Serine/Threonine Kinase 40 (Stk40) Functions as a Novel Regulator of Skeletal Muscle Differentiation*

Received for publication, February 4, 2016, and in revised form, November 8, 2016. Published, JBC Papers in Press, November 29, 2016, DOI 10.1074/jbc.M116.719849

Ke He‡†, Jing Hu‡†, Hongyao Yu‡, Lina Wang‡, Fan Tang‡, Junjie Gu‡, Laixiang Ge‡, Hongye Wang§, Sheng Li§, Ping Hu§, and Ying Jin‡¶

From the‡ Laboratory of Molecular Developmental Biology, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai 200025, China, the¶ Key Laboratory of Stem Cell Biology, Chinese Academy of Sciences Center for Excellence in Molecu lar Cell Science, Institute of Health Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China, and the§ Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200032, China

Edited by John M. Denu

Skeletal muscle differentiation is a precisely coordinated process, and the molecular mechanism regulating the process remains incompletely understood. Here we report the identification of serine/threonine kinase 40 (Stk40) as a novel positive regulator of skeletal myoblast differentiation in culture and fetal skeletal muscle formation in vivo. We show that the expression level of Stk40 increases during skeletal muscle differentiation. Down-regulation and overexpression of Stk40 significantly decreases and increases myogenic differentiation of C2C12 myoblasts, respectively. In vivo, the number of myofibers and expression levels of myogenic markers are reduced in the fetal muscle of Stk40 knockout mice, indicating impaired fetal skeletal muscle formation. Mechanistically, Stk40 controls the protein level of histone deacetylase 5 (HDAC5) to maintain transcriptional activities of myocyte enhancer factor 2 (MEF2), a family of transcription factor important for skeletal myogenesis. Silencing of HDAC5 expression rescues the reduced myogenic gene expression caused by Stk40 deficiency. Together, our study reveals that Stk40 is required for fetal skeletal muscle development and provides molecular insights into the control of the HDAC5-MEF2 axis in skeletal myogenesis.

Skeletal muscle differentiation occurs in both normal muscle development and muscle regeneration in the postnatal period. Skeletal muscle development in the mouse initiates from embryonic day 8.5/9 (E8.5/9) to birth (~19 days), followed by further maturation for about 2–3 weeks after birth (1, 2). Satellite cells are responsible for the regeneration of adult skeletal muscle and undergo activation, proliferation, and terminal differentiation after injury (3). Terminal differentiation of muscle cells during muscle development and regeneration consists of several processes, including cell cycle exit of mononucleated myoblasts, myogenic gene expression, and fusion of myocytes to multinucleated myotubes (4, 5).

The major transcription factors modulating skeletal myogenesis are myogenic regulatory factors (MRFs), a family sharing a common basic helix-loop-helix domain. The members of the family, including MyoD, Myogenin, Myf5, and MRF4, can form heterodimers with E proteins to bind the E box sequence present in the regulatory region of skeletal muscle-specific genes (6–8). Notably, MyoD can convert non-muscle cells, such as mesenchymal stem cells of the C3H10T1/2 line, into myotubes (9). Another important family of transcription factors regulating skeletal myogenesis is myocyte enhancer factor 2 (MEF2), which works as the coactivator of the MRF family to activate myogenic gene expression (10–13). The family of MEF2 contains four members, MEF2A, B, C, and D, in vertebrates and has a common DNA-binding MCM1, agamous, deficiens, and serum-response factor (MADS)/MEF2 domain, forming homo- and heterodimers with coactivators and corepressors as well as its own family members (11). Muscle-specific knockout of Mef2c causes some defects in skeletal muscle development, including myofiber disarray and sarcomere disorganization (14). Mef2a-null mice show delayed muscle regeneration (15). Conditional triple knockout of Mef2a, c, and d in satellite cells impairs muscle regeneration (16). Interestingly, Mef2c is the direct target of the MRF and MEF2 families. Hence, MEF2C regulates its own expression during skeletal muscle development (17), consistent with the autoregulatory activity of Drosophila MEF2 (18).

Numerous coactivators and corepressors of MEF2 have been reported. Class IIa histone deacetylases (HDACs), including HDAC4, 5, 7, and 9, control muscle gene expression, acting as corepressors of MEF2. Among these, cellular localization and protein levels of HDAC5 are known to influence its repressive effect on the transcriptional activity of MEF2. HDAC5 shuttles between the nucleus and cytoplasm, depending on its phosphorylation at the conserved serine residues. Calcium/calmodulin-dependent protein kinase phosphorylates HDAC5 at Ser-259

* This study was supported by grants from the Ministry of Science and Technology of China (2016YFA0100100 and 2013CB966801), the National Natural Science Foundation (31330105 and 91419309), and the Chinese Academy of Sciences (XDB19020100). The authors declare that they have no conflicts of interest with the contents of this article.

† This article contains supplemental Table 1.

‡ Both authors contributed equally to this work.

§ To whom correspondence should be addressed: Laboratory of Molecular Developmental Biology, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Rd., Shanghai 200025, China. E-mail: jyin@sibs.ac.cn. Tel.: 86-21-54923342.

¶ The abbreviations used are: E, embryonic day; MEF, myocyte enhancer factor; HDAC, histone deacetylase; MyHC, myosin heavy chain; P, postnatal day; MCK, muscle creatine kinase; TA, tibialis anterior; RT-qPCR, quantitative RT-PCR.
and Ser-498, resulting in the nuclear export of HDAC5 and, in turn, relieving its repression on MEF2 (19–22). Moreover, HDAC5 can be ubiquitinated and degraded by the proteasome pathway in the nucleus of C2C12 cells. MEF2 activation decreases when HDAC5 protein levels increase because of the block of proteasomes (23), indicating that the nuclear protein level of HDAC5 negatively controls MEF2 transcriptional activity. However, the regulatory mechanism for the control of the HDAC5 protein level is not clearly understood.

Stk40, a putative serine/threonine kinase, can activate the Erk/MAPK pathway to induce mouse embryonic stem cell differentiation into the extraembryonic endoderm (24). Stk40 knockout mice suffer from immature lung development and neonatal lethality at birth (25). Besides, Stk40 represses adipogenesis through controlling the translation of CCAAT/enhancer binding proteins (C/EBP) proteins (26). Thus, the function of Stk40 is multifarious. Here we find that the expression of Stk40 is positively related to MEF2 transcriptional activities but inversely correlated to the levels of HDAC5. Concomitantly, Stk40 is required for skeletal myogenic differentiation both in vitro and in vivo. Therefore, our study sheds light on the regulatory mechanism for the HDAC5 protein level, as well as for the MEF2 transcriptional activity and myogenesis. In addition, the findings uncover an important function of Stk40 in skeletal muscle development.

## Results

### Stk40 Regulates Skeletal Myogenesis

#### Stk40 Expression Levels Increase during Skeletal Muscle Differentiation and Regeneration—

To learn about the involvement of Stk40 in skeletal myogenesis, we began with an examination of Stk40 expression patterns in both in vivo and in vitro models of skeletal muscle differentiation. First, we used the C2C12 myoblast line, a well-established in vitro model for studying skeletal muscle differentiation (27). Efficient myogenic differentiation of C2C12 myoblasts was demonstrated by the induction of myogenic transcription factors, including Myogenin and MEF2C, as well as their downstream target myosin heavy chain (MyHC) (Fig. 1A). Simultaneously, protein expression of Stk40 significantly increased after the induction of differentiation (Fig. 1B). However, the mRNA level of Stk40 increased slightly (Fig. 1C). Second, we examined the expression of Stk40 in developing skeletal muscle in vivo. In this regard, hind limb muscles at the fetal (E16.5 and E18.5), perinatal (postnatal day 2, P2), and adult (postnatal week 8, P8 week) stages were isolated...

---

**FIGURE 1.** Stk40 expression levels increase during skeletal muscle differentiation in vitro and in vivo. A, protein levels of Stk40, MyHC, Myogenin, and MEF2C at the indicated time points of C2C12 cell differentiation (Diff) were analyzed by Western blotting. α-Tubulin was used as a loading control. The mean gray values of Stk40 analyzed by ImageJ are listed below the Stk40 blot. B, fold change of the mean gray values of the protein levels of Stk40 on differentiation days 0 and 2. The mean gray values were analyzed by ImageJ. Data were normalized to the level of α-tubulin. Error bars represent S.D; Student’s t test; ***, p < 0.001. C, the mRNA level of Stk40 at the indicated time points of C2C12 cell differentiation was detected by RT-qPCR assays. Data were normalized to the level of GAPDH. Error bars represent S.D. D, hind limb muscles were isolated from E16.5, E18.5, P2, and P8 week embryos. Protein levels of Stk40, Myogenin, and MEF2C in the hind limb muscle at the indicated time point of muscle development were analyzed by Western blotting. GAPDH was used as a loading control. E, protein levels of Stk40 in different tissues and organs isolated from mouse embryos at E18.5 were analyzed by Western blotting. GAPDH was used as a loading control. F, protein levels of Stk40, Myogenin, and MEF2C in TA muscle treated with CTX for 0, 3, 5, 9, and 14 days, respectively, were analyzed by Western blotting. GAPDH was used as a loading control.
from C57BL/6 mice. Similar to Myogenin and MEF2C, the steady-state levels of Stk40 proteins were highest in the muscle from E16.5 embryos and gradually declined afterward (Fig. 1D). By P8 week, little Stk40 could be detected in the muscle. Nevertheless, Stk40 was not specifically expressed in the muscle at fetal stage. It was ubiquitously expressed in various tissues and organs at E18.5, including skeletal muscle tissues such as the tongue, diaphragm, and hind limb muscle as well as the kidney, lung, heart, thymus, and so on (Fig. 1E). In addition, the myogenic process also takes place during muscle regeneration after injury. Cardiotoxin (CTX) treatment induces the muscle degeneration and regeneration program (28). Interestingly, expression of Stk40 was also induced during this process, in a manner similar to that of MEF2C (Fig. 1F). These results indicate that the expression of Stk40 is developmentally regulated in the skeletal muscle and that it is potentially involved in skeletal muscle differentiation.

**Stk40 Positively Regulates Skeletal Myogenic Differentiation**—To study the function of Stk40 in myogenesis, we knocked down Stk40 in C2C12 myoblasts by specific shRNA delivered via retroviral plasmids. Two independent shRNA sequences were used (Stk40 shRNA-1 and shRNA-2), and expression of

![Image](image-url)
Stk40 Regulates Skeletal Myogenesis

Stk40 Controls Myogenesis through a Cell Cycle- and Cell Survival-independent Mechanism—Having shown that Stk40 deficiency led to attenuated myogenesis, we explored whether Stk40 could control the cell cycle or cell survival during myogenesis. To address this question, we compared the percentage of cells in the S phase between control and Stk40-deficient C2C12 cells, as the cell cycle exit occurs at the very beginning of myoblast differentiation (4). As shown in Fig. 4, A and B, there was no significant difference in the percentage of the S phase cells between control and Stk40-deficient cells, although a substantial reduction in the percentage of cells in the S phase was observed on differentiation day 1 for both control and Stk40-deficient cells. Moreover, mRNA levels of the cyclin-dependent kinase inhibitor p21 were comparable between the two groups (Fig. 4C). Furthermore, protein levels of cleaved Caspase-3, a marker of cell apoptosis, were similar in control and Stk40-deficient cells, although they increased significantly on day 1 of myogenic differentiation of C2C12 myoblasts for cells of both types (Fig. 4D). Therefore, the impaired myogenesis observed in Stk40-deficient C2C12 cells was not caused by an altered cell cycle or cell survival.

Stk40 Modulates the Level of HDAC5 Proteins during Myogenic Differentiation of C2C12 Myoblasts—To search for the molecular mechanism by which Stk40 regulates skeletal myogenesis, we investigated the regulatory role of Stk40 in the transcriptional activity of important factors modulating myogenesis. Interestingly, overexpression of Stk40 enhanced the luciferase activity of the MEF2-responsive gene reporter (3 × MEF2) (Fig. 5A), which contained three MEF2 binding sites upstream of a c-fos minimal promoter, suggesting that Stk40 might play a role in the control of MEF2 transcriptional activities.

To know how Stk40 positively modulated MEF2 activity, we examined the expression levels of HDAC5, as it is known that HDAC5 represses MEF2 activity (19). Compared with control C2C12 cells, Stk40-deficient cells had a higher level of HDAC5 proteins during myogenic differentiation (Fig. 5B). As expected, Stk40-deficient cells had reduced levels of the MEF2 downstream target genes MEF2C and MyHC (Figs. 2E and 5B), in line with the previous finding that higher levels of HDAC5 correspond to lower transcriptional activity of MEF2 (30). In contrast, overexpression of Stk40 reduced the protein levels of HDAC5, accompanied by enhanced protein levels of MEF2C and MyHC on day 2 of differentiation (Fig. 5C), further indicating a negative regulatory role of Stk40 for HDAC5 protein levels during myogenesis.

HDAC5 carries out its suppressive effect on MEF2 transcriptional activities in the nucleus (22, 30). Moreover, HDAC5 was reported to be phosphorylated and undergo nuclear export during myogenic differentiation (22). Therefore, we examined whether Stk40 could modulate HDAC5 protein levels in the nucleus during myogenic differentiation of C2C12 cells. Two independent approaches were applied: one was cytoplasmic and nuclear protein fractionation and another was immunofluorescence staining. First, the efficient isolation of cytoplasmic and nuclear proteins was verified by the correct distribution of cytoplasmic GAPDH and nuclear H3 proteins, respectively. In addition, Stk40 proteins were mainly detected in the nuclear...
extract, consistent with its nuclear location observed by confocal microscopy (Fig. 2F). We found that Stk40 knockdown increased, whereas Stk40 overexpression decreased, HDAC5 proteins in the nuclei, respectively (Fig. 5, D and E). Second, immunofluorescence staining showed that HDAC5 proteins mainly located in the cytoplasm on differentiation day 4 in control C2C12 cells. However, Stk40-deficient cells had evidently more HDAC5 proteins located in the nuclei than control C2C12 cells (Fig. 5F). The results from both approaches indicate that Stk40 negatively modulates HDAC5 protein levels in the nuclei. It is worth mentioning that the level of Ser-259 phosphorylated HDAC5 proteins, mostly located in the cytoplasm, was also higher in Stk40-deficient cells (Fig. 5D), suggesting that Stk40 might control the steady-state levels of HDAC5 proteins regardless of their subcellular localization.

**HDAC5 Is an Important Factor for Stk40-controlled Myogenesis**—To validate the involvement of HDAC5 in Stk40-regulated myogenesis, we examined the expression pattern of HDAC5 during C2C12 differentiation. Its protein level decreased along with myogenic differentiation (Fig. 6A), similar to the HDAC4 expression pattern reported previously (31). Functionally, overexpression of HDAC5 blocked myogenesis and attenuated the expression of MEF2C and MyHC on differentiation day 2 (Fig. 6A, B, and C), resembling the phenotype observed in Stk40-deficient cells. Importantly, knockdown of HDAC5 reverted the reduction in MyHC protein levels caused by Stk40 deficiency (Fig. 6D). Therefore, HDAC5 represses skeletal myogenesis and functions as an important player in Stk40-controlled myogenic differentiation. As a member of class IIa HDACs, HDAC4 has been reported to inhibit skeletal myogenesis by repressing MEF2 activity similarly as HDAC5 (22, 30, 31). We examined whether HDAC4 was also involved in Stk40-controlled skeletal myogenesis. HDAC4 displayed a similar expression pattern as HDAC5 during the myogenic differentiation of C2C12 cells (Fig. 6, A and E). Also, Stk40-deficient cells had a higher level of HDAC4 proteins during myogenic differentiation (Fig. 6F). Therefore, it seems that Stk40 regulates the protein levels of both HDAC4 and HDAC5, which might both be involved in Stk40-controlled myogenesis.

**Stk40 Is Required for Normal Fetal Skeletal Muscle Development—Stk40−/− mice died at birth, which prevented us from studying its role in adult myogenesis (25). To investigate the physiological function of Stk40 in skeletal myogenesis in vivo, we examined whether there existed some defects in fetal skeletal muscle development at E18.5. Histological immunostaining showed that the hind limb muscle tissue of Stk40−/− mice was smaller and had fewer myofibers at E18.5, as indicated by the reduced number of Laminin-positive cells, compared with that of wild-type mice (Fig. 7, A and B). The ratio of numbers of Laminin-positive cells to body weight was significantly decreased in Stk40−/− mice, which suggested a specific role of Stk40 in the control of myofiber number in development. However, we did not detect any remarkable changes in the muscle pattern (Fig. 7A). The size of each myofiber was comparable in Stk40+/+ and Stk40−/− mice (Fig. 7C), suggesting that the smaller muscle tissue mainly resulted from the fewer number of myofibers but not the smaller size of myofibers. In addition, we examined the expression level of muscle-specific markers in the hind limb muscle at E18.5. As shown in Fig. 7D, the level of the neonatal MyHC isoform was significantly decreased in Stk40−/− mice, which was the major MyHC isoform in the fetal muscle (32). Also, downstream targets of MEF2 such as α-skeletal actin (Acta1), muscle creatine kinase (MCK), and desmin were all down-regulated in Stk40−/− muscle. These results support the notion that Stk40 is required for normal fetal skeletal muscle development.

**Discussion**

In this study, we show that Stk40 can positively modulate skeletal muscle differentiation. Stk40 expression levels in-
creased during skeletal muscle differentiation, whereas they were down-regulated in myotubes, implying that Stk40 has a potential role in the differentiation process rather than in the maintenance or the survival of mature myotubes. Moreover, the loss-of-function and gain-of-function studies conducted in C2C12 cells suggest that Stk40 is essential for skeletal myogenesis in vitro. Stk40 had higher expression levels in developing fetal muscle and muscle tissues in regeneration after injury compared with normal adult muscle. The hind limb muscle tissue from Stk40/H11002/H11002/embryos at E18.5 was smaller than that from wild-type embryos because of the reduced number of myofibers, providing in vivo evidence for the essential role of Stk40 in normal skeletal muscle differentiation. We did not investigate the role of Stk40 in muscle regeneration because of the lack of viable adult Stk40−/− mice (25). Nevertheless, the increased expression level of Stk40 during muscle regeneration, together with the function of Stk40 in myogenesis of C2C12 myoblasts derived from satellite cells and in fetal muscle development, implies that Stk40 might play a role in muscle regeneration as well. Identification of Stk40 as a new regulator for skeletal muscle differentiation is important for understanding how mammalian myogenesis is controlled at the molecular level.

Skeletal muscle development and regeneration are important biological processes and regulated by multiple transcription factors (2, 33). However, how the transcriptional activities of these factors are regulated is poorly elucidated. We showed that Stk40 could control the transcriptional activity of MEF2, a family of transcription factors required for the activation of myogenic gene expression. First, Stk40 enhanced MEF2-spe-
specific reporter activities; second, Stk40 deficiency led to a remarkable reduction in protein levels of the MEF2 down-stream targets MEF2C and MyHC. Thus, Stk40 is required for the appropriate activity of MEF2. Interestingly, further study showed that Stk40-deficient C2C12 cells had higher levels of HDAC5 proteins in both whole-cell lysate and nuclear lysate during myogenic differentiation. Consistent with the previous report that nuclear HDAC5 proteins repressed MEF2 transcriptional activity, we found that overexpression of HDAC5 disrupted myogenesis substantially and attenuated the expression of MEF2C and MyHC during C2C12 cell differentiation. We propose that Stk40 positively regulates MEF2 activities through controlling the protein level of HDAC5 in the nucleus. Indeed, we found that down-regulation of Stk40 in C2C12 cells gave rise to higher protein levels of HDAC5, which, in turn, repressed the MEF2 activity and resulted in down-regulation of MEF2 target genes as well as attenuated myogenesis. Supporting this proposal, silencing of HDAC5 partially rescued the phenotype induced by Stk40 deficiency. Our data indicate that Stk40 modulates myogenesis, at least partially, through controlling the HDAC5-MEF2 axis.

Currently, we are not clear about how Stk40 regulates the protein level of HDAC5 at a posttranscriptional level, as it did not affect the transcript level of HDAC5 (data not shown). Several studies demonstrate that class IIA HDACs are posttranscriptionally controlled (34–36). HDAC5 is posttranscriptionally regulated by miR-2861 during osteoblast differentiation (37). For myogenic differentiation, miR-1, miR-206, and miR-29 target HDAC4 to promote myogenesis (31, 38). However, whether HDAC5 is regulated by microRNA in myogenesis has not yet been elucidated. Another reported posttranscriptional regulation for HDAC5 is ubiquitination. Exogenous HDAC5 proteins can be ubiquitinated and undergo degradation in C2C12 cells (23). In addition, previous studies showed that protein kinases such as calcium/calmodulin-dependent protein kinase could promote skeletal myogenesis and MEF2 transcriptional activity via phosphorylating HDAC5 to induce HDAC5 nuclear export (20, 30). As Stk40 is a putative protein kinase and could control HDAC5 protein levels in the nucleus, we hypothesized that Stk40 might regulate the myogenesis of C2C12 myoblasts and MEF2 activities through enhancing nuclear export of HDAC5 proteins. Unexpectedly, the phos-
phorylation level of HDAC5 was higher in Stk40-deficient cells than in control cells (Fig. 5D), which might be caused by the increased whole-cell protein level of HDAC5. The result excludes the possibility that Stk40 controls the levels of nuclear HDAC5 by modulating HDAC5 nuclear export. Further investigations are needed to understand the molecular mechanisms underpinning the regulatory function of Stk40 for HDAC5 proteins.

Class IIa HDAC members are involved in many physiological processes, such as the formation of slow-twitch myofibers, regulation of cardiac hypertrophy, fibrosis, and pathological remodeling as well as cardiovascular growth and function (23, 39, 40). They also share mechanisms in the control of development, such as skeletal myogenesis. Our study raises the possibility of involvement of Stk40 in these processes and provides new clues to explore HDAC inhibitors for therapeutic application of the related diseases.

**Experimental Procedures**

**Animals and Muscle Regeneration**—The muscle tissue was obtained from C57BL/6 mice. All animals were raised under the conditions described previously and handled according to the guidelines approved by the Shanghai Jiao Tong University School of Medicine (26). The genotype of Stk40+/+ and Stk40−/− mice was determined as described previously (26). For muscle injury, 100 μl of CTX (10 μM) was injected into the tibialis anterior (TA) muscle of 12-week-old C57BL/6 mice. The injected TA muscle was isolated at the indicated time point after treatment with CTX.

**Cell Culture and Differentiation**—C2C12 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% i.-glutamine. C3H10T1/2 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% i.-glutamine. For myogenic differentiation, after confluence, the medium of C2C12 or C3H10T1/2 cells was changed to DMEM supplemented with 2% horse serum, 1% penicillin/streptomycin and 1% i.-glutamine.

**Virus Package and Transduction**—For Stk40 knockdown assays, shRNAs specific to Stk40 were cloned into the pSIREN plasmid. Plasmids for HDAC5 knockdown assays were purchased from the GIPZ Lentiviral Mouse shRNA Library (GE Dharmacon). The shRNA interference sequence for HDAC5 was 5′-CCGGGAAGGCTCTACAGAA-3′. The shRNA interference sequences for Stk40 were 5′-GGACCCATCGGATACTAT-3′ and 5′-TGATACCCGAGTACTCTCT-3′. The shRNA interference sequence for control was 5′-GTGCCGCCTGCTGGTGCAAC-3′. For overexpression assays, Stk40-GFP cDNA and HDAC5 cDNA were cloned into the pMXS plasmid. Retroviral and lentiviral packaging and transduction were performed as described previously (25).

**RNA Extraction and RT-qPCR**—Whole-cell RNA was prepared using TRIzol (Invitrogen). 2 μg of total RNA was used to perform reverse transcription by a Fastquant reverse kit (Tiangen). Quantitative PCR was performed on the ABI 7900 using RT-qPCR assays, shRNAs specific to Stk40 were cloned into the pSIREN plasmid. Plasmids for HDAC5 knockdown assays were purchased from the GIPZ Lentiviral Mouse shRNA Library (GE Dharmacon). The shRNA interference sequence for HDAC5 was 5′-CCGGGAAGGCTCTACAGAA-3′. The shRNA interference sequences for Stk40 were 5′-GGACCCATCGGATACTAT-3′ and 5′-TGATACCCGAGTACTCTCT-3′. The shRNA interference sequence for control was 5′-GTGCCGCCTGCTGGTGCAAC-3′. For overexpression assays, Stk40-GFP cDNA and HDAC5 cDNA were cloned into the pMXS plasmid. Retroviral and lentiviral packaging and transduction were performed as described previously (25).

**Immunoblotting**—Total protein extract from C2C12 cells or muscle tissue was prepared with lysis buffer consisting of 2 mM EDTA, 0.5% Nonidet P-40, 50 mM Tris (pH 7.5), 150 mM NaCl, and 10% glycerol and quantified with the BCA kit (Pierce). Nuclear and cytoplasmic extraction reagents (Thermo) were used to isolate the nuclear and cytoplasmic proteins from C2C12 cells according to the recommendations of the manu-
Stk40 Regulates Skeletal Myogenesis

Biressi, S., Molinaro, M., and Cossu, G. (2007) Cellular heterogeneity during vertebrate skeletal muscle development. Dev. Biol. 308, 281–293

Tajbakhsh, S. (2009) Skeletal muscle stem cells in developmental versus regenerative myogenesis. J. Int. Med. 266, 372–389

Yin, H., Price, F., and Rudnicki, M. A. (2013) Satellite cells and the muscle stem cell niche. Physiol. Rev. 93, 23–67

Walsh, K., and Perelman, H. (1997) Cell cycle exit upon myogenic differentiation. Curr. Opin. Genet. Dev. 7, 597–602

Bailey, P., Holowacz, T., and Lassar, A. B. (2001) The origin of skeletal muscle stem cells in the embryo and the adult. Curr. Opin. Cell Biol. 13, 679–689

Rudnicki, M. A., and Jaenisch, R. (1995) The MyoD family of transcription factors and skeletal myogenesis. BioEssays 17, 203–209

Weintraub, H., Davis, R., Tappcott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., and Hollenberg, S. (1991) The myoD gene family: nodal point during specification of the muscle cell lineages. Science 251, 761–766

Massari, M. E., and Murre, C. (2000) Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol. Cell Biol. 20, 429–440

Davis, R. L., Weintraub, H., and Lassar, A. B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51, 987–1000

Braun, T., and Gautel, M. (2011) Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis. Nat. Rev. Mol. Cell Biol. 12, 349–361

Black, B. L., and Olson, E. N. (1998) Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu. Rev. Cell Dev. Biol. 14, 167–196

Bentzinger, C. F., Wang, Y. X., and Rudnicki, M. A. (2012) Building muscle: molecular regulation of myogenesis. Cold Spring Harb. Perspect. Biol. 4, a008342

Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1995) Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell 83, 1125–1136

Potthoff, M. J., Arnold, M. A., McAnally, J., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2007) Regulation of skeletal muscle sarcomere integrity and postnatal muscle function by Mef2c. Mol. Cell Biol. 27, 8143–8151

Snyder, C. M., Rice, A. L., Estrella, N. L., Held, A., Kandarian, S. C., and Naya, F. J. (2013) MEF2A regulates the Gli2-Dio3 microRNA megacluster to modulate WNT signaling in skeletal muscle regeneration. Development 140, 31–42

Liu, N., Nelson, B. R., Bezprozvannaya, S., Shethon, J. M., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2014) Requirement of MEF2A, C, and D for skeletal muscle regeneration. Proc. Natl. Acad. Sci. U.S.A. 111, 4109–4114

Wang, D. Z., Valdez, M. R., McAnally, J., Richardson, J., and Olson, E. N. (2001) The Mef2c gene is a direct transcriptional target of myogenic bHLH and MEF2 proteins during skeletal muscle development. Development 128, 4623–4633

Cripps, R. M., Lovato, T. L., and Olson, E. N. (2004) Positive autoregulation of the Myoeyc enhancer factor-2 myogenic control gene during somatic muscle development in Drosophila. Developmental Biology 267, 536–547

Mckinsey, T. A., Zhang, C. L., and Olson, E. N. (2001) Control of muscle development by driving HATs and HDACs. Curr. Opin. Genet. Dev. 11, 497–504

Mckinsey, T. A., Zhang, C. L., and Olson, E. N. (2000) Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14–3–3 to histone deacetylase 5. Proc. Natl. Acad. Sci. U.S.A. 97, 14400–14405

Mckinsey, T. A., Zhang, C. L., and Olson, E. N. (2002) MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends Biochem. Sci. 27, 40–47

Mckinsey, T. A., Zhang, C. L., Lu, J., and Olson, E. N. (2000) Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. Nature 408, 106–111

Potthoff, M. J., Wu, H., Arnold, M. A., Sheltton, J. M., Backs, J., McAnally, J., Richardson, J. A., Bassel-Duby, R., and Olson, E. N. (2007) Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers. J. Clin. Invest. 117, 2459–2467

Li, L., Sun, L., Gao, F., Jiang, J., Yang, Y., Li, C., Gu, J., Wei, Z., Yang, A., Lu, R., Ma, Y., Tang, F., Kwon, S. W., Zhao, Y., Li, J., and Jin, Y. (2010) Stk40 links the pluripotency factor Oct4 to the Erk/Mapk pathway and controls extraembryonic endoderm differentiation. Proc. Natl. Acad. Sci. U.S.A. 107, 1402–1407

Yu, H., He, K., Li, L., Sun, L., Tang, F., Li, R., Ning, W., and Jin, Y. (2013) Deletion of Stk40 protein in mice causes respiratory failure and death at birth. J. Biol. Chem. 288, 5342–5352

Yu, H., He, K., Wang, L., Hu, J., Gu, J., Zhou, C., Lu, R., and Jin, Y. (2015) Stk40 represses adipogenesis through translational control of CCAAT enhancer-binding proteins. J. Cell Sci. 128, 2881–2890

Yaffe, D., and Saxel, O. (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 270, 725–727

Acknowledgments—We thank Dr. Guang Ning for providing cell lines (C3H10T1/2) and Dr. Xin Fu for technical support.
28. Chargé, S. B., and Rudnicki, M. A. (2004) Cellular and molecular regulation of muscle regeneration. *Physiol. Rev.* **84**, 209–238
29. Jeong, H., Bae, S., An, S. Y., Byun, M. R., Hwang, J. H., Yaffe, M. B., Hong, J. H., and Hwang, E. S. (2010) TAZ as a novel enhancer of MyoD-mediated myogenic differentiation. *FASEB J.* **24**, 3310–3320
30. Lu, J., McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2000) Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol. Cell* **6**, 233–244
31. Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L., and Wang, D. Z. (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat. Genet.* **38**, 228–233
32. Lyons, G. E., Ontell, M., Cox, R., Sassoon, D., and Buckingham, M. (1990) The expression of myosin genes in developing skeletal muscle in the mouse embryo. *J. Cell Biol.* **111**, 1465–1476
33. Schienda, J., Engleka, K. A., Jun, S., Hansen, M. S., Epstein, J. A., Tabin, C. J., Kunkel, L. M., and Kardon, G. (2006) Somitic origin of limb muscle satellite and side population cells. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 945–950
34. Weeks, K. L., and Avkiran, M. (2015) Roles and post-translational regulation of cardiac class Ila histone deacetylase isoforms. *J. Physiol.* **593**, 1785–1797
35. Wang, Z., Qin, G., and Zhao, T. C. (2014) HDAC4: mechanism of regulation and biological functions. *Epigenomics* **6**, 139–150
36. Swierczynski, S., Klieser, E., Illig, R., Alinger-Scharinger, B., Kiesslich, T., and Neureiter, D. (2015) Histone deacetylation meets miRNA: epigenetics and post-transcriptional regulation in cancer and chronic diseases. *Expert Opin. Biol. Ther.* **15**, 651–664
37. Li, H., Xie, H., Liu, W., Hu, R., Huang, B., Tan, Y. F., Xu, K., Sheng, Z. F., Zhou, H. D., Wu, X. P., and Luo, X. H. (2009) A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. *J. Clin. Invest.* **119**, 3666–3677
38. Winbanks, C. E., Wang, B., Beyer, C., Koh, P., White, L., Kantharidis, P., and Gregorevic, P. (2011) TGF- regulates miR-206 and miR-29 to control myogenic differentiation through regulation of HDAC4. *J. Biol. Chem.* **286**, 13805–13814
39. Haberland, M., Montgomery, R. L., and Olson, E. N. (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat. Rev. Genet.* **10**, 32–42
40. Chang, S., McKinsey, T. A., Zhang, C. L., Richardson, J. A., Hill, J. A., and Olson, E. N. (2004) Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. *Mol. Cell Biol.* **24**, 8467–8476