogenic lineage commitment, myoblast proliferation and terminal differentiation (Buckingham, 2001; Bentzinger *et al.* 2012). These steps are tightly controlled by transcription factor families, including the myogenic basic helix-loop-helix (bHLH) proteins and the myocyte enhancer factor 2 (MEF2). Members of the myogenic bHLH proteins, MyoD and Myf5, are required for myoblast specification and

differentiation, whereas myogenin and MRF4 play key roles in myoblast fusion (Hernandez-Hernandez *et al.* 2017). Myoblast differentiation requires the co-operative activation of members of the MyoD families and MEF2 (Dodou *et al.* 2003), which need to be dissociated from class II HDACs to release the repression function (McKinsey *et al.* 2000; Lu *et al.* 2000b); however, this regulatory mechanism is still not fully understood.

In the present study, we employed our newly generated Lipin1^{Myf5cKO} mice to investigate the role of lipin1 in myogenic differentiation. The results obtained suggest that lipin1 deficiency results in reduced SM mass. Lipin1 is a major determinant of skeletal myogenesis by the promotion of MEF2c and MyoD-regulated muscle differentiation. We evaluated the expression of the myogenic transcription factors and proteins involved in SM differentiation, and identified a lipin1-associated regulatory pathway that was further confirmed in lipin1-depleted myoblasts. Our experiments uncover the key role of lipin1 in a regulatory circuit that facilitates gene programme transition from proliferation to differentiation.

Methods

Animals

Lipin1^{Myf5cKO} mice were generated by crossing Lipin1^{fl/fl} mice (Nadra *et al.* 2008) with Myf5-Cre mice (Stock No: 007893; Jackson Laboratories, Bar Harbor, ME, USA). Both Lipin1^{fl/fl} and Myf5-Cre mice were in a C57/B6 background. Experiments were performed on 2–4-month-old mice. These mice had free access to drinking water and regular chow, unless otherwise noted. All animal experiments were performed in accordance with the relevant guidelines and regulations approved by the Animal Care and Use Committee of Wright State University (#1058).

Cell culture and differentiation of C2C12 myoblasts

Mouse C2C12 myoblasts were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (#10569-010; Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (#16000-044; Gibco) and 1% penicillin–streptomycin (#15140-122; Gibco) under humidified air containing 5% CO₂ at 37°C. Myotube formation was induced at confluence by replacing growth medium with differentiation medium consisting of DMEM supplemented with 2% horse medium (#16050-122; Gibco) and 1% penicillin–streptomycin.

Adenoviruses-mediated short hairpin RNA (shRNA) knockdown

Adenoviruses expressing shRNA to knockdown lipin1 (shLpin1) and control shLacZ were generous gifts from Dr Thurl Harris (University of Virginia, Charlottesville, VA, USA). For knockdown of lipin1 expression, C2C12 myoblasts were infected with shLpin1 and shLacZ control in growth medium containing 8 g mL^{-1} polybrene (#107689; Sigma, St Louis, MO, USA) for 24 h. The medium was then replaced with DMEM supplemented with 10% fetal bovine serum. The myoblasts were incubated for an additional 24 h until they were confluent. Differentiation was then initiated by switching the medium to DMEM differentiation medium.

Immunofluorescence, microscopy and image processing

Cells were fixed with 4% formaldehyde and permeabilized in PBS containing 0.1% Triton X-100 for 20 min followed by blocking in PBS containing 1% BSA (#BP9704; Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. The cells were then incubated with the Mf20 monoclonal antibody (#AB_2147781; dilution 1:10; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) to detect MHC-expressing cells overnight at 4°C and subsequently with an Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary antibody (#A-21411; dilution 1:500; Thermo Fisher Scientific) for 1 h at room temperature. Nuclei of cells were detected by staining with 4',6-diamidino-2-phenylindole (DAPI) (#D1306; $2.5 \mu\text{g mL}^{-1}$; Thermo Fisher Scientific) for 10 min. Images were obtained using an inverted microscope (IX70; Olympus, Tokyo, Japan) equipped with a DFC7000T camera (Leica Microsystems, Wetzlar, Germany). The fusion index was calculated as the ratio of nuclei in MyHC-positive myotubes to the total number of nuclei in the field for five random fields.

Cos7 cells were seeded onto 13 mm round glass coverslips. Cells were transfected with HDAC5-GFP (#32211; Addgene, Cambridge, MA, USA) with/without lipin1-HA, lipin1 D712A-HA mutant or PKC μ -HA (#10808; Addgene) plasmids. After 24 h, cells were fixed in 4% paraformaldehyde. The cells were then permeabilized with 0.1% Triton X-100 (#28314; Thermo Fisher Scientific) made with PBS for 10 min at room temperature. After washes, the cells were blocked with 1% BSA, incubated with rabbit anti-HA primary antibody (#Ab9110; dilution 1:1000; Abcam, Cambridge, MA, USA) using 1% BSA blocking reagent overnight at 4°C . The next day, the cells were incubated with secondary antibody, goat anti-rabbit 555 (#A21428; dilution 1:1000; Thermo Fisher Scientific) with 1% BSA blocking reagent. Nuclei were then labeled with DAPI.

Co-localization of HDAC5 and lipin1, lipin1D712A or PKC μ was examined.

Quantitative real-time PCR

Total RNA from C2C12 myoblasts and muscle tissues was extracted using TRIzol reagent (#15596018; Thermo Fisher Scientific). The concentration and quality of RNA were assessed with a NanoDrop 2000 (Thermo Fisher Scientific). One microgram of total RNA was used for reverse transcription with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The quantitative real-time PCR reaction was performed in a QuantStudio Real-Time PCR System (Thermo Fisher Scientific) using SYBR Green Real-time PCR Master Mix (#A25742; Thermo Fisher Scientific). The sequence of primers is provided in Table 1. The C_t ($2^{-\Delta\Delta C_t}$) method was used to analyse the relative gene expression data.

Western blotting

Cells and muscle tissues were lysed in RIPA buffer containing 10 mM Tris-HCL (pH 7.4), 30 mM NaCl, 1 mM EDTA and 1% Nonidet P-40, supplemented with proteinase inhibitors and phosphatase inhibitors before use. Protein concentration was determined for each sample and equal amounts of proteins were used, boiled at 95°C for 5 min in $1 \times$ SDS sample buffer and separated by 7.5–15% SDS-PAGE. Thereafter, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using a Mini Trans-Blot Cell System (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 1% casein buffer for 1 h, and incubated with the primary antibodies overnight at 4°C . After probing with secondary antibodies for 1 h at 25°C , protein bands were detected by using Amersham Imager 600 (GE Healthcare Life Sciences, Little Chalfont, UK). β -actin (#A1978; dilution 1:5000; Sigma) or GAPDH (Ab181602; dilution 1:20000; Abcam) antibodies were used as a loading control. The densitometry values were normalized by the corresponding loading control densitometry values obtained from the same sample. Antibodies used include lipin1 (#14906; dilution 1:1000; Cell Signaling Technology, Beverly, MA, USA), phospho-PKC μ (#2054; dilution 1:1000; Cell Signaling Technology), MyoD (#Ab16148; dilution 1:1000; Abcam), MEF2c (#5030; dilution 1:1000; Cell Signaling Technology), phospho-PKC α (#sc-377565 dilution 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-PKC θ isoforms (#sc-271922; dilution 1:1000; Santa Cruz Biotechnology) and Pax7 (#AB_528428; dilution 1:1000; Developmental Studies Hybridoma Bank) and (#Ab34360; dilution 1:1000; Abcam), along with goat anti-mouse IgG-HRP (#w402B; dilution 1:5000; Promega, Madison, WI, USA) and

Table 1. Primer sequence used for RT-PCR

Primer	Forward 5'- to 3'	Reverse 5'- to 3'
Lipin1	CCTTCTATGCTGCTTTTGGGAACC	GTGATCGACCACTTCGCAGAGC
Myf-5	TGTATCCCCTCACCAGAGGAT	GGCTGTAATAGTTCTCCACCTGTT
MyoD	GCAGGCTCTGCTGCGCGACC	TGCAGTCGATCTCTCAAAGCACC
Mef2c	TCTGTCTGGCTCAACACTG	TGGTGGTACGGTCTCTAGGA
Gapdh	CCAATGTGTCCGTCGTGGATCT	GTTGAAGTCGAGGAGACAACC

goat anti-rabbit IgG-HRP (#w401B; dilution 1:5000; Promega).

Statistical analysis

Unless noted otherwise, data are provided as the mean \pm SD number (n) of independent experiments. Statistical significance was calculated using a two-tailed Student's t test. $P < 0.05$ was considered statistically significant.

Results

Depletion of lipin1 in Myf5-expressing precursors causes suppression of SM development

To investigate the role of lipin1 in SM development, we generated Lipin1^{Myf5cKO} mice to specifically deplete lipin1 expression in Myf5-expressing progenitors. Myf5 is the

first muscle regulatory factor (i.e. Myf5, MyoD, myogenin and Mrf4) expressed during embryonic development (Ott *et al.* 1991; Buckingham, 1992; Bentzinger *et al.* 2012). Loss of lipin1 in Myf5-expressing progenitors did not induce neurological phenotypes such as hind-limb clasp reflexes, tremors or an unsteady gait, as observed in global lipin1-deficient *fld* mice shortly after 10 days of age. However, compared to control mice, selectively deleting lipin1 in Myf5-expressing precursors caused the inhibition of SM development, characterized by a 24% reduction in muscle mass of both TA and GAS muscle in Lipin1^{Myf5cKO} mice (Fig. 1A–C). Haematoxylin and eosin staining exhibited a reduction in mean fibre cross-sectional area in both TA and GAS muscle of Lipin1^{Myf5cKO} mice compared to WT mice (Fig. 1D–E). Consistent with recent reports in skeletal muscle-specific lipin1 deficient (MCK promoter) (Schweitzer *et al.* 2018) and *fld* (Zhang *et al.* 2014)

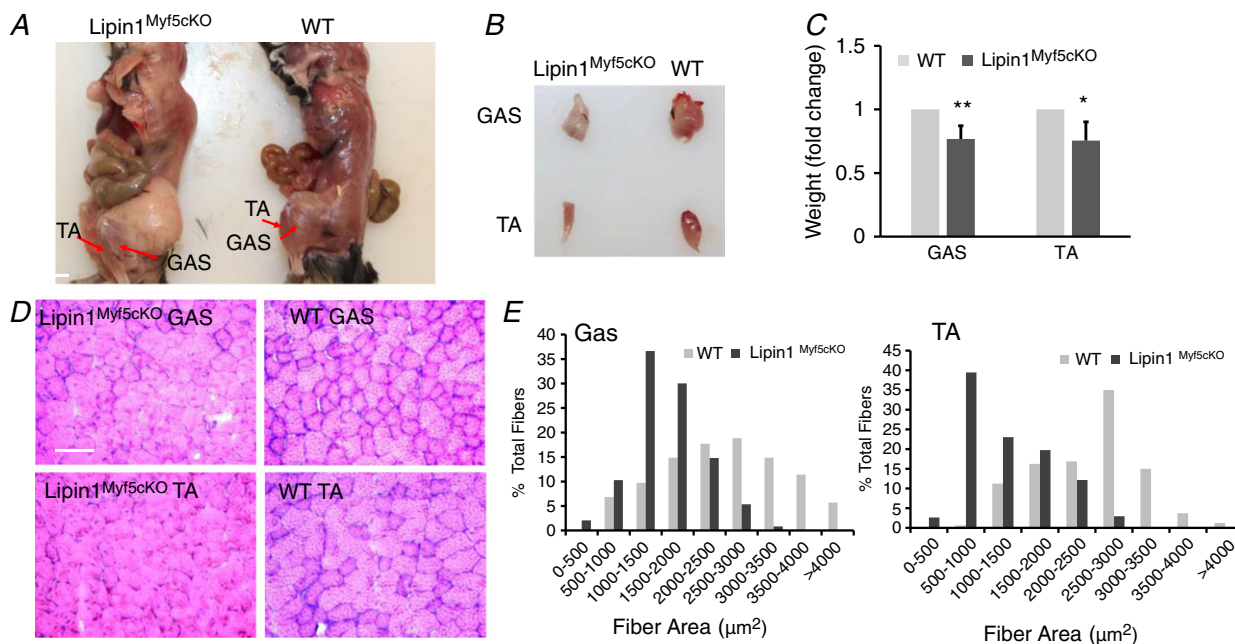


Figure 1. Lipin1 deficiency in Myf5-expressing cells results in reduced SM mass

Appearance of TA and GAS muscles in WT and Lipin1^{Myf5cKO} mice at 3 months of age (TA and GAS are indicated by arrows) (A). Isolated TA and GAS muscle (B) and the muscle weight (C) in WT and Lipin1^{Myf5cKO} mice. (WT vs. Lipin1^{Myf5cKO}, * $P < 0.05$; ** $P < 0.01$ Student's t test). Histology examination (D) and quantification of reduced myofibre area (E) of GAS and TA muscle from WT and Lipin1^{Myf5cKO} mice (scale bar = 100 μ m).

mice, SM of Lipin1^{Myf5cKO} mice also displayed a large number of centrally nucleated fibres compared to control mice, suggesting that lipin1 deficiency results in myopathy. The centrally positioned nuclei are associated with excessive degeneration/regeneration processes similar to those reported in patients with lipin1 deficiency (Zeharia *et al.* 2008; Michot *et al.* 2012). However, we did not observe any obvious body weight difference in these knockout mice. These mice also have altered fat deposition and metabolism (Fig. 1A) and increased intramuscular lipid accumulation (data not shown). We are currently exploring the impact of lipin1 deficiency on fat-associated phenotypes in a separate project.

We next examined the expression levels of lipin1 in the TA and GAS muscles of Lipin1^{Myf5cKO} mice. As shown in Fig. 2A, lipin1 was expressed in the control WT mice but depleted in Lipin1^{Myf5cKO} mice. We also measured the protein expression levels of Myf5, MyoD and MEF2c in GAS and TA muscles of Lipin1^{Myf5cKO} and control mice. Although lipin1 was depleted in Myf5-expression progenitor cells, the expression level of Myf5 was not affected. Interestingly, the expression levels of MyoD and MEF2c were reduced by ~48% and 60%, respectively, in GAS and TA muscle of Lipin1^{Myf5cKO} mice compared to the control group (Fig. 2B). We also measured mRNA expression levels, which were consistently decreased, whereas the mRNA expression levels of Myf5 were not (Fig. 2C).

Lipin1 deficiency diminishes PKC activation

Lipin1 catalyses diacylglycerol (DAG) biosynthesis (Han *et al.* 2006). Overexpression of lipin1 increases cellular DAG, whereas a deficiency contributes to decreased DAG (Ren *et al.* 2010). DAG can recruit and activate a variety of downstream effector molecules by phosphorylation, including PKC μ (renamed as protein kinase D; PKD) and other PKC isoforms, including classical (PKC α , PKC β I, PKC β II and PKC γ) and novel (PKC δ , PKC ϵ , PKC η and PKC θ) isoforms (Brose *et al.* 2004; Toker, 2005; Gallegos & Newton, 2008; Schmitz-Peiffer & Biden, 2008; Newton, 2009; Leonard & Hurley, 2011). PKC activation has been suggested to be involved in MyoD expression, although the detailed mechanisms of its action are unknown (Brunelli *et al.* 2007). To investigate whether any particular isoform was specifically implicated in the signalling mechanisms by which lipin1 deficiency induces its myogenesis defects, we assessed the activation of conventional PKC α and novel θ isoforms, as well as PKC μ and PKA, in the GAS and TA muscle of WT vs. Lipin1^{Myf5cKO} mice. The activation of PKC isoforms was measured by performing western blotting using antibodies directed against the phosphorylated proteins. As shown in Fig. 3, both phosphorylated PKC μ at Ser744/748 decreased by more than 70%, and PKC α at Ser657 reduced by 63%, in GAS and TA muscle, suggesting an inhibition of PKC μ and PKC α activation in the SM of Lipin1^{Myf5cKO} mice. Phosphorylated novel PKC θ isoform was also reduced by

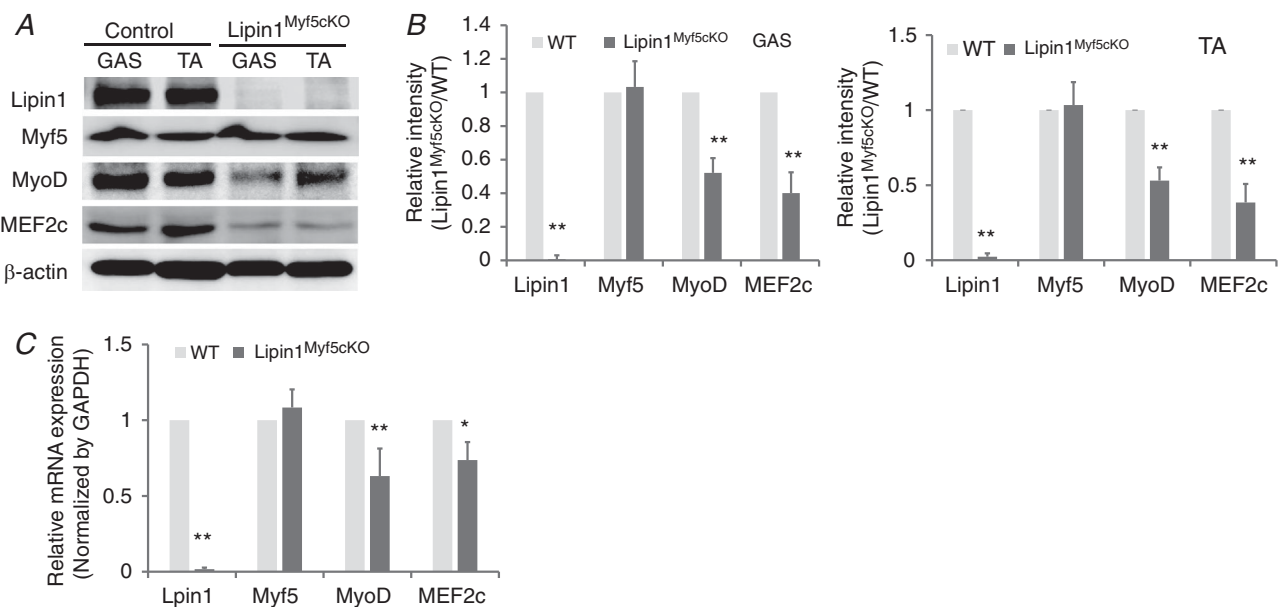


Figure 2. Lipin1 depletion in Myf5-expressing progenitor cells inhibits MEF2 and MyoD expression but does not alter Myf5 expression

Western blotting (A), densitometry graphs (B) and mRNA expression (C) of lipin1, myogenic regulatory factors (Myf5 and MyoD) and of myogenic enhancer factor (MEF2c) in GAS and TA muscles of 3-month-old WT and Lipin1^{Myf5cKO} mice. Unless otherwise indicated, data are from one representative ($n = 3$) of at least three independent experiments (* $P < 0.05$; ** $P < 0.01$ Student's t test).

40% in the TA muscle of Lipin1^{Myf5cKO} mice compared to WT mice. By contrast, total levels of PKA and PKC did not exhibit significant differences.

Lipin1 is required for myoblast differentiation in cell culture

To clarify the role of lipin1 in the myoblast differentiation process, we knocked down lipin1 expression in C2C12 cells using adenovirus-driven shRNA against the *Lpin1* gene and evaluated the effect of lipin1 deficiency on myoblast differentiation at days 0, 4 and 6 after differentiation treatment. At day 6 post-differentiation treatment, we fixed the differentiated cells and examined myotube formation using Mf20 immunostaining to detect myosin heavy chain-positive myotubes. We found that lipin1 deficiency in C2C12 myoblasts induced a significantly reduced myoblast differentiation and myotube formation compared to shLacZ-treated cells (Fig. 4A). Consistently, the lipin1 shRNA-treated myotubes had a reduced fusion index, which is calculated as the percentage of nuclei contained in myosin-positive myotubes. This index was reduced from 49% to 30% in shRNA-treated myotubes compared to the control (Fig. 4B). This is consistent with a previous study in which we revealed that lipin1 is required for myoblast differentiation using lipin1 depleted myoblasts and primary myoblasts (Jiang *et al.* 2015).

We also examined the expression levels of myogenic regulatory proteins after differentiation treatment (Fig. 4C–D). The expression level of Myf5 was not altered, although MyoD and MEF2c were dramatically reduced

at day 6 after differentiation treatment. PKC μ activation indicated by phosphorylated PKC μ levels was reduced by 60% at day 6. In addition, the expression levels of the myogenic transcriptional factor, MyoD, were also decreased by 75% at day 6 after differentiation treatment. These results are consistent with our findings obtained *in vivo* and confirm that lipin1 is essential for effective differentiation during the early-stage myogenesis.

Inhibition of PKC μ activity suppresses myoblast differentiation by inhibiting MyoD and MEF2c expression *in vitro*

Next, we determined whether PKC μ activity is required for MyoD and MEF2c expression during myogenesis. C2C12 cells were differentiated for 6 days by fetal bovine serum withdrawal in the presence of CID755673 to selectively inhibit PKC μ activity. As shown in Fig. 5A, treatment with CID755673 reduced the degree of C2C12 differentiation, as indicated by visualizing MHC-positive myotubes detected by Mf20 immunostaining (Fig. 5A). The fusion index, which is calculated by the percentage of nuclei inside the myotube, was decreased from 50% to 31% after CID755673 treatment (Fig. 5B). To assess whether myogenesis was regulated by the activation of PKC, myoblasts were shifted from a growth medium to a differentiation medium (DMEM containing 2% horse serum) and differentiation was examined at days 0, 4 and 6. The phosphorylation of PKC μ , MyoD and MEF2c expression in differentiated myoblasts was reduced by 70%, 75% and 63%, respectively, at day 6 after differentiation treatment (Fig. 5C–D).

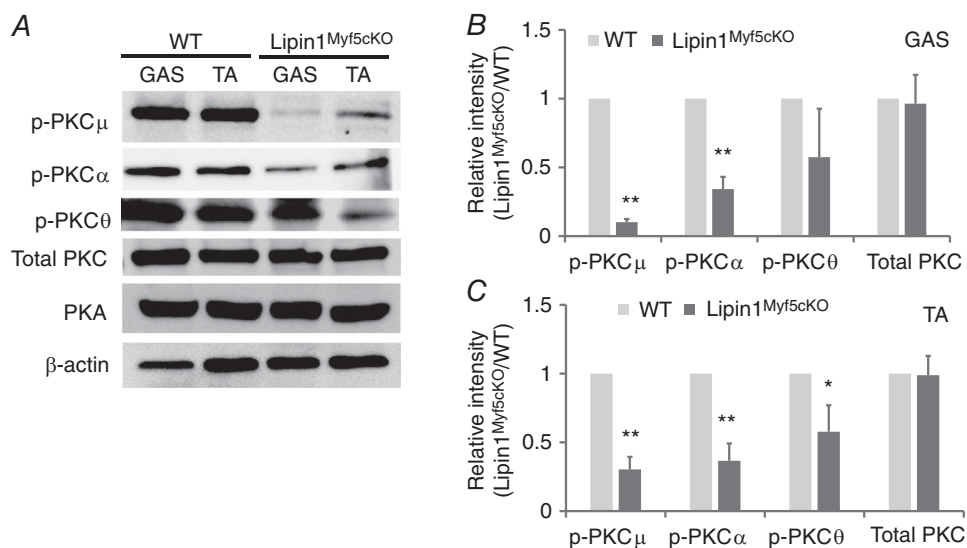


Figure 3. Lipin1 deficiency diminishes PKC activation

Representative immunoblots (A) and densitometry graphs of lipin1 and different protein kinase expression and phosphorylation in GAS (B) and TA (C) muscles in WT and Lipin1^{Myf5cKO} mice at 3 months. Unless otherwise indicated, data are from one representative ($n = 3$) of at least three independent experiments. (* $P < 0.05$; ** $P < 0.01$ Student's t test).

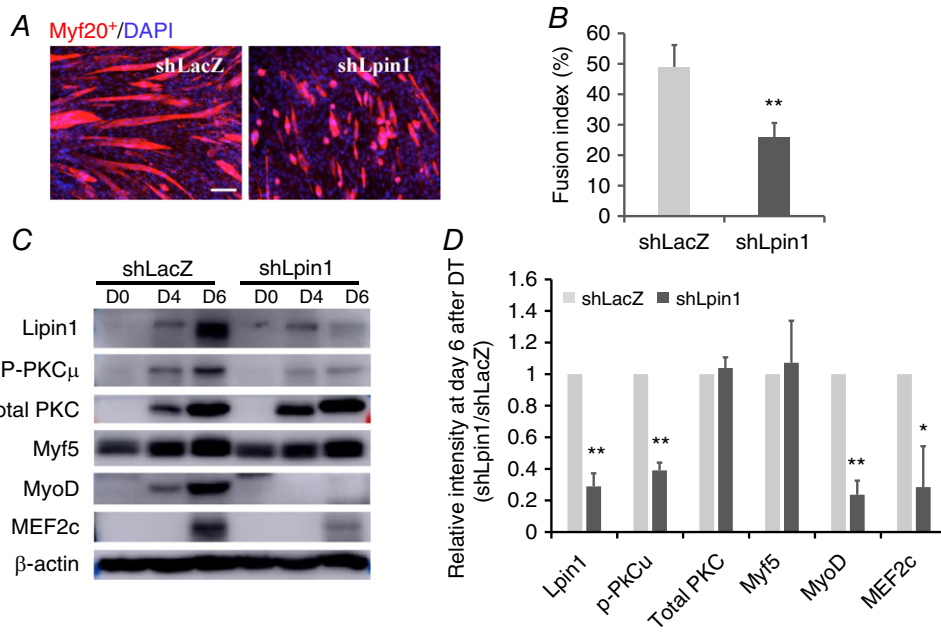


Figure 4. Lipin1 is required for myoblast differentiation in cell culture

C2C12 myoblasts were infected with adenovirus to knockdown lipin1 (*shLpin1*) or control *shLacZ* before differentiation. *A*, cells were fixed and stained for Mf20⁺ and DAPI (scale bars = 50 μm). *B*, fusion index was determined by counting the percentage of nuclei within Mf20⁺ myotubes over total nuclei within five randomly selected fields per sample. Protein expression levels (*C*) and densitometric analysis (*D*) of PKC, myogenic differentiation markers and MEF2c in differentiated myoblasts. **P* < 0.05; ***P* < 0.01.

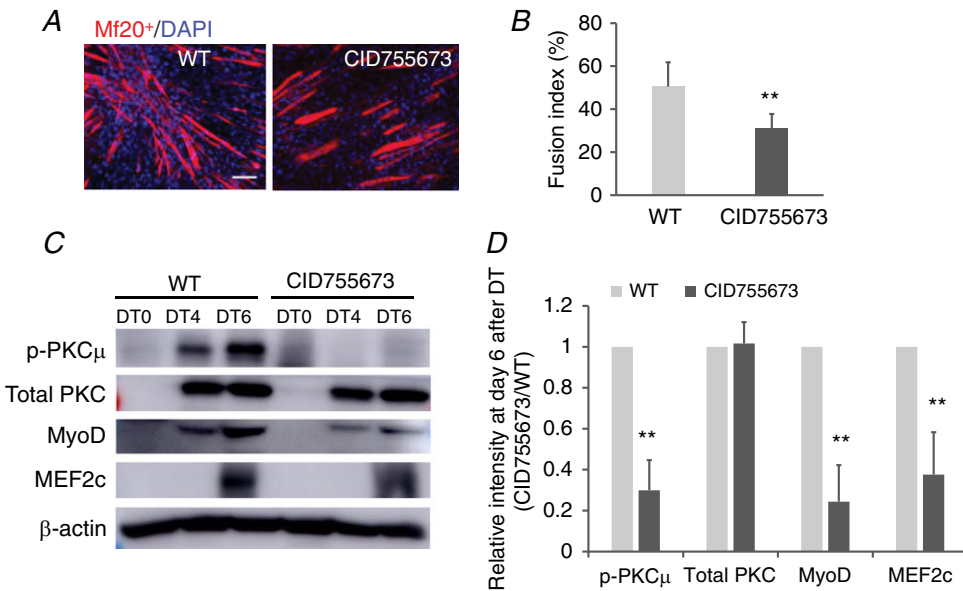


Figure 5. Inhibition of PKCμ activity suppresses myoblast differentiation by inhibiting MyoD and MEF2c expression in vitro

C2C12 myoblasts were treated with PKCμ inhibitor, CID 755673, for 6 days, and subjected to myoblast differentiation. Six days after differentiation, cells were fixed and stained with Mf20 and DAPI (scale bars = 50 μm) (*A*) and the fusion index was calculated (*B*). *C*, cell lysate was harvested, and protein expression was measured by western blot. *D*, protein expression levels in (*C*) were quantified by densitometric analysis and normalized to β-actin. ***P* < 0.01.

Lipin1 deficiency decreases MEF2 and MyoD by inhibiting the nuclear export of HDAC5

MyoD and MEF2c have been reported to interact with each other via their DNA-binding and dimerization motifs to synergistically activate transcription and myogenic programming (Kaushal *et al.* 1994; Molkenin *et al.* 1995; Molkenin *et al.* 1996; Black *et al.* 1998). The MEF2c protein also interacts with the class II deacetylase, HDAC5, resulting in the repression of MEF2c-dependent genes (Lu *et al.* 2000a). The release of MEF2c from repression by HDAC5 is controlled by the nuclear export of HDAC5, which can be regulated by CaMK (Ginnan *et al.* 2012), protein kinase A (Ha *et al.* 2010) and salt inducible kinase (Takemori *et al.* 2009). Because MyoD–MEF2c interaction and activation require the nuclear export of Class II HDAC to release the suppression function, we also

examined the protein expression of HDAC5. As shown in Fig. 6A, we did not observe any obvious differences in the expression levels of total HDAC5, although the phosphorylated HDAC5 was substantially suppressed in the SM of Lipin1^{Myf5^{CKO}} mice. To further confirm our observation in tissue samples, we measured HDAC5 and phosphorylated HDAC5 expression levels in CID755673 treated differentiated C2C12 as shown in Fig. 5. As a result of a low transfection efficiency in myoblasts, we used Cos7 cells for this experiment. We consider that the overexpression of the indicated plasmids in C2C12 myoblasts should have a similar effect in Cos7 cells. PKC μ suppression consistently did not alter protein expression levels of the total HDAC5, although it inhibited its phosphorylated form, suggesting that PKC μ activation is required during myogenesis to translocate HDAC5

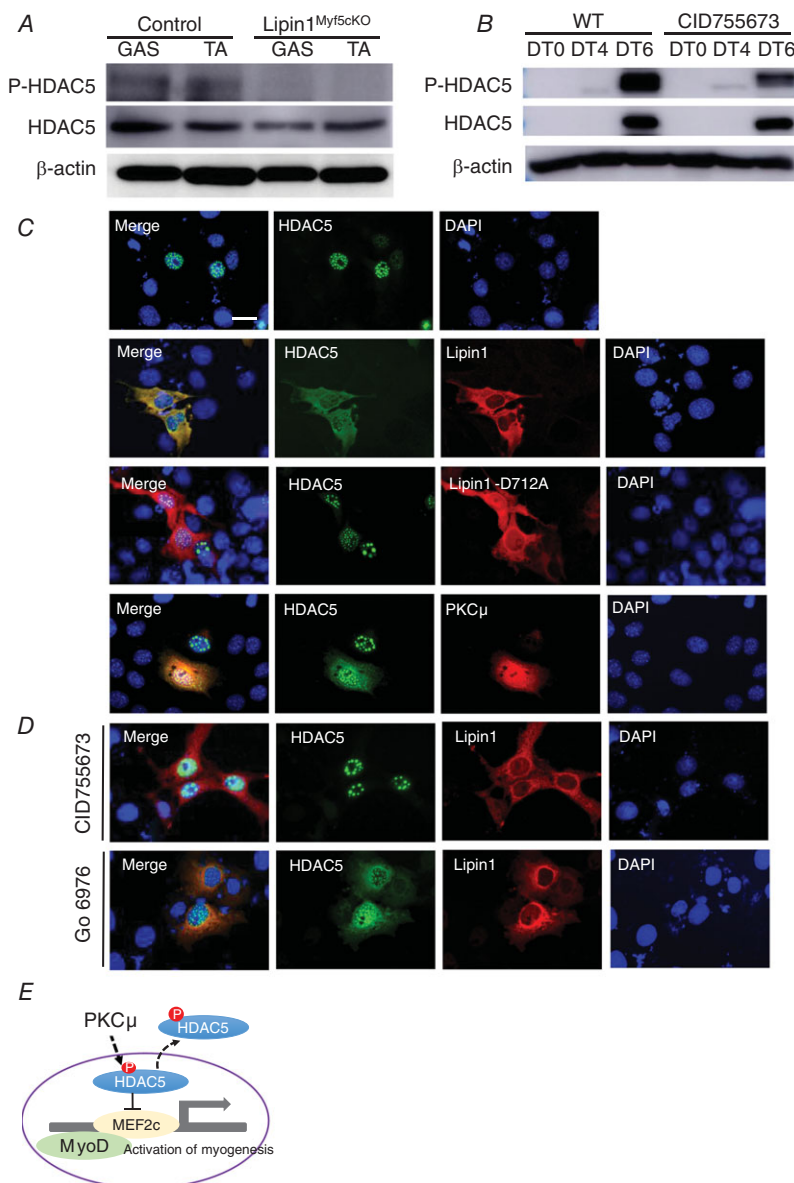


Figure 6. Lipin1 regulates HDAC5 nuclear export by activation of PKC μ

A, western blot of HDAC5 and phosphorylated HDAC5 (Ser259) in the GAS and TA muscle of WT and Lipin1^{Myf5^{CKO}} mice. **B**, C2C12 myoblasts were treated with CID 755673 for 6 days and subjected to myoblast differentiation. Six days after differentiation, cell lysate was harvested, the protein expression levels of HDAC5 and phosphorylated HDAC5 were measured by western blotting. **C**, Cos7 cells were transfected with either HDAC5-GFP or along with lipin1-HA, catalytic mutant lipin1-D712A-HA or PKC μ -HA. Cells were fixed 24 h after transfection, and subcellular localization of lipin1 and HDAC5 were examined by anti-HA (red), anti-GFP (green) antibodies. Nuclei were detected by DAPI (blue). Co-localization of HDAC5 and lipin1 or PKC μ was determined. Scale bars = 50 μ m. **D**, cells co-transfected with HDAC5-GFP and lipin1-HA were treated with either selective PKC μ inhibitor, CID755673 or a pan-PKC inhibitor against for PKC α , PKC β and PKC γ isoforms, Go 6976. After treatment for 6 h, the subcellular localization of lipin1 and HDAC5 was evaluated by immunostaining using anti-HA and anti-GFP antibodies. **E**, a model for regulating HDAC5 nuclear export.

from the nucleus to release its transcriptional repressor function.

We next examined the subcellular localization of HDAC5 and found that overexpressed HDAC5 was almost exclusively localized in the nucleus (Fig. 6C). Interestingly, co-overexpression of lipin1 and HDAC5 induced HDAC5 nuclear export. HDAC5 nuclear export was dependent on lipin1 catalytic activity because the catalytic inactive mutant, D712A, lost its capability to induce HDAC5 nuclear translocation. Co-transfection of PKC μ with HDAC5 also induced HDAC5 nuclear export. Selective PKC μ inhibitor, CID755673, inhibited HDAC5 nuclear export in the presence of lipin1 (Fig 6D). Interestingly, inhibition of Ca²⁺-dependent isoforms of PKC (PKC α , PKC β and PKC γ) by Go 6976 (Guh *et al.* 1998) failed to inhibit HDAC5 nuclear export in the presence of lipin1, suggesting that lipin1 regulates HDAC5 nuclear export via the activation of PKC μ . These results suggest that PKC μ -mediated phosphorylation of class IIa HDACs is required to release the suppression function of

MEF2 transcription to activate genes that govern muscle differentiation and growth (Fig 6E). Lipin1 deficiency failed to induce HDAC5 nuclear export that releases the inhibition function of HDAC5 to MEF2c via PKC μ activity.

Restoring DAG-mediated signalling response by phorbol 12-myristate 13-acetate (PMA) treatment transiently activated PKC μ /HDAC5 and upregulated MEF2c expression

PMA, an analogue of DAG, activates PKC by mimicking the activating ligand DAG (Way *et al.* 2000). To assess whether restoration of DAG-mediated signalling could alter HDAC5 subcellular localization, we treated cells transfected with HDAC5 with 10 μ M PMA for 30 min, and then evaluated the HDAC5 subcellular localization (Fig. 7A). We found that PMA treatment drives HDAC5 subcellular translocation from the nucleus to cytoplasm.

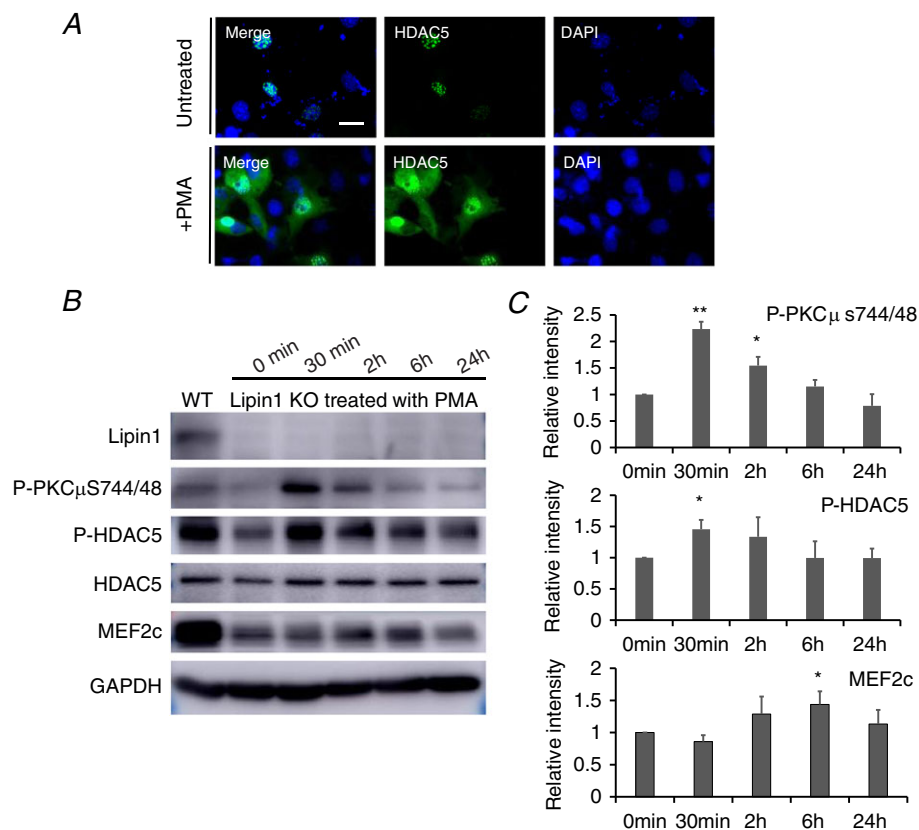


Figure 7. PMA treatment activates PKC/HDAC5 and promotes the upregulation of MEF2c

A, Cos7 cells transfected with HDAC5 treated with/without PKC agonist, PMA, for 30 min. Subcellular localization of HDAC5 was examined by immunostaining (scale bars = 50 μ m). B, lipin1 deficient C2C12 myoblasts were subjected to myogenic differentiation for 6 days followed by PMA treatment for 0 min, 30 min, 2 h, 6 h and 24 h. The activation of PKC μ , HDAC5 and the expression of MEF2c were measured by western blotting at differentiate time points followed by PMA treatment. C, densitometry analysis of western blots. Data are shown as the fold change in the absolute fluorescence intensity at various time points after PMA treatment compared to zero time point (0 min). Data were from three independent experiments. * $P < 0.05$.

To evaluate whether restoring DAG-mediated PKC activation could rescue myoblast differentiation defect induced by lipin1-deficiency, lipin1 deficient myoblasts were subjected to myogenic differentiation for 6 days. Cells were then treated with 10 μM of PMA for 0 min, 30 min, 2 h, 6 h and 24 h. After treatment, the activation of PKC μ and HDAC5 and the expression level of MEF2c were assessed (Fig. 7B and C). We found that PKC μ and HDAC5 were activated after 30 min of PMA treatment. However, the activation of PKC μ and HDAC5 was terminated immediately after 30 min of treatment, as indicated by downregulation of phospho-PKC μ and phosphor-HDAC5 and MEF2c, and it returned to baseline levels 24 h later. Moreover, the expression of MEF2c was upregulated at 2 and 6 h of PMA treatment, most probably as a result of translational delay, returning to basal levels by 24 h.

Discussion

The present study suggests that lipin1 plays a critical role in SM development (Fig. 8). Lipin1 deficiency resulted in reduced SM mass by suppressing the activity of PKC μ , which is required for regulating HDAC5 nuclear-cytoplasmic shuttling. HDAC5 phosphorylation-induced cytoplasm translocation may release the suppression function of MEF2c and activate MEF2c and MyoD expression, resulting in myoblast differentiation *in vitro*.

In the present study, we found that the absence of lipin1 resulted in inhibited myoblast differentiation *in vitro*. The observed reduction was associated with the inhibition of phosphorylation/activation of PKC μ . PKC μ selective inhibitor treatment suppressed MyoD and MEF2c expression *in vitro* (Fig. 5) and inhibited

lipin1-mediated HDAC5 nucleo-cytoplasmic translocation (Fig. 6), suggesting that lipin1 regulates myogenesis mainly by activation of PKC μ . Our results are consistent with a previous study showing that PKC μ (PKD2) depletion by shRNAs inhibits C2C12 myoblast differentiation *in vitro*, and the ectopic expression of PKC μ increases myogenic differentiation (Kleger *et al.* 2011). We cannot rule out that other PKC isoforms, including PKC α and θ , may also play an important role in myogenesis. Capiati *et al.* (1999) reported that PKC α promote chick myoblast differentiation. Additional studies using isoform specific inhibitors or their specific shRNA are needed to comprehensively evaluate the role of PKC in myogenesis.

MyoD is a master regulator of skeletal myogenesis. Overexpression of MyoD in mouse embryonic fibroblasts converts cells into SM (Yao *et al.* 2013) and MyoD deficiency suppresses myoblast differentiation potential (Sabourin *et al.* 1999). The activation of MyoD is dependent on its association with members of the MEF2 family of transcription factors. SM-specific deletion of MEF2c isoform in mice results in disorganized myofibres and perinatal lethality demonstrating a critical role of MEF2 proteins in SM development (Potthoff *et al.* 2007). By contrast, neither MEF2a, nor MEF2d isoform is required for normal SM development *in vivo*. The combinatorial activity of MyoD and MEF2c transcription factors promotes the expression of myogenic genes during the transition from undifferentiated myoblasts to differentiated myotubes (Lu *et al.* 2000b). Our results suggest that a lipin1 deficiency induces decreased MEF2c and MyoD expression, and also that their respective expression impacts upon each other. Although previous work suggests that MyoD and MEF2c interact during skeletal myogenesis throughout the DNA binding domains (Tapscott, 2005; Potthoff & Olson, 2007), further research is needed to understand how they participate in a regulatory loop by inducing and maintaining the expression of each other during skeletal myogenesis.

We found that lipin1 regulates MEF2c expression by the manipulation of HDAC5 nuclear export. Lipin1 regulates phosphorylated-associated HDAC5 nucleocytoplasmic trafficking depending on its catalytic activity because the lipin1 inactive mutant D712A lost its capacity to mediate HDAC5 nuclear export. In the present study, treatment with the PKC agonist, PMA, also promoted HDAC5 nuclear export. Treatment with specific PKC μ inhibitor, CID755673, blocked HDAC5 nuclear export in the presence of lipin1, whereas treatment with a pan-PKC inhibitor against for PKC α , PKC β and PKC γ isoforms failed to block HDAC5 nuclear export induced by lipin1. Based on our findings, we propose that lipin1 regulates HDAC5 nucleocytoplasmic trafficking via DAG/PKC μ . In the nucleus, HDAC5 binds to MEF2c via the HDAC-interaction domain and represses its activity (Lemerrier *et al.* 2000). During myogenesis, MEF2c

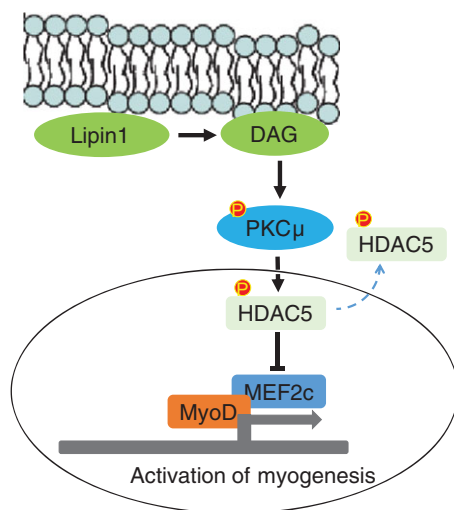


Figure 8. Lipin1 in SM differentiation

Lipin1 is suggested to play a critical role in SM development

repression is relieved in response to phosphorylation-induced translocation of HDAC5 to the cytoplasm. Therefore, lipin1 deficiency increases nuclear-localized HDAC5 and represses MEF2 expression. HDAC7 has also been reported to affect MEF2c transcriptional function (Dressel *et al.* 2001). Further studies will be conducted to examine the role of lipin1 on HDAC7 expression. A previous study also suggested that lipin1 could directly interact with MEF2c (Liu & Gerace, 2009). It is also possible that, in addition to activating MEF2c via the DAG/HDAC5 mediated pathway, lipin1 may also be able to activate MEF2c via its transcriptional regulation. The nuclear transcription regulation of MEF2c by lipin1 requires further investigation.

We also explored whether restoration of DAG by treating cells with PMA could rescue the myoblast differentiation defect in lipin1 deficient cells. We found that PMA treatment can activate PKC μ and HDAC5, in turn upregulating MEF2c expression. However, PKC μ and HDAC5 activation by PMA was rapid and transient, showing a robust activation at 30 min and returning to baseline by 24 h. PKC μ activation requires membrane recruitment by DAG and other PKC isoforms for its catalytic activity. PKC μ has to be phosphorylated by novel PKC isoforms within its activation loop at Ser744 and Ser748 to promote its activity (Brandlin *et al.* 2002; Yuan *et al.* 2002). The phosphorylated and activated PKC μ dissociates from the plasma membrane, translocates to the cytosol, and subsequently enters into the nucleus to activate HDAC5. The activation of PKC μ has been suggested to be sustained for a few hours once it leaves the membrane and is dependent on its phosphorylation status (Matthews *et al.* 2000). An ubiquitin E3 ligase, RINCK, has been reported to be able to bind the C1 domain of PKC and lead to the ubiquitination and degradation of PKCs (Chen *et al.* 2007). However, the detailed mechanisms that govern the termination of the cellular action of PKC μ , as well as how PKC μ is regulated spatially and temporally in biological systems, are largely unknown and remain to be determined.

In summary, we identified an essential role of lipin1 in regulating SM development via the activation of PKC, which regulates MyoD and MEF2c expression by manipulating HDAC5 nuclear export. Because patients with a lipin1 deficiency are associated with severe rhabdomyolysis and muscle atrophy in type 2 muscle fibres, the regulatory circuitry identified in the present study provides an insight into how the dysfunction of lipin1 causes muscle rhabdomyolysis.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

HR designed the experiments. RC provided Lipin1^{fl/fl} mice. AJ, DH and AA performed the research. AJ, DH, AA and HR analysed the data. HR wrote the manuscript. All authors read and approved the final version of the manuscript submitted for publication.

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