Control of MEF2 Transcriptional Activity by Coordinated Phosphorylation and Sumoylation*

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A eukaryotic protein is often subject to regulation by multiple modifications like phosphorylation, acetylation, ubiquitination, and sumoylation. How these modifications are coordinated in vivo is an important issue that is poorly understood but is relevant to many biological processes. We recently showed that human MEF2D (myocyte enhancer factor 2D) is sumoylated on Lys-439. Adjacent to the sumoylation motif is Ser-444, which like Lys-439 is highly conserved among MEF2 proteins from diverse species. Here we presented several lines of evidence to demonstrate that Ser-444 of MEF2D is required for sumoylation of Lys-439. Histone deacetylase 4 (HDAC4) stimulated this modification by acting through Ser-444. In addition, phosphorylation of Ser-444 by Cdk5, a cyclin-dependent kinase known to inhibit MEF2 transcriptional activity, stimulated sumoylation. Opposing the actions of HDAC4 and Cdk5, calcineurin (also known as protein phosphatase 2B) dephosphorylated Ser-444 and inhibited sumoylation of Lys-439. This phosphatase, however, exerted minimal effects on the phosphorylation catalyzed by ERK5, an extracellular signal-regulated kinase known to activate MEF2D. These results identified an essential role for Ser-444 in MEF2D sumoylation and revealed a novel mechanism by which calcineurin selectively “edits” phosphorylation at different sites, thereby reiterating that interplay between different modifications represents a general mechanism for coordinated regulation of eukaryotic protein functions in vivo.

In higher eukaryotes, each cell type has a unique gene expression pattern that is ultimately determined by a specific network of transcription factors. The question how cell signaling regulates activities of transcription factors is thus of central importance to many biological processes. The MEF2 family of transcription factors comprises four members in mammals, MEF2A, -B, -C, and -D. They were originally identified as major transcriptional activators for muscle differentiation. The MEF2 family proteins also have important roles in non-muscle cells by regulating programs. At the molecular level, MEF2 is composed of a highly conserved N-terminal domain responsible for DNA recognition and a C-terminal domain with trans-acting function. Regulation of MEF2 function is complex and occurs at multiple levels, including tissue-specific expression (1, 5), alternative pre-mRNA splicing (1, 17–19), caspase cleavage (20, 21), modulation of DNA-binding affinity (22–24), and association with transcriptional coregulators (25–29). There is also evidence suggesting the involvement of cytoplasmic sequestration (30). In addition, covalent modifications such as phosphorylation (see below), acetylation (31), and sumoylation (32) are important for regulating MEF2 function.

Phosphorylation of MEF2 occurs at multiple sites (18, 33). Casein kinase II and protein kinase A phosphorylate the DNA-binding domain of MEF2 and modulate its DNA-binding affinity (22, 24). Three proline-directed kinases, on the other hand, phosphorylate the trans-acting domain. The mitogen-activated protein kinase p38 specifically phosphorylates MEF2A and MEF2C and enhances their transcriptional activity (2, 34, 35). Similarly, extracellular signal-regulated kinase 5 (ERK5) phosphorylates MEF2A, -C, and -D to up-regulate transcription (36–39). Paradoxically, calcineurin (also known as protein phosphatase 2B or PP2B) stimulates MEF2-dependent transcription (8, 40, 41). Although the underlying mechanism remains to be determined, one possibility is that some phosphorylation events inhibit the activity of MEF2, and calcineurin blocks this inhibition. Related to this, cyclin-dependent kinase 5 (Cdk5) phosphorylates Ser-444 of human MEF2D and reduces its transcriptional potential during neurotoxicity-induced apoptosis (17, 42). Most intriguingly, just four residues away from Ser-444 in Lys-439, whose sumoylation also serves as an inhibitory mechanism (32, 43). Both residues are highly conserved among different MEF2 proteins and form a portable repression domain (17, 19, 32). Therefore, an interesting question to be addressed is how modifications of Lys-439 and Ser-444 are coordinated for the regulation of MEF2 function in vivo.

Here we show that Ser-444 is required for sumoylation of Lys-439. By phosphorylating Ser-444, Cdk5 stimulated the sumoylation. Ser-444 was also found to be crucial for HDAC4 to stimulate the sumoylation. Moreover, HDAC4 promoted phosphorylation of MEF2. Opposing the
actions of Cdk5 and HDAC4, calcineurin selectively edited phosphorylation of MEF2D to block its sumoylation. We thus conclude that phosphorylation of Ser-444 interplays with sumoylation of Lys-439 to regulate transcriptional and myogenic activities of MEF2 in a coordinated fashion.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK293 and C3H10T1/2 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), penicillin (Invitrogen), and streptomycin.

**Plasmid Constructs**—Expression plasmids for FLAG- or HA-tagged human MEF2D proteins have been described previously (32). Point mutants were generated by PCR with Expand thermotable DNA polymerase (Roche Applied Science) and subcloned into derivatives of pCDNA3.1 (Invitrogen). Mutations were verified by automatic DNA sequencing. Expression plasmids for Cdk5 and p35 were kindly provided by L. H. Tsai (see Ref. 44), and a GSK3β expression construct was a gift from J. R. Woodgett (see Ref. 45). Expression plasmids for HA-tagged wild-type and dominant-negative calcineurin (46), untagged constitutively active calcineurin (46), HA-SUMO proteins (47, 48), Ub9 (49), FLAG-ERK5 (36), HA-MEK5(D) (36), and HDAC proteins (32, 50–52) have been described.

**In Vivo Sumoylation Assays**—For analysis of MEF2 sumoylation in vivo, HEK293 cells were lysed in buffer S (15 mM Tris-HCl, pH 6.7, 0.5% SDS, 3% glycerol, 0.8× PBS, 4% Nonidet P-40, 0.1% mercaptoethanol, 25 mM N-ethylmaleimide, and protease inhibitors) and processed as described (32, 53).

**Reconstituted Sumoylation Assays**—MEF2D and its point mutants K439R, S444A, and S444E were produced in the presence of [35S]methionine by use of the Tnt® T7 coupled reticulocyte lysate system (Promega). 2 μl of [35S]-labeled MEF2D or its point mutant was mixed with 0.15 μg of purified human SaeI/SaeII, 1 μg of purified human Ubc9, and 1 μg of purified human SUMO1 or SUMO3 (LAe Biotech) in the in vitro sumoylation buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM dithiothreitol, and 2.5 mM ATP). The mixture was incubated at 37 °C for 1 h. To determine the potential SUMO E3 ligase activity of HDAC4, 1.5 or 3 μl of unlabeled in vitro translated HDAC4 protein was added to the sumoylation reactions. Alternatively, the HDAC4 protein was replaced with that expressed in and purified from S99 cells (50). Sumoylation reactions were stopped with an SDS sample buffer for subsequent SDS-PAGE and autoradiography.

**Alkaline Phosphatase Treatment**—The FLAG-MEF2D expression plasmid was transfected into 4 × 10⁵ HEK293 cells (in a 10-cm dish) with or without the expression plasmid for HA-HDAC4. 48 h after transfection, cells were lysed in 0.5 ml of buffer S. Soluble extracts were used for affinity purification on M2-agarose (Sigma). Purified proteins (8 μl in buffer S) were incubated with 10 units of calf intestinal alkaline phosphatase (Roche Applied Science) in a total volume of 20 μl of the dephosphorylation buffer (50 mM Tris-HCl, pH 8.5, and 1 mM EDTA) at 37 °C for 1 h. The reactions were stopped by addition of an SDS sample buffer for subsequent SDS-PAGE and Western blotting.

**MEF2D Dephosphorylation by Calcineurin in Vitro**—FLAG-MEF2D (5 μl), expressed in and affinity-purified from HEK293 cells as above, was incubated with 12.5 units of bovine calcineurin A/B subunits (Sigma) in 20 μl of the reaction buffer (50 mM HEPES, pH 7.4, 0.5 mM EDTA, 3 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, and 1 μg/ml leupeptin) at 30 °C for 1 h. As specified, 0.3 μg of calmodulin (Sigma) and/or 0.1 mM CaCl₂ was added. Reaction mixtures were stopped for separation by SDS-PAGE, followed by Western blotting analysis with anti-FLAG and anti-phospho-Ser-444 MEF2D antibodies. The phospho-specific antibody has been described (42).

**LiCl Treatment**—To analyze effects of LiCl on MEF2 phosphorylation, HEK293 cells were transfected with expression plasmids as specified in the figure legends. 48 h post-transfection, HEK293 cells were incubated with regular media containing 20 mM LiCl for 6 h and then lysed in buffer K (20 mM sodium phosphate, pH 7.0, 150 mM KCl, 30 mM sodium pyrophosphate, 0.1% Nonidet P-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na₃VO₄, 1 mM phenethylsulfonyl fluoride, and protease inhibitors) supplemented with 20 mM LiCl. Soluble extracts were used for immunoprecipitation with an anti-FLAG M2-agarose and immunoblotting with anti-FLAG antibody.

**Cyclosporin A Treatment**—For analysis of effects of cyclosporin A on MEF2 sumoylation, HEK293 cells were transfected with expression plasmids as specified in the figure legends. 24 h post-transfection, HEK293 cells were treated with 3.4 or 5 μM cyclosporin A for 24 h and lysed in buffer S for extract preparation. Extracts were used for immunoprecipitation with anti-MEF2D antibody conjugated to protein A-agarose or anti-FLAG M2-agarose, followed by immunoblotting with anti-SUMO2 and anti-MEF2D antibodies.

**Immunoprecipitation**—For analysis of interaction of calcineurin with MEF2D and class Ia HDACs, an expression plasmid for HA-tagged calcineurin was transfected into HEK293 cells along with constructs expressing FLAG-tagged MEF2D or class Ia HDACs. 48 h post-transfection, cells were lysed in buffer K for extract preparation and affinity purification on M2-agarose. Purified proteins were separated by SDS-PAGE and detected by immunoblotting with anti-HA and anti-FLAG antibodies.

**Reporter Gene Assays**—The assays were performed with the luciferase reporter Gal4-tk-luc, which contains five tandem copies of the Gal4-responsive element to drive the expression of the luciferase gene (50, 54). Briefly, 100–200 ng of this reporter was transfected into 4 × 10⁴ HEK293 cells (in a well of a 12-well plate) using SuperFect transfection reagent (2 μl; Qiagen), along with mammalian expression plasmids for wild-type and mutant Gal4-MEF2 fusion proteins, Ubc9, HDAC4, and/or calcineurin as specified in the figure legends. As an internal control for normalization of transfection efficiency, the β-galactosidase expression plasmid CMV-β-Gal (50 ng) was cotransfected. pBlueScript KSII(−) was used to supplement the plasmid mixture so that its total amount in each transfection was 1.5 μg. After 48 h, cells were lysed in situ at room temperature, and luciferase reporter activities were determined by using d-(−)-luciferin (Roche Applied Science) as the substrate. β-Galactosidase activities were determined with Galacto-Light Plus (Tropix) as the substrate. The chemiluminescence from activated luciferin or Galacto-Light Plus was measured on a luminometer plate reader (Dynex). Each transfection was performed at least three times.

**Myogenesis Assays**—C3H10T1/2 cells were seeded at 5 × 10⁴ cells per well on glass coverslips in 12-well plates and transfected with expression plasmids as specified. Coverslips were pretreated by incubation with 0.01% polylysine (Sigma) at 37 °C in a CO₂ incubator for 30 min and washed once with PBS. 48 h post-transfection, cells were washed with PBS and fed with the differentiation medium (2% horse serum, penicillin, and streptomycin). On day 7, coverslips were processed for immunofluorescence microscopy with anti-myosin heavy chain MF-20 antibody (Developmental Studies Hybridoma Bank, Iowa) to detect differentiated cells. A green fluorescent protein expression plasmid was cotransfected to normalize transfection efficiency.
RESULTS

Critical Role of Ser-444 in Sumoylation of MEF2D—We postulated that Ser-444 of MEF2D regulates sumoylation of Lys-439 (32). To test this hypothesis, Ser-444 was substituted with alanine to engineer the point mutant S444A, which was then subjected to in vivo sumoylation assays. For the assays, expression plasmids for FLAG-tagged wild-type MEF2D, K439R, and S444A were transfected into HEK293 cells along with an HA-SUMO2 expression construct. Cell extracts were prepared for affinity purification on anti-FLAG M2-agarose, and bound proteins were eluted with FLAG peptide and analyzed by Western blotting (WB) with the indicated antibodies. An asterisk marks non-specific bands (~66 kDa) because of a specific lot of horse serum used for Western blotting (A, lanes 1–3). D and E, wild-type MEF2D and point mutants were labeled with [35S]methionine and subjected to in vitro sumoylation assays with SUMO3. Sumoylation was analyzed by SDS-PAGE and autoradiography. Although SUMO2 and SUMO3 are preferred donors of its potential E3 ligase activity, FLAG-HDAC4 (1.5 and 3 μl), translated in vitro with nonradioactive methionine, was added to sumoylation reactions for wild-type MEF2D (D, lanes 4 and 5). Western blotting with anti-FLAG antibody confirmed the expression of FLAG-HDAC4 (D, lanes 4 and 5, bottom). FLAG-HDAC4, expressed in and purified from SF9 cells, yielded similar results (data not shown). S-MEF2D, sumoylated MEF2D.

MEF2 Regulation by Phosphorylation and Sumoylation

FIGURE 1. Requirement of Ser-444 for MEF2D sumoylation. A–C, expression plasmids for indicated proteins were transfected into HEK293 cells with (A and C) or without (B) a construct expressing HA-SUMO2. Extracts were prepared in buffer S for immunoprecipitation (IP) on M2-agarose, and bound proteins were eluted with FLAG peptide and analyzed by Western blotting (WB) with the indicated antibodies. An asterisk marks non-specific bands (~66 kDa) because of a specific lot of horse serum used for Western blotting (A, lanes 1–3). D and E, wild-type MEF2D and point mutants were labeled with [35S]methionine and subjected to in vitro sumoylation assays with SUMO3. Sumoylation was analyzed by SDS-PAGE and autoradiography. Although SUMO2 and SUMO3 are preferred donors of its potential E3 ligase activity, FLAG-HDAC4 (1.5 and 3 μl), translated in vitro with nonradioactive methionine, was added to sumoylation reactions for wild-type MEF2D (D, lanes 4 and 5). Western blotting with anti-FLAG antibody confirmed the expression of FLAG-HDAC4 (D, lanes 4 and 5, bottom). FLAG-HDAC4, expressed in and purified from SF9 cells, yielded similar results (data not shown). S-MEF2D, sumoylated MEF2D.
targets a subgroup of Cdk5 including Cdk5 (55), decreased the sumoylation level (Fig. 2C), suggesting the involvement of Cdk5 and/or related Cdk5. Ser-444 of MEF2D and the equivalent serine of MEF2C are known to be phosphorylated in vivo (17, 42). Moreover, Cdk5 is a major kinase to phosphorylate Ser-444 of MEF2D (42). To verify this, we performed Western blotting with an anti-phospho-Ser-444 antibody (42). As shown in Fig. 2D, Cdk5 specifically phosphorylated Ser-444. Moreover, this kinase had minimal effects on sumoylation of mutant S444E (Fig. 2A, lane 6, and Fig. 1C). These results indicate that through Ser-444, Cdk5 positively regulates MEF2D sumoylation.

Role of Ser-444 in HDAC4-stimulated MEF2 Sumoylation—Two recent studies showed that HDAC4 potentiates MEF2 sumoylation in vitro (32, 43), but the underlying mechanisms remain unclear. One possibility is that HDAC4 acts as a SUMO E3 ligase. To address this, we asked whether HDAC4 stimulates MEF2D sumoylation. As shown in Fig. 1D (lanes 4 and 5), HDAC4 failed to promote SUMO addition to MEF2D in vitro, indicating that HDAC4 may not be an authentic E3 ligase for MEF2D sumoylation.

Given the importance of Ser-444 in MEF2D sumoylation (Figs.1 and 2), we asked whether HDAC4 acts through this residue to promote sumoylation (32). To test this, we determined how HDAC4 affects sumoylation of mutants S444A and S444E. As reported (32), HDAC4 stimulated sumoylation of MEF2D in vivo (Fig. 3A, lanes 1, 2, 7, and 8). However, HDAC4 did not alter the sumoylation status of mutant S444A (Fig. 3A, lanes 3, 4, 9, and 10) and only slightly stimulated sumoylation of mutant S444E (lanes 5, 6, 11, and 12). These results indicate that Ser-444 is critical for HDAC4 to stimulate MEF2 sumoylation.

**Stimulation of MEF2D Phosphorylation by HDAC4**—As noted previously (32), HDAC4 caused migration shifts of MEF2D (Fig. 3, A and B). To determine whether the shifts are due to phosphorylation (32), we treated affinity-purified MEF2D with an alkaline phosphatase. This treatment eliminated the slowly migrating species (Fig. 3B), indicating that the shifts are indeed due to phosphorylation. HDAC4 induced similar shifts on mutant S444A (Fig. 3A, lanes 3, 4, 9, and 10) and only slightly stimulated sumoylation of mutant S444E (lanes 5, 6, 11, and 12). These results further support that HDAC4 potentiates sumoylation via promoting phosphorylation.

Next we investigated how HDAC4 induces phosphorylation and subsequent sumoylation of MEF2D. One possibility is that HDAC4 associates with a kinase(s) to target MEF2D. Because Ser-444 is critical for HDAC4 to potentiates MEF2D sumoylation (Fig. 3A), we first consid-
ered Cdk5, which is able to phosphorylate Ser-444 (Fig. 2D) (42) and stimulate Lys-439 sumoylation (Fig. 2, A–C). As shown in Fig. 2E, HDAC4 weakly interacted with Cdk5. Consistent with this, roscovitine treatment reduced the ability of HDAC4 to stimulate MEF2 sumoylation (Fig. 2F). We also analyzed how HDAC4 affects Ser-444 phosphorylation. As shown in Fig. 2G, HDAC4 stimulated this modification, and roscovitine inhibited the stimulation (lanes 1–3). Most interestingly, this inhibitor had minimal effects on the migration shifts of MEF2D (Fig. 2G, lane 3, bottom), supporting the involvement of additional phosphorylation events. Together, these results suggest that HDAC4 recruits Cdk5 to phosphorylate Ser-444 and promote Lys-439 sumoylation.

Because Ser-444 is dispensable for MEF2 mobility shifts induced by HDAC4 (Fig. 3A, lanes 3, 4, 9, and 10), we wondered whether HDAC4 recruits an additional kinase. This kinase might phosphorylate MEF2D at sites close to Lys-439, so we inspected the surrounding sequence for potential phosphorylation motifs. Two serine residues (Ser-440 and Ser-446) match the consensus site of GSK3β/H9252 (45). To investigate whether this kinase phosphorylates MEF2D, we utilized LiCl, a general GSK3β inhibitor (45). As shown in Fig. 3C, treatment with LiCl blocked
the migration shifts induced by HDAC4. Substitution of Ser-440 or Ser-446 with alanine neither affected sumoylation of MEF2D nor altered the migration shifts induced by HDAC4 (data not shown). Other preliminary data also suggest that GSK3β/H9252 may not be involved. Related to this, it was reported that a novel Li1/sensitive kinase phosphorylates MEF2D (56). Together, these results suggest that HDAC4 recruits Cdk5, as well as an unknown Li1-sensitive kinase, to phosphorylate MEF2D and stimulate sumoylation.

Negative Regulation of MEF2D Sumoylation by Calcineurin—HDAC4 may also target phosphatases to affect MEF2 phosphorylation. Calcineurin (or PP2B) is known to activate MEF2-dependent transcription (8, 40, 41), so it was first considered as a logical candidate. It may counteract the inhibitory phosphorylation induced by HDAC4. To test this, we transfected FLAG-MEF2D and HA-HDAC4 expression constructs into HEK293 cells along with those for constitutively active and dominant-negative forms of calcineurin (referred to as ca- and dn-CaN, respectively). Extracts were prepared for affinity purification on M2-agarose and immunoblotting with anti-FLAG antibody. As shown in Fig. 3D, ca-CaN but not dn-CaN abrogated the migration shifts induced by HDAC4, indicating that the shifts are due to serine/threonine phosphorylation. Because Ser-444 is dispensable for the shifts, we directly tested whether calcineurin targets this residue. As shown in Fig. 2, G (lanes 4–6) and H, calcineurin could reverse Ser-444 phosphorylation.

Activated by MEK5, ERK5 phosphorylates MEF2D and potentiates its transcriptional activity (36–39), so this modification activates MEF2D-dependent transcription. Most interestingly, calcineurin exerted minimal effects on the shifts caused by ERK5 and MEK5 (Fig. 3E). Moreover, these shifts are different from those induced by HDAC4 (Fig. 3E, compare lanes 1 and 3), raising the interesting possibility that different shifts represent distinct phosphorylation events. These findings thus reveal an unexpected ability of calcineurin to “edit” the phosphorylation status of MEF2 by selectively removing inhibitory phosphate groups.

We next investigated whether calcineurin modulates MEF2 sumoylation. For this, in vivo sumoylation assays were performed. As shown in Fig. 3F, ca-CaN abolished sumoylation of MEF2D, whereas dn-CaN slightly increased the modification. The positive effect of dn-CaN suggests that endogenous calcineurin may regulate sumoylation. To confirm this, HEK293 cells were treated with cyclosporin A, a known inhibitor of calcineurin. Endogenous MEF2D was immunoprecipitated with anti-MEF2D antibody, and its sumoylation status was determined by immunoblotting with anti-SUMO2 antibody. As illustrated in Fig. 3G, cyclosporin A treatment increased the sumoylated pool of endogenous MEF2D. Thus, calcineurin is involved in regulating MEF2D sumoylation.

Then we analyzed how calcineurin affects sumoylation in the presence of exogenous HDAC4. Under such conditions, ca-CaN blocked MEF2D sumoylation, whereas expression of dn-CaN or cyclosporin A treatment potentiated the stimulatory effect of HDAC4 on MEF2D sumoylation (Fig. 3, H and I). These results indicate that calcineurin counteracts HDAC4-mediated phosphorylation of MEF2D and inhibits its sumoylation.

Interaction of Calcineurin with MEF2D and HDAC4—To gain further insight into how calcineurin regulates phosphorylation and sumoylation of MEF2D, we investigated whether they interact with each other. As reported (46), interaction of MEF2D with calcineurin was barely detectable (Fig. 4A). By contrast, the interaction of calcineurin with HDAC4 could be easily detected (Fig. 4B). To map which region of HDAC4 mediates the interaction, we analyzed mutants 1–666 and 621–1084, corresponding to the N-terminal and C-terminal fragments...
of HDAC4, respectively (50). As shown in Fig. 4C, mutant 621–1084 interacted with calcineurin more strongly than mutant 1–666. We also analyzed other class IIa HDACs (57). Like HDAC4, HDAC5, HDAC7, and the MEF2-interacting transcription repressor MITR (an HDAC9 isoform) associated with calcineurin (Fig. 4D). These findings suggest that calcineurin regulates MEF2 sumoylation by directly targeting HDAC4 and homologs.

Role of Ser-444 in Mediating Effects of Sumoylation and Calcineurin on Transcriptional and Myogenic Activities of MEF2D—Both Lys-439 sumoylation and Ser-444 phosphorylation are known to inhibit the transcriptional activity of MEF2 (17, 32, 42, 43), but their potential interplay at the functional level has not been investigated. To address this, we first determined how Ubc9 regulates transcriptional activities of wild-type and mutant MEF2D. To avoid interference and complication of endogenous MEF2 proteins, MEF2D and mutants were expressed as proteins fused to the DNA-binding domain of the yeast transcription factor Gal4. Expression levels of these fusion proteins were similar (Fig. 5A). Consistent with our previous findings (32), mutant K439R was more active than wild-type MEF2D, and Ubc9 reduced the transcriptional activity of wild-type MEF2D but not that of mutant K439R (Fig. 5B), confirming that Ubc9 acts through Lys-439. In the assays, mutant S444A behaved just like K439R. Although mutant S444A possessed an intact sumoylation site, Ubc9 had minimal effects on its transcriptional activity (Fig. 5B). This finding is consistent with the conclusion that mutant S444A is resistant to SUMO modification (Fig. 1).

We then analyzed the functional effects of Cdk5. As reported (17, 42), this kinase inhibited the transcriptional activity of MEF2D (Fig. 5C). By contrast, it had minimal effects on the activity of mutant K439R, S444A, or S444E, suggesting that this kinase acts sequentially through Ser-444 and Lys-439. Consistent with this suggestion, phosphorylation of Ser-444 by Cdk5 facilitated sumoylation of Lys-439 (Fig. 2, A–C). The double mutant K439R/S444A exhibited a similar level of activity as the corresponding single mutants (Fig. 5C), suggesting that the two modifications function in the same pathway. Compared with others, mutant S444E was much less active (Fig. 5C), further supporting that phosphorylation of Ser-444 inhibits the activity of MEF2D.

We also assessed how calcineurin affects transcriptional activities of MEF2D and mutants. As shown in Fig. 5D, this phosphatase activated wild-type MEF2D to a level similar to those of the point mutants but had minimal effects on the mutants, indicating that under the assay conditions this phosphatase acts primarily through Ser-444 and Lys-439 to activate MEF2D.

To substantiate these results from reporter gene assays, we utilized MyoD-dependent myogenic conversion assays to compare myogenic activities of MEF2D and its mutants (58). For this, pluripotent C3H10T1/2 cells were cotransfected with a MyoD expression construct along with expression plasmids for MEF2D or its mutants. As reported (32, 43), wild-type MEF2D stimulated the myogenic potential of MyoD, and mutant K439R was more effective in this stimulation (Fig. 6). Consistent with the data from reporter gene assays (Fig. 5), mutant S444A was as active as K439R. The S444E mutation reduced the ability of MEF2D to potentiate myogenic conversion (Fig. 6). Calcineurin stimulated the myogenic activity of wild-type MEF2D but not that of S444A (Fig. 6). Together, these assays provide functional data to support that Ser-444 phosphorylation stimulates Lys-439 sumoylation to control the transcriptional and myogenic activities of MEF2D.

**DISCUSSION**

Ser-444 of MEF2D Is Essential for Lys-439 Sumoylation—MEF2D is sumoylated at a highly conserved motif (32). Adjacent to this motif is

![FIGURE 5. Role of Ser-444 in MEF2D-dependent transcription.](image-url)
Thus, this serine residue plays a leading role in controlling sumoylation. In vivo, Ubc9; (iii) promoting the transfer of SUMO from Ubc9 to the substrate; (ii) interacting with the SUMO-conjugating enzyme fulfill the first three requirements as it binds to MEF2, localizes with the substrate; and (iv) promoting this SUMO transfer to the substrate. One possibility is that it is not the hydroxyl group per se but rather its phosphorylation that is required for sumoylation. Consistent with this, the phosphorylation-mimicking mutation S444E recovered sumoylation (Fig. 1). As reported (42), Cdk5 phosphorylated Ser-444 and potentiated sumoylation on Lys-439 (Fig. 2). Furthermore, calcineurin reversed the phosphorylation (Fig. 2, G and H) and inhibited sumoylation in a phosphatase activity-dependent manner (Fig. 3, F and G). Therefore, it is likely that phosphorylation of Ser-444 is a prerequisite for sumoylation of Lys-439, thereby highlighting a novel cross-talk between these two neighboring modifications (Fig. 7A). Lys-439 and Ser-444 are part of a small portable repression domain (17, 19), to act like a "modification or signaling cassette" to transmit and process molecular cues from cellular signaling networks (Fig. 7A) (59, 60). As discussed below, Cdk5, HDAC4, and calcineurin act through this "signaling cassette" to regulate the activity of MEF2D (Fig. 7A). Because Lys-439 and Ser-444 of MEF2D are highly conserved among other MEF2 proteins (17, 32), it is tempting to propose that such a cross-talk also plays an important role in inhibiting activities of other MEF2 proteins.

Cdk5 Positively Regulates Sumoylation of MEF2—Phosphorylation of MEF2D at Ser-444 by Cdk5 is known to inhibit the transcriptional activity of MEF2D during neuronal apoptosis (42), but the underlying molecular mechanism remains unclear. Our results suggest that Cdk5 imparts this inhibitory effect by promoting sumoylation of Lys-439 (Fig. 2). Consistent with this, both Lys-439 and Ser-444 are required for Cdk5 to negatively regulate the activity of MEF2D (Fig. 5C).

Sumoylation has been shown to down-modulate activities of many DNA-binding transcription factors (61–63). Different models have been proposed to explain how this modification leads to transcriptional repression (63, 64). According to one model, SUMO addition creates docking sites for transcriptional corepressors (59, 64–66). Another model suggests that SUMO-modified transcription factors are recruited into repressive subnuclear domains (67–69). In the case of MEF2, there is a third possibility: Cdk5 induces the cleavage of MEF2 by caspases (19) and may act through sumoylation to promote the cleavage. Further investigation is needed to elucidate how these models operate to control MEF2 activities in vivo.

Calcineurin Counteracts Stimulatory Effects of HDAC4 on Phosphorylation and Sumoylation of MEF2—HDAC4 stimulate MEF2 sumoylation in mammalian cells (32, 43). One possibility is that HDAC4 functions as a SUMO E3 ligase. The following four criteria are often used to determine whether a candidate is a genuine SUMO E3 ligase: (i) binding to the substrate; (ii) interacting with the SUMO-conjugating enzyme Ubc9; (iii) promoting the transfer of SUMO from Ubc9 to the substrate in vitro; and (iv) promoting this SUMO transfer in vitro (61). HDAC4 fulfills the first three requirements as it binds to MEF2, localizes with Ubc9 in HeLa cells, and stimulates MEF2 sumoylation in different mammalian cells (26, 32, 51, 70–72). However, HDAC4 did not promote sumoylation of MEF2D in vitro (Fig. 1D, lanes 4 and 5). Therefore, HDAC4 may not be an authentic E3 ligase for MEF2 sumoylation.

If so, how does HDAC4 potentiate MEF2 sumoylation in vivo? When coexpressed, HDAC4 caused mysterious mobility shifts on MEF2 (32). More importantly, calf intestinal alkaline phosphatase treatment and calcineurin expression abolished these shifts (Fig. 3, B–D), supporting that the shifts are due to phosphorylation. Therefore, HDAC4 is able to

Ser-444, which like Lys-439 is invariant among MEF2 proteins (Fig. 7) (17, 32, 43). Substitution of Ser-444 with alanine abolished sumoylation (Fig. 1), so this serine residue is critical for the modification. Related to this, mutant S444A was as active as mutant K439R in reporter and myogenic conversion assays (Figs. 5 and 6). Moreover, Ubc9-dependent inhibition of MEF2D transcriptional activity required Ser-444 (Fig. 5B). Thus, this serine residue plays a leading role in controlling sumoylation of Lys-439 and is important for modulating inhibitory effects of this modification on transcriptional and myogenic activities of MEF2D.
stimulate MEF2 phosphorylation. Mechanistically, there are at least three possibilities to explain this. First, HDAC4 may associate with kinases and bridge their interaction with MEF2. Our results indicate that Cdk5 and a Li\(^+\)-sensitive kinase (Figs. 2 and 3) are involved. Of relevance, it has been suggested that a Li\(^+\)-sensitive kinase distinct from GSK-3\(\beta\) phosphorylates MEF2D (56). Second, HDAC4 may inhibit the activity of a phosphatase that acts on MEF2. Consistent with this, HDAC4 associates with calcineurin (Fig. 4B). Third, HDAC4 may stimulate the synthesis or activity of a kinase that can phosphorylate MEF2. At this moment, it is unclear whether this mechanism really operates.

Our data support that the phosphorylation induced by HDAC4 promotes MEF2 sumoylation. First, this deacetylase stimulated Ser-444 phosphorylation, and this modification was required for sumoylation (Figs. 2 and 3). Second, calcineurin not only reversed specific MEF2D phosphorylation (Figs. 2G and 3D) but also inhibited the sumoylation (Fig. 3F). Third, LiCl treatment, which inhibited the HDAC4-mediated phosphorylation of MEF2D (Fig. 3C), also abrogated the sumoylation (data not shown).

Calcineurin has been characterized as an important positive regulator of MEF2D-dependent transcription (8, 40). Different mechanisms have been proposed. One group demonstrated that calcineurin activates MEF2A by stabilizing its association with promoters in cerebellar granule neurons (23), whereas others showed that this phosphatase promotes MEF2 interaction with cofactors like NF-AT and p303 (73). Calcineurin has also been found to bind and dephosphorylate MEF2A (8, 40, 41). A recent report indicated that this phosphatase dephosphorylates murine MEF2C at Ser-412 to promote nuclear localization (74). This mechanism may be specific to MEF2C because calcineurin does not regulate the nuclear localization of MEF2A (74) and the serine residue is not conserved in either MEF2A or MEF2D. We found that calcineurin reversed the phosphorylation of MEF2D stimulated by HDAC4 (Figs. 2 and 3). HDAC4 but not MEF2D interacted with calcineurin (Fig. 4), suggesting that this phosphatase may be recruited to MEF2 via HDAC4. Our results also indicate that calcineurin activates MEF2 by dephosphorylating Ser-444 and inhibiting sumoylation on Lys-439 (Figs. 2, 3, and 7). In agreement with this, calcineurin had minimal effects on transcriptional activities of sumoylation-deficient MEF2D mutants (Fig. 5D).

Multiple kinases have been shown to phosphorylate MEF2 transcription factors (1, 2). Dependent on sites of action, functional consequences can be completely different. Although p38 and ERK5 induce stimulatory phosphorylation events (2, 34–39), Cdk5 promotes inhibitory ones (17, 42). Phosphorylation induced by HDAC4 belongs to the latter category. Most interestingly, calcineurin had minimal effects on phosphorylation mediated by ERK5 (Fig. 3E). Therefore, calcineurin is able to specifically remove inhibitory phosphate groups while leaving the stimulatory ones intact. Such an “editing” role is really unexpected.

Interplay between Different Modifications as a General Regulatory Mechanism—As proposed in Fig. 7A, signaling events may act through phosphorylation of Ser-444 to control Lys-439 sumoylation. The cross-talk between these two modifications is reminiscent of what has been reported for the stress-inducible heat shock factor HSF1 and the nuclear receptor PPAR\(\gamma\). Upon cellular stress, HSF1 is phosphorylated at Ser-303, which in turn stimulates sumoylation of Lys-298 (75). Similarly, PPAR\(\gamma\) is sumoylated at Lys-107, and phosphorylation of Ser-112 promotes the sumoylation (76). As illustrated in Fig. 7B, additional transcription factors possess serine residues at equivalent positions. Therefore, the cross-talk may represent a general regulatory mechanism. Related to this, phosphorylation-mimicking residues like aspartate or glutamate are often found at equivalent positions for many sumoylated transcriptional repressors (77). Different from MEF2, HSF1, and PPAR\(\gamma\), phosphorylation of c-Jun inhibits sumoylation (78). Sumoylation has also been shown to interplay with other lysine modifications; ubiquitin and SUMO1 compete for the same lysine residue on I\(\kappa\)B (53), and deacetylation of p300 is required for subsequent sumoylation (79). Together, these findings are consistent with the notion that many eukaryotic proteins are subject to multiple modifications, and different modifications can synergize, or counteract each other, to regulate protein function in a coordinated fashion (59, 60, 80).
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Conclusion—The experiments described herein demonstrate that Ser-444 phosphorylation of human MEF2D plays an important role in regulating sumoylation of Lys-439. Although phosphorylation of Ser-444 by Cdk5 up-regulates the sumoylation, calcineurin dephosphorylates Ser-444 to inhibit SUMO modification. Lys-439 and Ser-444 of MEF2D are highly conserved among MEF2 proteins from different organisms (18, 32), so such a cross-talk should also be important for regulating functions of other MEF2 proteins. This study thus identifies a novel regulatory mechanism for sumoylation of MEF2 proteins and provides further support for the notion that interplay between different modifications is an important mechanism for coordinated regulation of protein functions in vivo.

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