MOLECULAR AND CELLULAR BIOLOGY, Nov. 2007, p. 7623–7630 Vol. 27, No. 21
0270-7306/07/$08.00+0 doi:10.1128/MCB.01481-07

Nemo-Like Kinase–Myocyte Enhancer Factor 2A Signaling Regulates Anterior Formation in *Xenopus* Development▽

Kiyotoshi Satoh,[1] Junji Ohnishi,[1] Atsushi Sato,[1,2] Michio Takeyama,[1] Shun-ichiro Iemura,[3] Tohru Natsume,[3] and Hiroshi Shibuya[1,4]*

Department of Molecular Cell Biology, Medical Research Institute and School of Biomedical Science, Tokyo Medical and Dental University, and SORST, JST, Chiyoda-ku, Tokyo 101-0062, Japan[1]; Medical Top Track Program, Medical Research Institute, Tokyo Medical and Dental University, Chiyoda, Tokyo 101-0062, Japan[2]; National Institutes of Advanced Industrial Science and Technology, Biological Information Research Center (JBIRC), Kohtoh-ku, Tokyo 135-0064, Japan[3]; and Center of Excellence Program for Research on Molecular Destruction and Reconstruction of Tooth and Bone, Tokyo Medical and Dental University, Chiyoda, Tokyo 101-0062, Japan[4]

Received 16 August 2007/Accepted 21 August 2007

The development of anterior neural structure in *Xenopus laevis* requires the inhibition of bone morphogenic protein 4 and Wnt signaling. We previously reported that Nemo-like kinase (NLK) negatively regulates Wnt signaling via the phosphorylation of T-cell factor/lymphoid enhancer factor. However, the molecular events occurring downstream of NLK pathways in early neural development remain unclear. In the present study, we identified the transcription factor myocyte enhancer factor 2A (MEF2A) as a novel substrate for NLK. NLK regulates the function of *Xenopus* MEF2A (xMEF2A) via phosphorylation, and this modification can be inhibited by the depletion of endogenous NLK. In *Xenopus* embryos, the depletion of either NLK or MEF2A results in a severe defect in anterior development. The endogenous expression of anterior markers was blocked by the depletion of endogenous *Xenopus* NLK (xNLK) or xMEF2A but, notably, not by the depletion of other xMEF2 family proteins, xMEF2C and xMEF2D. Defects in head formation or the expression of the anterior marker genes caused by the depletion of endogenous xMEF2A could be eliminated by the expression of wild-type xMEF2A, but not xMEF2A containing mutated xNLK phosphorylation sites. Furthermore, the expression of xNLK-induced anterior markers was efficiently blocked by the depletion of endogenous xMEF2A in animal pole explants. These results show that NLK specifically regulates the MEF2A activity required for anterior formation in *Xenopus* development.

The decision to form neural tissue or not is a binary choice that requires the repression of the alternative fate of ventral epidermal cells and that depends on threshold activity levels of several growth factors, including bone morphogenic protein 4 (BMP4) (8). In *Xenopus laevis*, this fate is determined by the Spemann's organizer, located on the dorsal side of the gastrula, which is a source of secreted neural inducers such as Chordin, Noggin, and Follistatin. These molecules create a gradient of BMP activity by antagonizing BMP signaling in the anterior ectoderm of the embryo, and this BMP gradient establishes a dorsoventral pattern underlying the neural structure, cement gland, and epidermis (5). The organizer region also secretes a number of other antagonists of growth factor signaling, including Wnt inhibitors Frzb-1, crescent, and Dkk-1 and the Nodal inhibitor Cerberus. In the current model, the simultaneous inhibition of both BMP (5) and Wnt (18) signaling pathways is required for anterior neural induction. Additionally, instructive cues from fibroblast growth factor (12) and insulin-like growth factor (32) signaling pathways have been reported to actively contribute to anterior neural specification. In *Xenopus*, signals from the dorsal mesoderm induce the anteroposterior pattern of the overlying ectoderm during gastrulation, which is converted into the central nervous system as well as the non-neural cement gland. These structures are organized along an anterior-posterior axis in which the cement gland is the most anterior ectodermal structure. The development of all neural tissue initially proceeds toward anterior fates, and all such tissue would develop into forebrain and midbrain tissue and cement glands if not subjected to inhibition. However, these anterior fates are diverted toward more-posterior fates: development into hindbrain and spinal chord tissue (5, 8, 37).

Nemo-like kinase (NLK) is an evolutionarily conserved serine-threonine protein kinase that was originally isolated as a murine orthologue of the *Drosophila melanogaster* Nemo, which plays a role in diverse signaling processes (2). Studies of Nemo null mutants of *Drosophila* revealed that Nemo plays a role in head development and the epithelial planar cell polarity pathway during eye development by controlling programmed cell death (25). In our previous studies, we demonstrated that NLK is involved in the suppression of Wnt/β-catenin signaling pathways. NLK inactivates a transcriptional unit composed of β-catenin–T-cell factor/lymphoid enhancer factor (TCF/LEF) by phosphorylating TCF/LEF, and this inactivation results in the inhibition of its binding to target gene sequences (15, 40). NLK functions downstream of transforming growth factor β-activated kinase 1, a member of the mitogen-activated protein kinase (MAPK) kinase kinase family (15, 29); Wnt1 (14); and Wnt5a (16). The loss of NLK/Nemo function results in a lethal phenotype in *Drosophila* (25), *Caenorhabditis elegans* (33), and mouse (19) embryos, strongly implicating NLK/Nemo as a very important regulator of cell growth, patterning, and death. We previously demonstrated that in *Xenopus* embryos, the expression of NLK is restricted to the central

* Corresponding author. Mailing address: Department of Molecular Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, Chiyoda, Tokyo 101-0062, Japan. Phone and fax: 81-3-5280-8062. E-mail: shibuya.mcb@mri.tmd.ac.jp.

▽ Published ahead of print on 4 September 2007.

nervous system, eye field, and anterior neural crest cell populations. *Xenopus* NLK (xNLK) induces the expression of anterior neural marker genes, such as those for Otx2 and the neural cell adhesion molecule (N-CAM), and associates with the high-mobility group domain transcription factor xSox11 (13). Our recent data indicate that, in addition to TCF/LEF and xSox11, NLK associates with and modulates the activities of other transcription factors, including STAT3 (29) and HMG2L1 (39). This finding suggests that NLK contributes to various signaling pathways via its ability to interact with a diverse collection of transcription factors.

Myocyte enhancer factor 2 (MEF2) proteins are members of the MADS (MCMI, agamous, deficiens, and serum response factor) box family of transcription factors. MEF2, originally identified as a transcription factor present at high levels in muscle tissue, binds to an A/T-rich DNA sequence in the control regions of numerous muscle-specific genes and, in particular, cooperates with members of the MyoD family in specifying the differentiation of skeletal muscle (1, 3, 38). Emerging evidence has shown that MEF2 proteins constitute a family of transcription factors that play critical roles in the processes of cell differentiation during the development of multicellular organisms (24). Mammalian MEF2 proteins are encoded by four genes (those for MEF2A, MEF2B, MEF2C, and MEF2D), which exhibit isoform-specific or overlapping patterns of expression in both embryonic and adult tissues. Structurally, the MEF2 proteins comprise highly homologous amino termini that mediate MEF2 homo- and heterodimerization and DNA binding, while the carboxyl termini are more divergent and contain the transactivation domain (1). All MEF2 family members are expressed at high levels in neurons in the central nervous system. The expression of all MEF2s increases in differentiating neurons during brain development (22). Although recent in vitro findings support the hypothesis that MEF2 family members regulate neuronal survival and development, little is known about the exact functions of MEF2s during early neuronal development in embryos.

MEF2 family molecules are the targets for several key intracellular signaling pathways. In this regard, the involvement of p38 MAPK (41), extracellular signal-regulated kinase 5 (ERK5) (17), cyclin-dependent kinase 5 (CDK5) (9), and protein kinase A (36) in these processes has been well known. However, the dynamic changes in MEF2 phosphorylation during diverse cellular processes such as neuronal survival and apoptosis strongly suggest that the regulation of MEF2 is more complex, raising the possibility of the involvement of yet-unidentified kinases and phosphatases (7, 10, 23, 30, 35).

In the present study, we first identified the MEF2A transcription factor as a novel partner for NLK. xNLK directly phosphorylated *Xenopus* MEF2A (xMEF2A) at Thr^{297} and Ser^{340}, and the endogenous phosphorylation of MEF2A was inhibited by the depletion of endogenous NLK. The depletion of either xNLK or xMEF2A resulted in a defect in anterior neural development, including the loss of eye and head structures in *Xenopus* embryos. The phenotypes induced by endogenous xMEF2A depletion were reversed by the overexpression of wild-type xMEF2A but not by that of mutant xMEF2A harboring mutations in the NLK phosphorylation sites. These results reveal a new mechanism for regulating anterior formation by NLK-MEF2A signaling that involves the phosphorylation of MEF2A.

MATERIALS AND METHODS

Plasmid construction. The xMEF2A cDNA was kindly provided by N. Ueno (National Institute for Basic Biology). The human MEF2A and xMEF2C and xMEF2D cDNAs were amplified by reverse transcription-PCR (RT-PCR) from cDNA templates prepared from 293 cells and *Xenopus* embryo tissue, respectively, and were subcloned into the CS2+ vector.

Embryo handling and MO. Capped mRNAs were synthesized from linearized vectors using the mMessage machine kit (Ambion). mRNAs were then injected into the animal blastomeres of embryos. Animal cap explants were removed at the late blastula stage and allowed to grow until control sibling embryos reached the neurula stage. The morpholino oligonucleotides (MO; Gene Tools, LLC) used here were 5′-GCCCTTCCCTACACGGATGTCCCCC-3′ (xNLK MO) (28), 5′-CATGGTTGCGTTGTTCTCTCCC-3′ (xMEF2A MO), 5′-CCATAGT CCCCGTTTTTCTGTCTTC-3′ (xMEF2C MO), and 5′-AATCTGGATCTTTT TTCTGCCCATG-3′ (xMEF2D MO). MO for xMEF2A, xMEF2C, or xMEF2D specifically inhibited the translation of mRNA for xMEF2A, xMEF2C, or xMEF2D, respectively.

RT-PCR analysis. Total RNA was prepared using TRIzol (Invitrogen). cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcriptase (Invitrogen). The sequences of the primer pairs were as described previously (13) and as follows: Pax6, 5′-CAGAACATCTTTTACCCAGGA-3′ and 5′-ACTACTGCTAATGGGAATGTG-3′; Lhx2, 5′-GTCTTCAGCTTGCC CCCTCTCCTTCT-3′ and 5′-GGTCGGGTTCCTGTGTTGCTTCTT-3′; Rx1, 5′-CCCCAACAGGAGCATTTAGAAGAC-3′ and 5′-AGGGCACTCATGGC AGAAGGTT-3′; and Six3, 5′-ATGGTGTTCAGGTCCCCTCT-3′ and 5′-GG ATGGACTCGTGTTTATTAATGG-3′.

Antibodies. The following antibodies were used for immunoprecipitation and/or Western blotting analysis: anti-FLAG (M2; Sigma), anti-Myc (9B11; Cell Signaling), anti-MEF2A (Santa Cruz) and phospho-Thr312 anti-MEF2A (Signal Antibody Technology), and anti-NLK (16).

Immunoprecipitation and Western blotting analysis. 293 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. 293 cells were transfected with the plasmids indicated below by the calcium phosphate precipitation method. After 24 h, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, 10 μg of pepstatin/ml, and 20 μg of leupeptin/ml). Lysates were precleared with protein G-Sepharose beads (Amersham Biosciences) and immunoprecipitated with the appropriate antibodies. For Western blotting analysis, whole-cell lysates or immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences). The membranes were probed with the appropriate antibody, and proteins of interest were visualized with horseradish peroxidase-conjugated mouse or rabbit immunoglobulin G by using Western Lightning chemiluminescence reagent Plus (PerkinElmer Life Science).

Protein identification by LC-MS-MS analysis. FLAG-NLK was expressed in 293 cells, and NLK and associated proteins were recovered from cell extracts by immunoprecipitation with anti-FLAG antibody. The NLK-associated complexes were digested with *Achromobacter* protease I, and the resulting peptides were analyzed using a nanoscale liquid chromatography-tandem mass spectrometry (LC-MS-MS) system as described previously (26).

In vitro kinase assay. 293 cells were transfected with the FLAG-NLK expression plasmid. The lysates were prepared from the transfected cells by using lysis buffer and immunoprecipitated with anti-FLAG antibody. Immunoprecipitates were incubated with 1 μg of bacterially expressed glutathione *S*-transferase fusion proteins corresponding to amino acids 217 to 470 of xMEF2A in 20 μl of kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM dithiothreitol, 5 mM $MgCl_2$, and 5 μCi of [γ-^{32}P]ATP at 25°C for 5 min. Phosphorylated substrates were subjected to SDS-PAGE and quantitated using a BAS 2500 image analyzer (Fujifilm).

RNA interference. Neuro2A cells were grown in 12% fetal bovine serum–DMEM with 4.5 g of glucose/liter and 25 mM HEPES (pH 7.4). For differentiation experiments, Neuro2A cells were subjected to serum depletion by replacing the medium with fresh serum-free DMEM and further incubated for the periods indicated below (6). We designed small interfering RNAs (siRNAs) targeting mouse NLK mRNA (sense, 5′-CAGATCCAAGAGATGGAAA-3′) along with the corresponding antisense RNA oligonucleotide with two thymidine residues (dT-dT) at the 3′ end of the sequence (Dharmacon). The control siRNA si*CONTROL* nontargeting siRNA no. 2 (Dharmacon) was used for the negative control. Neuro2A cells were transfected with these siRNAs by using Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). Thirty-six hours posttransfection, the medium was replaced with fresh serum-free DMEM to induce the differentiation of Neuro2A cells.

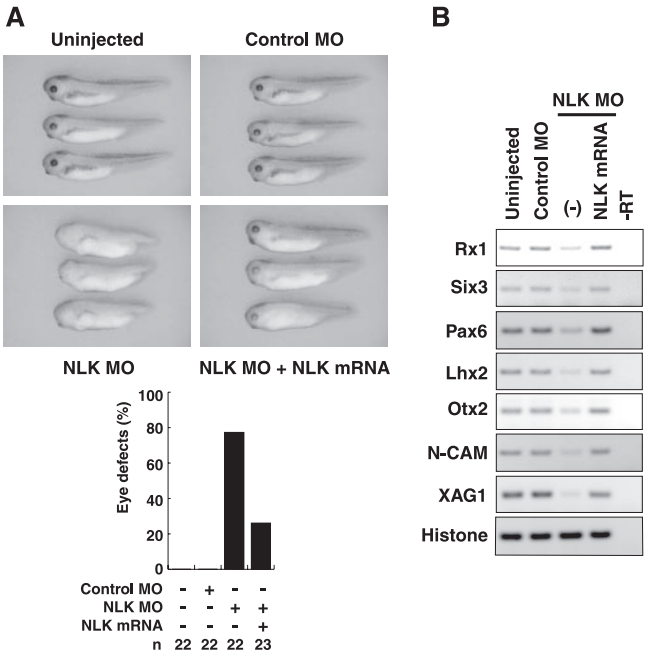

FIG. 1. Anteroneural defects caused by NLK depletion. (A) Control MO (8 ng), NLK MO (8 ng), and/or NLK mRNA (100 pg) was injected into the four animal blastomeres of eight-cell-stage embryos. The embryos were cultured to stage 37. The injection of NLK MO resulted in incomplete formation of the head structure, including the eyes and the cement gland. The NLK MO-induced phenotype was reversed by the coinjection of NLK mRNA. The number of embryos analyzed is indicated in the bottom panel below each bar (*n*). The results shown are representative of results from three independent experiments. +, injected; −, not injected. (B) Control MO (20 ng), NLK MO (20 ng), and NLK mRNA (100 pg) were injected into the four animal blastomeres of eight-cell-stage embryos. Total RNA from the heads of embryos at stage 20 (neurula) was prepared and analyzed by RT-PCR. Histone H4 was used as a loading control. (−), NLK MO injected without NLK mRNA; −RT, no RT step.

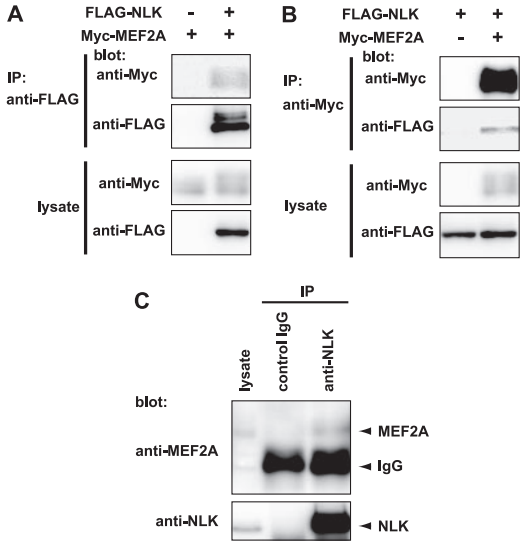

FIG. 2. MEF2A associates with NLK. (A and B) 293 cells were transfected as indicated. Immunoprecipitates (IP) obtained using anti-FLAG (A) or anti-Myc (B) antibody were subjected to Western blotting with the indicated antibodies. +, present; −, absent. (C) Immunoprecipitates obtained from P17 mouse brain extracts using control immunoglobulin G (IgG) or anti-NLK antibody were subjected to Western blotting with anti-MEF2A antibody or anti-NLK antibody.

Whole-mount in situ hybridization. pBluescript vectors containing a fragment of xNLK cDNA encoding the C-terminal region (nucleotides 945 to 1509; GenBank accession no. AB071285) and the 3′ untranslated region of xMEF2A cDNA (nucleotides 1799 to 2352; GenBank accession no. BC046368) were used as templates to generate digoxigenin-labeled RNA probes with a digoxigenin RNA labeling kit according to the protocol of the manufacturer (Roche). Whole-mount in situ hybridization analysis of embryos of various stages was performed with digoxigenin-labeled RNA probes essentially as described by Hemmati-Brivanlou et al. (11) with the following modifications. After the manual removal of the vitelline membranes, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline and dehydrated by gradual washing in methanol. Embryos were rehydrated with phosphate-buffered saline containing 0.1% Triton X-100 and then treated with proteinase K (2 μg/ml) for 10 min at ambient temperature, followed by postfixation with 4% paraformaldehyde for 20 min. Hybridization was performed at 68°C with a solution of 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 2× Denhardt's solution, 200 μg of tRNA/ml, 0.1% Triton X-100, and 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} containing 200 ng of digoxigenin-labeled RNA probe/ml. Color detection was carried out with BM purple (Roche).

RESULTS

NLK is essential for anterior neural development. Our recent studies have shown that xNLK is expressed in neural tissues and induces the expression of the panneural marker N-CAM and the anterior neural marker Otx2 (13). To examine the in vivo role of xNLK, an antisense MO targeting xNLK (xNLK MO) was injected into *Xenopus* embryos to inhibit the expression of NLK (data not shown). We injected xNLK MO into the four animal blastomeres of eight-cell-stage *Xenopus* embryos and examined its effect on neuroectodermal tissue formation. The depletion of endogenous xNLK caused severe defects in the formation of head structures, including the loss of both eyes and the cement gland at the tadpole stages (Fig. 1A), and reduced the expression of the genes for the neural markers N-CAM and Otx2, cement gland marker XAG1, and the anterior markers Pax6, Lhx2, Rx1, and Six3 (42) (Fig. 1B). No phenotype was detected upon the injection of up to 20 ng of control MO. The observed defects induced by xNLK MO were eliminated by the coinjection of xNLK mRNA (Fig. 1), indicating that xNLK is involved in head development.

MEF2A is a novel NLK-associating protein. To further clarify the functions of NLK during head formation in *Xenopus* development, we analyzed proteins that immunoprecipitated with FLAG-tagged NLK from 293 cells by LC-MS-MS. We identified the transcription factor MEF2A as a candidate protein that may physically interact with NLK (data not shown). To confirm this interaction, we performed coimmunoprecipitation using tagged versions of each protein. We found that FLAG-xNLK could be immunoprecipitated with Myc-tagged xMEF2A, and vice versa, when both proteins were expressed in 293 cells (Fig. 2A and B). To prove the existence of an endogenous NLK and MEF2A complex in vivo, we prepared extracts from mouse brain and performed coimmunoprecipitation analysis using anti-NLK and anti-MEF2A antibodies. Figure 2C shows that endogenous MEF2A in mouse brain was coimmunoprecipitated with NLK. We conclude that NLK can associate with MEF2A in mammalian cells.

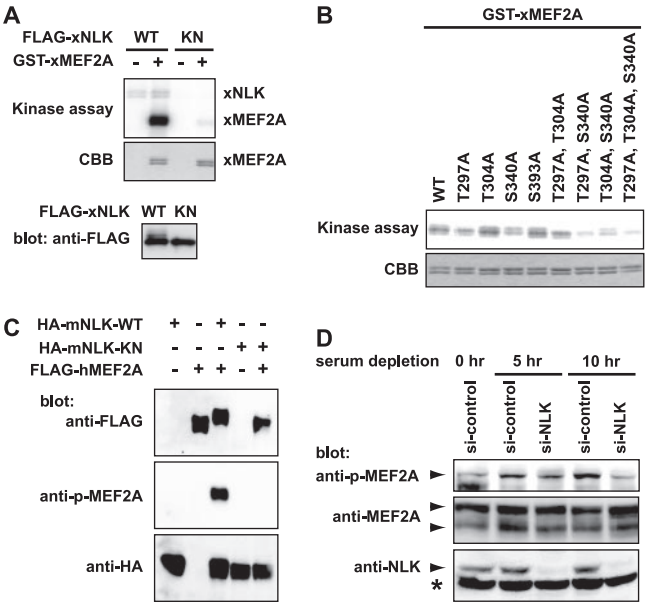

FIG. 3. NLK phosphorylates the activating sites of MEF2A. (A) 293 cells were transfected with FLAG-tagged wild-type (WT) xNLK or kinase-negative (KN) xNLK (13). Immunoprecipitates obtained with anti-FLAG antibody were incubated with bacterially expressed xMEF2A and [γ-^{32}P]ATP. The phosphorylation of xMEF2A and the autophosphorylation of NLK are shown (top panel). The amount of xMEF2A was visualized by Coomassie brilliant blue (CBB) staining (middle panel). The expression of FLAG-NLK was monitored by Western blotting with anti-FLAG antibody (bottom panel). GST, glutathione *S*-transferase; +, present; −, absent. (B) Immunoprecipitated wild-type NLK was incubated with wild-type or mutant xMEF2A. The phosphorylation of xMEF2A is shown (top panel). The amount of xMEF2A was monitored by Coomassie brilliant blue staining (bottom panel). (C) 293 cells were transfected as indicated, and whole-cell lysates were subjected to Western blotting using the indicated antibodies. The phosphorylation of human MEF2A (hMEF2A) was detected by Western blotting with antibody that specifically recognizes the form of hMEF2A that is phosphorylated at the Thr^{312} residue (Thr^{297} in *Xenopus*; p-MEF2A) (middle panel). HA-mNLK-WT, hemagglutinin-tagged wild-type mouse NLK; HA-mNLK-KN, hemagglutinin-tagged kinase-negative mouse NLK; HA, hemagglutinin. (D) Neuro2A cells were transfected with the control siRNA (si-control) or NLK siRNA (si-NLK). After 36 h, the medium was replaced with fresh serum-free DMEM. Whole-cell lysates were subjected to Western blotting by using the indicted antibodies. The asterisk indicates nonspecific bands.

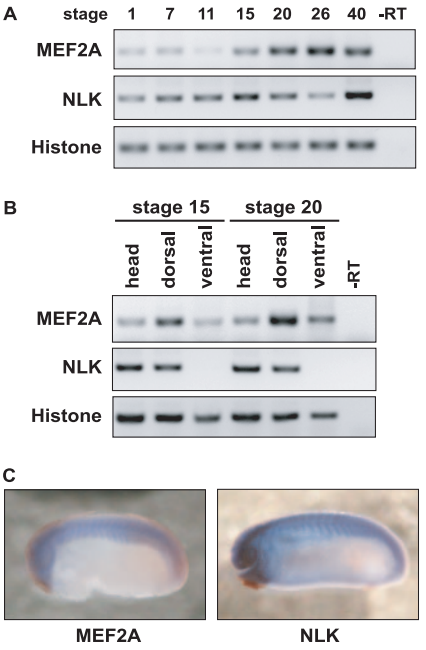

FIG. 4. Developmental expression of MEF2A and NLK in *Xenopus* embryos. (A) RT-PCR analysis revealed that mRNAs of MEF2A and NLK are stored maternally and increase after stage 15. Lane numbers correspond to developmental stages: 1, one-cell stage; 7, blastula; 11, gastrula; 15 and 20, neurula; 26, tail bud tadpole; and 40, swimming tadpole. −RT, no RT step. (B) Total RNAs from the heads, the dorsal side of the bodies, or the ventral side of the bodies of stage 15 or 20 embryos were prepared. MEF2A and NLK were expressed in the head during neurogenesis. (C) Whole-mount in situ hybridization showed that MEF2A and NLK are expressed in the head and on the dorsal side. Images are lateral views with the anterior ends toward the right.

NLK phosphorylates the activating sites of MEF2A. The coexpression of MEF2A and NLK in 293 cells caused the mobility of MEF2A protein to be retarded upon SDS-PAGE (Fig. 2A). To examine whether NLK directly phosphorylates MEF2A, an in vitro kinase assay was performed using bacterially expressed MEF2A protein. Figure 3A shows that glutathione *S*-transferase–MEF2A was phosphorylated by NLK in a kinase activity-dependent manner. It is known that three phosphorylation residues within the *trans*-activation domain of MEF2A, Thr^{312}, Thr^{319}, and Ser^{355} in the human protein and Thr^{297}, Thr^{304}, and Ser^{340} in xMEF2A, are involved in the transcriptional activity of MEF2A (17, 41). In contrast, the phosphorylation of a C-terminal serine residue of MEF2A (Ser^{408} in human MEF2A and Ser^{393} in xMEF2A) inhibits transcriptional activity (9). We thus tested whether NLK phosphorylates any of these residues. The replacement of Thr^{297} or Ser^{340} in xMEF2A with alanine (T^{297}A or S^{340}A) significantly reduced the level of phosphorylation by xNLK (Fig. 3B). The replacement of both Thr^{297} and Ser^{340} with alanine (T^{297}A, S^{340}A) led to a further reduction in the level of phosphorylation (Fig. 3B). On the other hand, the replacement of Thr^{304} or Ser^{393} with alanine had no effect on the levels of xMEF2A phosphorylation. These results indicate that xNLK directly phosphorylates both the Thr^{297} and Ser^{340} residues of xMEFA.

We next examined whether NLK endogenously phosphorylates MEF2A in vivo. The phosphorylation of MEF2A was monitored by Western blotting using an antibody that specifically recognizes the form of MEF2A phosphorylated at the Thr^{312} residue in the case of human MEF2A (Thr^{297} in xMEF2A). We confirmed that MEF2A was phosphorylated at the Thr^{312} residue depending on the kinase activity of NLK when 293 cells were transfected with Myc-tagged human MEF2A with or without FLAG-tagged mouse NLK (Fig. 3C). We next examined endogenous Thr^{312} phosphorylation levels during the differentiation of cells of the Neuro2A line, a mouse neuroblastoma cell line, and found that the phosphorylation of MEF2A at Thr^{312} increased during serum depletion-induced differentiation (Fig. 3D). To assess whether endogenous NLK is involved in this phosphorylation, we performed a knockdown of NLK protein by using siRNA. When Neuro2A cells were transfected with NLK siRNA, the level of endogenous phosphorylation of MEF2A at Thr^{312} was significantly lower than

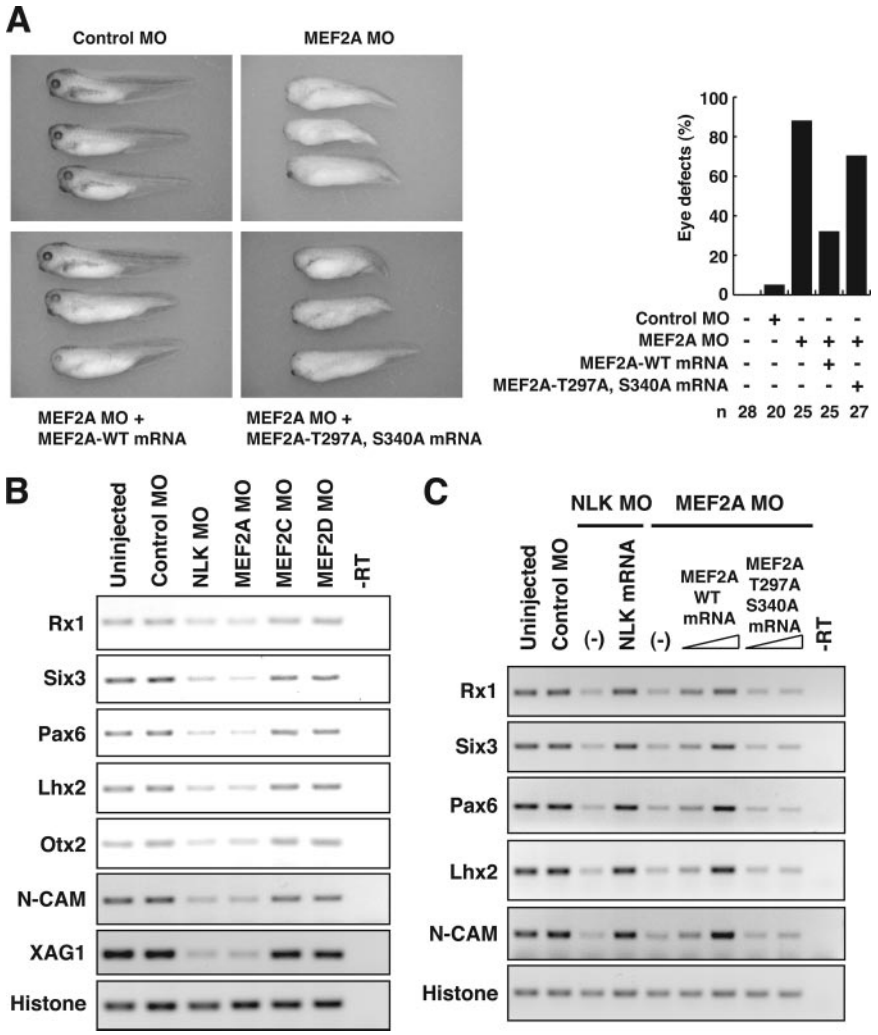

FIG. 5. NLK-MEF2A signaling regulates anterior formation in *Xenopus*. (A) Control MO (8 ng), MEF2A MO (8 ng), and wild-type xMEF2A (MEF2A-WT) mRNA (20 pg) or xMEF2A($T^{297}A$, $S^{340}A$) mRNA (20 pg) were injected into the four animal blastomeres of eight-cell-stage embryos. The embryos were cultured to stage 37. The injection of MEF2A MO resulted in incomplete formation of the head structure, including defects of the eyes and the cement gland. The number of embryos analyzed is indicated in the far-right panel below each bar (*n*). +, injected; −, not injected. The results shown are representative of three independent experiments. (B) Control MO (20 ng), NLK MO (20 ng), MEF2A MO (10 ng), MEF2C MO (10 ng), and MEF2D MO (10 ng) were injected into the four animal blastomeres of eight-cell-stage embryos. Total RNAs from the heads of embryos at the neurula stage were prepared. −RT, no RT step. (C) Control MO (20 ng), NLK MO (15 ng), MEF2A MO (15 ng), and NLK mRNA (100 pg), wild-type xMEF2A mRNA (20 or 100 pg), or xMEF2A($T^{297}A$, $S^{340}A$) mRNA (20 or 100 pg) were injected into the four animal blastomeres of eight-cell-stage embryos. Total RNAs from the heads of embryos at the neurula stage were prepared. (−), NLK MO injected without NLK mRNA or MEF2A MO injected without MEF2A mRNA, as indicated.

that of the control. These results demonstrate that endogenous NLK phosphorylates MEF2A.

xNLK-xMEF2A signaling regulates anterior formation in *Xenopus*. To analyze the function of xNLK-xMEF2A signaling in embryonic development, we first confirmed the expression pattern of xMEF2A during *Xenopus* embryonic development by RT-PCR analysis. We found that xMEF2A was expressed from maternally deposited mRNA and that its zygotic expression was induced after the neurula stage (Fig. 4A). It is noteworthy that xMEF2A expression in the head region of *Xenopus* embryos was detected during neural stages and that it parallels the expression of xNLK (Fig. 4B and C). To address the physiological relationship between xNLK and xMEF2A in the anterior tissues, we synthesized an antisense MO targeting xMEF2A. By Western blotting analysis, we confirmed that the injection of the designed xMEF2A MO specifically reduced the expression of xMEF2A protein (data not shown). When xMEF2A MO was injected into a *Xenopus* region that develops mainly into neuroectodermal tissues and head structures, the phenotype caused by the depletion of xMEF2A was mainly similar to the phenotype resulting from the injection of NLK MO: incomplete formation of the eyes (Fig. 5A) and reduced expression of anterior markers such as Pax6, Lhx2, Rx, and Six3; cement gland marker XAG1; and panneural marker N-CAM (Fig. 5B). The anterior defects induced by xMEF2A MO were abolished by the coinjection of wild-type xMEF2A mRNA but not by the coinjection of mRNA of the nonphosphorylatable mutant protein [xMEF2A($T^{297}A$, $S^{340}A$)] (Fig.

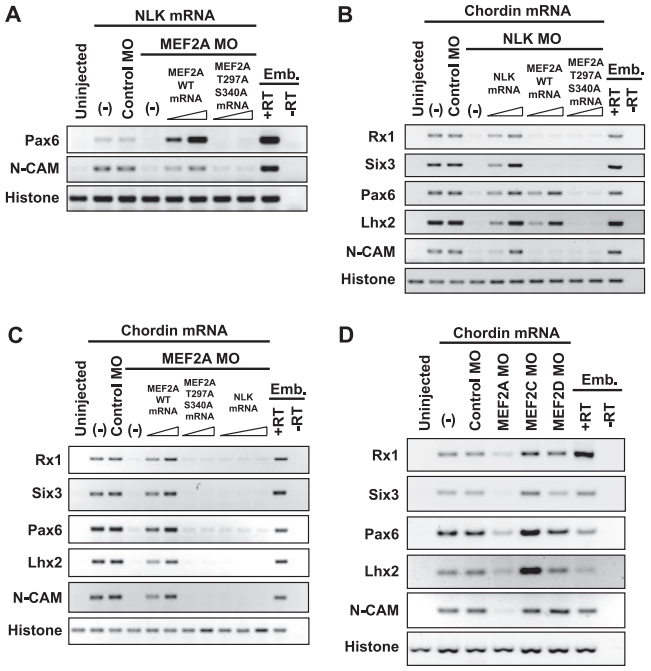

FIG. 6. MEF2A functions downstream of NLK. (A) NLK mRNA (2 ng), control MO (6 ng), MEF2A MO (6 ng), MEF2A mRNA (20 or 100 pg), and/or MEF2A($T^{297}A$, $S^{340}A$) mRNA (20 or 100 pg) was injected into the animal poles of two-cell embryos. Animal caps were cultured until stage 20 (neurula), and total RNA was prepared. The expression of Pax6 and N-CAM was analyzed by RT-PCR. Emb indicates the whole-embryo control with (+RT) or without (−RT) the RT step. (−), no additional mRNA or MO, as indicated. (B) Chordin mRNA (800 pg), control MO (10 ng), NLK MO (10 ng), NLK mRNA (50 or 100 pg), wild-type MEF2A (MEF2A-WT) mRNA (50 or 100 pg), and/or MEF2A($T^{297}A$, $S^{340}A$) mRNA (50 or 100 pg) was injected into the animal poles of two-cell embryos as indicated. Animal caps were cultured until stage 20 (neurula), and total RNA was prepared. (C) Chordin mRNA (800 pg) and control MO (8 ng), MEF2A MO (8 ng), wild-type MEF2A mRNA (20 or 100 pg), MEF2A($T^{297}A$, $S^{340}A$) mRNA (20 or 100 pg), and/or NLK mRNA (100, 500, or 2,000 pg) were injected into the animal poles of two-cell embryos. Animal caps were cultured until stage 20 (neurula), and total RNA was prepared. (D) Chordin mRNA (1,000 pg) and control MO (20 ng), MEF2A MO (20 ng), MEF2C MO (20 ng), and MEF2D MO (20 ng) were injected into the animal poles of two-cell embryos. Animal caps were cultured until stage 20 (neurula), and total RNA was prepared.

5A and C). These results suggest that MEF2A is involved in anterior formation and that the phosphorylation of xMEF2A at Thr^{297} and Ser^{340} is indispensable for normal anterior development in *Xenopus*.

xMEF2A functions downstream of xNLK. To examine whether xMEF2A functions downstream of xNLK, we performed animal cap assays. When xNLK mRNA was injected into the animal poles of two-cell embryos, expression of the panneural marker N-CAM was induced in the animal caps, as we have previously reported. Additionally, the expression of the eye marker *Xenopus* Pax6 was induced by the injection of xNLK mRNA (Fig. 6A). We found that the induction of both of these markers was inhibited by the injection of xMEF2A MO and restored by coinjection with wild-type xMEF2A mRNA but not with mRNA of the nonphosphorylatable mutant protein. These results suggest that the xNLK-induced expression of N-CAM and Pax6 is mediated by xMEF2A via its phosphorylation. To further confirm that xMEF2A acts downstream of xNLK, we tested whether the overexpression of xMEF2A reversed the effect of xNLK knockdown. When mRNA of *Xenopus* Chordin, a BMP4 inhibitor, was injected into the animal poles of two-cell embryos, the expression of N-CAM and anterior markers such as *Xenopus* Pax6, Lhx2, Rx1, and Six3 was induced (Fig. 6B and C). The injection of xNLK MO or xMEF2A MO inhibited the induction of these markers, and coinjection with NLK or MEF2A mRNA eliminated the defects caused by NLK MO or MEF2A MO, respectively (Fig. 6B and C). Interestingly, coinjection with wild-type xMEF2A mRNA also restored the expression of *Xenopus* Pax6 and Lhx2 but not the expression of *Xenopus* Rx1, Six3, and N-CAM (Fig. 6B). On the other hand, coinjection with xMEF2A($T^{297}A$, $S^{340}A$) mRNA failed to restore the expression of any anterior neural marker examined (Fig. 6B and C). Moreover, coinjection with xNLK mRNA could not reverse the inhibition of Chordin-induced anterior markers by the depletion of xMEF2A (Fig. 6C). These results suggest that xMEF2A is partially responsible for the downstream effects of xNLK in *Xenopus* anterior formation.

xMEF2A functions in a subtype-specific manner. In *X. laevis*, three isoforms of MEF2, xMEF2A, xMEF2C, and xMEF2D, have been isolated, and we found that the overexpression of xNLK in 293 cells caused electrophoretic mobility retardation of xMEF2A, xMEF2C, and xMEF2D, suggesting that xNLK could phosphorylate any isoforms of the xMEF2 family (data not shown). To examine whether xMEF2A functions specifically in anterior formation, we injected xMEF2C MO or xMEF2D MO into *Xenopus* embryos. Neither the injection of xMEF2C MO nor that of xMEF2D MO resulted in marked effects on the endogenous expression of anterior markers (Fig. 5B). Moreover, Chordin-induced expression of *Xenopus* Pax6, Lhx2, and N-CAM was inhibited by coinjection with xMEF2A MO but not by coinjection with xMEF2C MO or xMEF2D MO (Fig. 6D). These results suggest that xMEF2A regulates anterior neural development, including eye formation, in a subtype-specific manner.

DISCUSSION

The results of our previous overexpression experiments with animal pole explants indicated that xNLK induces the expression of neural marker genes, including anterior marker genes (13). In the present study, we identified the transcription factor MEF2A as a novel substrate of the MAPK-like serine-threonine kinase NLK and showed that NLK-MEF2A signaling controls anterior neural development in *Xenopus*. The depletion of either xNLK or xMEF2A resulted in severe defects in anterior neural development, including the loss of eye formation. These observed defects induced by xMEF2A depletion were abolished by wild-type xMEF2A but not by the nonphosphorylatable mutant protein [xMEF2A($T^{297}A$, $S^{340}A$)], indicating that xMEF2A functions downstream of xNLK in the development of anteroneural structures. Our results demonstrate new roles of xNLK-xMEF2A signaling in the regulation of endogenous anterior tissue development, including eye formation, in *Xenopus*.

It is well known that the phosphorylation of MEF2 is critical

for its tissue-specific functions. In this regard, p38 MAPK is the best-characterized kinase acting on MEF2 (4, 41); however, other kinases, such as ERK5 (17), CDK5 (9), and protein kinase A (36), have also been proposed to phosphorylate MEF2. Increasing evidence indicates that the MEF2 phosphorylation state plays a critical role at the branch point that determines neuronal survival versus apoptosis. For example, the phosphorylation of MEF2 by p38 MAPK activates the transcriptional activity of MEF2, thereby promoting neuronal survival (30). On the other hand, neurotoxic stress or the depletion of neuronal activity leads to the direct cleavage of MEF2A, MEF2C, and MEF2D by activated caspases, and the resulting MEF2 cleavage fragment induces neuronal apoptosis by dominantly interfering with the transcriptional activity of intact MEF2 (35). During neuronal apoptosis, the inactivation of MEF2 is associated with the hyperphosphorylation of MEF2 mediated by CDK5 (35). Here, we have found that NLK plays a novel role in regulating the activity of MEF2 via direct phosphorylation during early development in *Xenopus* embryos. The phosphorylation of the MEF2A residues Thr^{312}, Thr^{319}, and Ser^{355} (Thr^{297}, Thr^{304}, and Ser^{340} in *Xenopus*) by p38 MAPK (41) and/or ERK5 (17) is known to be important for MEF2A transcriptional activity, whereas the phosphorylation of Ser^{255} and Ser^{408} by CDK5 leads to the degradation of MEF2A (9, 35). Our findings demonstrate that xNLK phosphorylates Thr^{297} and Ser^{340}, which are residues in xMEF2A critical for its activation (Fig. 3). The observed eye defects induced by the depletion of endogenous xMEF2A could be abolished by coinjection with wild-type xMEF2A mRNA but not with mRNA of the nonphosphorylatable mutant MEF2A($T^{297}A$, $S^{340}A$). Moreover, the endogenous phosphorylation of MEF2A was inhibited by the depletion of endogenous NLK in neuronal cells (Fig. 3D). These findings provide the first evidence that xNLK regulates anterior development via the phosphorylation of xMEF2A. By contrast, xNLK did not phosphorylate Thr^{304} of xMEF2A, which is a potential target of p38α and ERK5. Of note, Nishimoto et al. recently reported that the depletion of MEK5 or ERK5 in *Xenopus* embryos resulted in defects in anterior head structures, including the loss of the eyes (28), apparently similar to the phenotypes we observed following the depletion of xNLK or xMEF2A. Therefore, it will be necessary to examine whether xNLK and *Xenopus* ERK5 may cooperatively regulate anterior development via the activation of xMEF2A.

Since we injected xNLK MO or xMEF2A MO into the animal regions of eight-cell-stage embryos, we cannot rule out the possible interference of xNLK or xMEF2A depletion with the activity of the Spemann organizer and anterior endomesoderm. These are two regions involved in the patterning of the dorsoanterior axis that could affect the formation of the anterior head structure. However, in the whole embryos and Chordin-injected ectodermal explants, we observed that the depletion of endogenous xNLK or xMEF2A reduced the expression of the specific anterior marker genes (those for *Xenopus* Pax6, Lhx2, Rx1, and Six3) and the panneural marker N-CAM (Fig. 5B and 6B, C, and D). In addition, embryos depleted of xMEF2A exhibited no apparent effects on the expression of mesodermal markers such as Brachyury (Xbra) or α-actin (data not shown). Moreover, endogenous NLK is required for the phosphorylation of MEF2A in both cultured cells and *Xenopus* embryos (Fig. 3D). The NLK- or Chordin-induced anterior markers were inhibited by the injection of xMEF2A MO and restored by coinjection with wild-type xMEF2A mRNA but not with mRNA of the nonphosphorylatable mutant protein (Fig. 6A and C). Furthermore, coinjection with xNLK mRNA could not reverse the inhibition of Chordin-induced anterior markers by the depletion of xMEF2A (Fig. 6C). Taken together, these results suggest that MEF2A mediates in part the effect of NLK on anterotissue development. On the other hand, the inhibition of *Xenopus* Pax6 and Lhx2 expression could be rescued by the wild-type xMEF2A but not by the nonphosphorylated mutant protein in the Chordin-injected animal caps (Fig. 6B) in which the endogenous NLK was depleted. This may be due to the existence of other potential MEF2A activators, such as p38α (41) and ERK5 (17, 28), that could compensate for NLK activity. In contrast, the overexpression of wild-type xMEF2A failed to induce the expression of *Xenopus* Rx1, Six3, and N-CAM genes (Fig. 6), suggesting that the endogenous expression of *Xenopus* Rx1, Six3, and N-CAM genes is absolutely dependent on the presence of NLK but not other kinases.

In *Xenopus*, three isoforms of the MEF2 family have been identified: xMEF2A, xMEF2C, and xMEF2D. Studies of mammalian MEF2 molecules suggest that different isoforms may overlap in their biological functions (1, 10, 24). Therefore, we examined whether other isoforms of xMEF2, xMEF2C and xMEF2D, may also be involved in xNLK-xMEF2A-mediated anterior formation. We found that neither the depletion of xMEF2C nor that of xMEF2D affected the expression of any anterior marker genes, whereas the depletion of endogenous xMEF2A blocked the induction of these markers (Fig. 5B and 6D). Thus, xMEF2A appears to be selectively responsible for regulating certain anterior marker genes downstream of xNLK. Other studies have also demonstrated possible functional specificity among different isoforms of the MEF2 family (20, 21, 27). For example, MEF2A and MEF2D, but not MEF2C or MEF2B, function as prosurvival factors with high levels of transcriptional activity in postmitotic cerebellar granule neurons (20). Studies of MEF2A and MEF2C null mice have indicated that MEF2C plays an essential role in cardiomyogenesis in early development (prior to looping morphogenesis) and that MEF2A fulfills specific roles in maintaining appropriate mitochondrial content and cytoarchitectural integrity in the postnatal heart (21, 27). However, studies of these null mice have reported no apparent defects in early neural development, and this may be due to the isoform complexity (31, 34) and possible redundancy of function in the mouse. Thus, our studies of *Xenopus* may provide the first clues to isoform-specific functions of MEF2 family members during embryogenesis.

To date, two regulatory pathways for head development have been proposed. One is a default state that is triggered by inhibiting growth factor signals required for trunk development, such as those induced by Wnt, BMP, and Nodal (5, 8, 18). The other is an insulin-like growth factor-mediated active pathway involved in anterior head formation (12, 32). These pathways function in a parallel and/or cooperative manner to promote anteriorizing activity and to suppress the trunk mesoderm. Although the actual ligands that initiate NLK-MEF2A signaling are unclear at present, it is known that NLK is acti-

vated by many ligands and stimuli and is involved in mesoderm induction and axis formation through Wnt and transforming growth factor β signaling pathways in early *Xenopus* development. It is possible that NLK activates MEF2A through the action of a yet-to-be-identified ligand(s) that regulates anterior tissue development, and further studies will be needed to identify the precise ligand(s) involved in this process. It will also be important to determine whether there is cross talk among NLK-MEF2A and other signaling pathways to mediate anterior tissue development.

ACKNOWLEDGMENTS

We thank K. Matsumoto for valuable discussions, N. Ueno for reagents, M. Lamphier for critical reading of the manuscript, and K. Nakamura for technical assistance.

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. **Black, B. L., and E. N. Olson.** 1998. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu. Rev. Cell Dev. Biol. **14:**167–196.
2. **Brott, B. K., B. A. Pinsky, and R. L. Erikson.** 1998. Nlk is a murine protein kinase related to Erk/MAP kinases and localized in the nucleus. Proc. Natl. Acad. Sci. USA **95:**963–968.
3. **Chambers, A. E., S. Kotecha, N. Towers, and T. J. Mohun.** 1992. Muscle-specific expression of SRF-related genes in the early embryo of *Xenopus laevis*. EMBO J. **11:**4981–4991.
4. **Cox, D. M., M. Du, M. Marback, E. C. Yang, J. Chan, K. W. Siu, and J. C. McDermott.** 2003. Phosphorylation motifs regulating the stability and function of myocyte enhancer factor 2A. J. Biol. Chem. **278:**15297–15303.
5. **De Robertis, E. M., and H. Kuroda.** 2004. Dorsal-ventral patterning and neural induction in *Xenopus* embryos. Annu. Rev. Cell Dev. Biol. **20:**285–308.
6. **Evangelopoulos, M. E., J. Weis, and A. Krüttgen.** 2005. Signaling pathways leading to neuroblastoma differentiation after serum withdrawal: HDL blocks neuroblastoma differentiation by inhibition of EGFR. Oncogene **24:**3309–3318.
7. **Flavell, S. W., C. W. Coan, T.-K. Kim, P. L. Greer, Y. Lin, S. Paradis, E. C. Griffith, L. S. Hu, C. Chen, and M. E. Greenberg.** 2006. Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. Science **311:**1008–1012.
8. **Gamse, J., and H. Sive.** 2000. Vertebrate anteroposterior patterning: the *Xenopus* neurectoderm as a paradigm. Bioessays **22:**976–986.
9. **Gong, X., X. Tang, M. Wieldmann, X. Wang, J. Peng, D. Zheng, L. A. C. Blair, J. Marshall, and Z. Mao.** 2003. Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis. Neuron **38:**33–46.
10. **Heidenreich, K. A., and D. A. Linseman.** 2004. Myocyte enhancer factor-2 transcription factors in neuronal differentiation and survival. Mol. Neurobiol. **29:**155–165.
11. **Hemmati-Brivanlou, A., D. Frank, M. E. Bolce, B. D. Brown, H. L. Sive, and R. M. Harland.** 1990. Localization of specific mRNAs in *Xenopus* embryos by whole-mount *in situ* hybridization. Development **110:**325–330.
12. **Hongo, I., M. Kengaku, and H. Okamoto.** 1999. FGF signaling and the anterior neural induction in *Xenopus*. Dev. Biol. **216:**561–581.
13. **Hyodo-Miura, J., S. Urushiyama, S.-I. Nagai, M. Nishita, N. Ueno, and H. Shibuya.** 2002. Involvement of NLK and Sox11 in neural induction in *Xenopus* development. Genes Cells **7:**487–496.
14. **Ishitani, T., J. Ninomiya-Tsuji, and K. Matsumoto.** 2003. Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related nemo-like kinase-dependent phophorylation in Wnt/β-catenin signaling. Mol. Cell. Biol. **23:**1379–1389.
15. **Ishitani, T., J. Ninomiya-Tsuji, S.-I. Nagai, M. Nishita, M. Meneghini, N. Baker, M. Waterman, B. Bowerman, H. Clevers, H. Shibuya, and K. Mastumoto.** 1999. The TAK1-NLK-MAPK-related pathway antagonizes signaling between β-catenin and transcription factor TCF. Nature **399:**798–802.
16. **Ishitani, T., S. Kishida, J. Hyodo-Miura, N. Ueno, J. Yasuda, M. Waterman, H. Shibuya, R. T. Moon, J. Ninomiya-Tsuji, and K. Matsumoto.** 2003. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca^{2+} pathway to antagonize Wnt/β-catenin signaling. Mol. Cell. Biol. **23:**131–139.
17. **Kato, Y., M. Zhao, A. Morikawa, T. Sugiyama, D. Chkravortty, N. Koide, T. Yoshida, R. I. Tapping, Y. Yang, T. Yokochi, and J.-D. Lee.** 2000. Big mitogen-activated kinase regulates multiple members of the MEF2 protein family. J. Biol. Chem. **275:**18534–18540.
18. **Kiecker, C., and C. Niehrs.** 2001. A morphogen gradient of Wnt/β-catenin signaling regulates anteroposterior neural patterning in *Xenopus*. Development **128:**4189–4201.
19. **Kortenjann, M., M. Nehls, A. J. H. Smith, R. Carsetti, J. Schüler, G. Köhler, and T. Boehm.** 2001. Abnormal bone marrow stroma in mice deficient for nemo-like kinase, Nlk. Eur. J. Immunol. **31:**3580–3587.
20. **Li, M., D. A. Linseman, M. P. Allen, M. K. Meintzer, X. Wang, T. Laessing, M. E. Wierman, and K. A. Heidenreich.** 2001. Myocyte enhancer factor 2A and 2D undergo phosphorylation and caspase-mediated degradation during apoptosis of rat cerebellar granule neurons. J. Neurosci. **21:**6544–6552.
21. **Lin, Q., J. Schwarz, C. Bucana, and E. N. Olson.** 1997. Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. Science **276:**1404–1407.
22. **Lin, X., S. Shah, and R. F. Bulleit.** 1996. The expression of MEF2 genes is implicated in CNS neuronal differentiation. Mol. Brain Res. **42:**307–316.
23. **Mao, Z., A. Bonni, F. Xia, M. Nadal-Vicens, and M. E. Greenberg.** 1999. Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science **286:**785–790.
24. **McKinsey, T. A., C. L. Zhang, and E. N. Olson.** 2002. MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends Biochem. Sci. **27:**40–47.
25. **Mirkovic, I., K. Charish, S. M. Gorski, K. McKnight, and E. M. Verheyen.** 2002. *Drosophila* nemo is an essential gene involved in the regulation of programmed cell death. Mech. Dev. **119:**9–20.
26. **Natsume, T., Y. Yamauchi, H. Nakayama, T. Shinkawa, M. Yanagida, N. Takahashi, and T. Isobe.** 2002. A direct nanoflow liquid chromatography-tandem mass spectrometry system for interaction proteomics. Anal. Chem. **74:**4725–4733.
27. **Naya, F. J., B. L. Black, H. Wu, R. Bassel-Duby, J. A. Richardson, J. A. Hill, and E. N. Olson.** 2002. Mitochondrial deficiency and cardiac sudden death in mice lacking the MEF2A transcription factor. Nat. Med. **8:**1303–1309.
28. **Nishimoto, S., M. Kusakabe, and E. Nishida.** 2005. Requirement of the MEK5-ERK5 pathway for neural differentiation in *Xenopus* embryonic development. EMBO Rep. **6:**1064–1069.
29. **Ohkawara, B., K. Shirakabe, J. Hyodo-Miura, R. Matsuo, N. Ueno, K. Matsumoto, and H. Shibuya.** 2004. Role of the TAK1-NLK-STAT3 pathway in TGF-β-mediated mesoderm induction. Genes Dev. **18:**381–386.
30. **Okamoto, S.-I., D. Krainc, K. Sherman, and S. A. Lipton.** 2000. Antiapoptotic role of the p38 mitogen-activated protein kinase-myocyte enhancer factor 2 transcription factor pathway during neuronal differentiation. Proc. Natl. Acad. Sci. USA **97:**7561–7566.
31. **Ornatsky, O. I., and J. C. McDermott.** 1996. MEF2 protein expression, DNA binding specificity and complex composition, and transcriptional activity in muscle and non-muscle cells. J. Biol. Chem. **271:**24927–24933.
32. **Pera, E. M., O. Wessely, S.-Y. Li, and E. M. De Rebertis.** 2001. Neural and head induction by insulin-like growth factor signals. Dev. Cell **1:**655–665.
33. **Rocheleau, C. E., J. Yasuda, T. H. Shin, R. Lin, H. Sawa, H. Okano, J. R. Priess, R. J. Davis, and C. C. Mello.** 1999. Wrm-1 activates the lit-1 protein kinase to transduce anterior/posterior polarity signals in *C. elegans*. Cell **97:**717–726.
34. **Suzuki, E., K. Guo, M. Kolman, Y.-T. Yu, and K. Walsh.** 1995. Serum induction of MEF2/RSRF expression in vascular myocytes is mediated at the level of translation. Mol. Cell. Biol. **15:**3415–3423.
35. **Tang, X., X. Wang, X. Gong, M. Tong, D. Park, Z. Xia, and Z. Mao.** 2005. Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. J. Neurosci. **25:**4823–4834.
36. **Wang, X., X. Tang, M. Li, J. Marshal, and Z. Mao.** 2005. Regulation of neuroprotective activity of myocyte-enhancer factor 2 by cAMP-protein kinase A signaling pathway in neuronal survival. J. Biol. Chem. **280:**16705–16713.
37. **Wardle, F. C., and H. L. Sive.** 2003. What's your position? The *Xenopus* cement gland as a paradigm of regional specification. Bioessays **25:**717–726.
38. **Wong, M.-W., M. Pisegna, M.-F. Lu, D. Leibham, and M. Perry.** 1994. Activation of *Xenopus* MyoD transcription by members of the MEF2 protein family. Dev. Biol. **166:**683–695.
39. **Yamada, M., B. Ohkawara, N. Ichimura, J. Hyodo-Miura, S. Urushiyama, K. Shirakabe, and H. Shibuya.** 2003. Negative regulator of Wnt signaling by HMG2L1, a novel NLK-binding protein. Genes Cells **8:**677–684.
40. **Yamada, M., J. Ohnishi, B. Ohkawara, S. Iemura, K. Satoh, J. Hyodo-Miura, K. Kwachi, T. Natsume, and H. Shibuya.** 2006. NARF, an Nemo-like kinase (NLK)-associated ring finger protein regulates the ubiquitylation and degradation of T cell factor/lymphoid enhancer factor (TCF/LEF). J. Biol. Chem. **281:**20749–20760.
41. **Zhao, M., L. New, V. V. Kravchenko, Y. Kato, H. Gram, F. di Padova, E. N. Olson, R. J. Ulevitch, and J. Han.** 1999. Regulation of the MEF2 family of transcription factors by p38. Mol. Cell. Biol. **19:**21–30.
42. **Zuber, M. E., G. Gestri, A. S. Viczian, G. Barsacchi, and W. A. Harris.** 2003. Specification of the vertebrate eye by a network of eye field transcription factors. Development **130:**5155–5167.