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Possible Involvement of Nemo-like Kinase 1 in *Xenopus* Oocyte Maturation As a Kinase Responsible for Pumilio1, Pumilio2, and CPEB Phosphorylation

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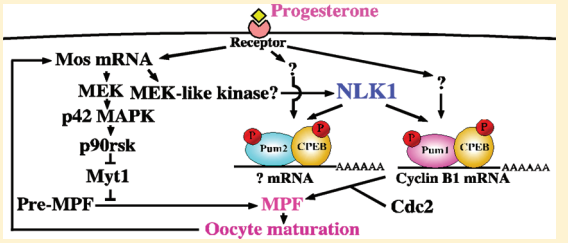
ABSTRACT: Members of the mitogen-activated protein kinase (MAPK) family play important roles in *Xenopus* oocyte maturation. Nemo-like kinase (NLK), an atypical MAPK, is known to function in multiple developmental processes in vertebrates and invertebrates, but its involvement in gametogenesis and gamete maturation is unknown. In this study, we biochemically examined NLK1 during *Xenopus* oocyte maturation. NLK1 is expressed in immature oocytes, and its protein level remains constant during maturation. NLK1 is inactive in immature oocytes but is activated during maturation, depending on Mos protein synthesis but not on p42 MAPK activation. Overexpression of NLK1 by injection of 5 ng of mRNA accelerates progesterone-induced oocyte maturation by enhancing Cyclin B1 protein synthesis through the translational activation of its mRNA, in accordance with precocious phosphorylation of Pumilio1 (Pum1), Pumilio2 (Pum2), and cytoplasmic polyadenylation element-binding protein (CPEB), key regulators of the translational control of mRNAs stored in oocytes. A higher level of NLK1 expression by injection of 50 ng of mRNA induces Pum1/Pum2/CPEB phosphorylation, CPEB degradation, Cyclin B1 protein synthesis, and oocyte maturation in the absence of progesterone. NLK1 phosphorylates Pum1, Pum2, and CPEB in vitro. These findings provide the first evidence for the involvement of NLK1 in *Xenopus* oocyte maturation. We suggest that NLK1 acts as a kinase downstream of Mos and catalyzes phosphorylation of Pum1, Pum2, and CPEB to regulate the translation of mRNAs, including Cyclin B1 mRNA, stored in oocytes.



Oocytes of most vertebrates are arrested at meiotic prophase I. These oocytes are unfertilizable and are thus called immature oocytes. When prophase I-arrested oocytes are stimulated by a maturation-inducing hormone, for example, progesterone in frogs, they resume meiosis and proceed to metaphase II, at which stage they are arrested again. (The metaphase II-arrested oocytes are now fertilizable and are referred to as mature oocytes or eggs.) The process in which oocytes acquire fertilizability is called oocyte maturation.[1] The final trigger of oocyte maturation is the maturation-promoting factor (MPF), a protein complex of Cdc2 and Cyclin B.[2−4] Immature *Xenopus* oocytes have an inactive form of MPF, named pre-MPF, and its activation induces oocyte maturation,[5−7] in striking contrast to immature oocytes of many vertebrate species having distinct breeding seasons, in which Cdc2 is present but Cyclin B is absent in immature oocytes and thus Cyclin B must be synthesized de novo from a stored mRNA for the initiation of oocyte maturation.[8,9]

A protein kinase cascade consisting of Mos, MEK, and p42 mitogen-activated protein kinase (MAPK), the Mos-MEK-MAPK pathway, plays a crucial role in pre-MPF activation in *Xenopus* oocytes.[10,11] Mos is newly synthesized after progesterone stimulation from a dormant mRNA stockpiled in immature oocytes and functions as a MAP kinase kinase kinase; Mos activates MEK, which in turn activates p42 MAPK.[12−14] A target of p42 MAPK is p90 ribosomal S6 kinase that inactivates Myt1, a kinase maintaining pre-MPF in an inactive state, therefore inducing pre-MPF activation and oocyte maturation.[15,16] In addition to p42 MAPK, it has been reported that two members of the MAPK family, c-Jun N-terminal kinase and p38γ kinase, are involved in *Xenopus* oocyte maturation.[17,18] It is thus plausible that members of the MAPK family contribute to various aspects of *Xenopus* oocyte maturation; however, their actual roles have not been fully elucidated except for that of p42 MAPK. (Actually, there are paradoxical results for the roles of MAPK itself that remain to be resolved.[19])

Besides Mos mRNA, many mRNAs including Cyclin B1 mRNA are stockpiled in immature oocytes as dormant forms. Translational activation of these mRNAs is initiated during oocyte maturation at a timing specific to each mRNA,[20−24] which ensures normal progression of oocyte maturation.[25,26] It is well-known that a *cis*-acting element called cytoplasmic polyadenylation element (CPE) in the 3′ untranslated region (UTR) of mRNA and its binding protein (CPEB) regulate the translational activation of dormant mRNAs during oocyte maturation.[27−29] The other *cis*-elements in 3′UTR, the Musashi-binding element (MBE),[30−32] the Pumilio-binding element (PBE)[33−37] and the translational control sequence (TCS),[38] have also been reported to participate in the temporal

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control of mRNA translation during oocyte maturation.[30,36,38,39] However, *trans*-acting factors recognizing these *cis*-elements, Musashi for MBE, Pumilio for PBE, and an unidentified protein for TCS, are not fully characterized. We previously showed that Pumilio1 (Pum1) and Pumilio2 (Pum2) interact with CPEB in immature oocytes and that they are phosphorylated during oocyte maturation, coincident with dissociation of CPEB from Pum1 or Pum2 and with translational activation of Cyclin B1 mRNA, a target mRNA of Pum1.[36] Thus, it is conceivable that Pumilio phosphorylation is one of the essential events for translational activation of mRNAs stored in oocytes at correct timings during maturation. Nonetheless, kinases that catalyze Pumilio phosphorylation have not yet been identified.

Nemo-like kinase (NLK) is an evolutionarily conserved atypical MAPK[40,41] and have been reported to contribute to the regulation of multiple developmental processes, including establishment of epithelial planar cell polarity during eye formation in *Drosophila*,[41] endoderm induction in *Caenorhabditis elegans*,[42] patterning of the midbrain and forebrain and neurogenesis in zebrafish,[43,44] anterior formation in *Xenopus*,[45,46] and hematopoiesis in mice.[47] In addition, a recent study has shown that levels of NLK expression in human cancer cells are higher than those in normal cells and that suppression of NLK expression arrests the cell cycle of cancer cells.[48] The fact that NLK regulates various cellular processes, such as the cell cycle, cell growth, tissue patterning and cell death, raises the possibility that this kinase also participates in the formation and maturation of gametes. To date, however, there has been no report on the existence of NLK in germ cells and its involvement in gametogenesis and gamete maturation.

In this study, we provide evidence that NLK is involved in gamete maturation (*Xenopus* oocyte maturation) by demonstrating that NLK1 is activated in a Mos-dependent manner during oocyte maturation and that NLK1 overexpression not only accelerates progesterone-induced maturation but also induce maturation without progesterone by the enhancement of Cyclin B1 protein synthesis via the translational activation of its mRNA, in accordance with Pum1, Pum2, and CPEB phosphorylation. We suggest that NLK1, as a kinase downstream of Mos, catalyzes Pum1, Pum2, and CPEB phosphorylation to regulate the translation of mRNAs, including Cyclin B1 mRNA, stored in oocytes.

EXPERIMENTAL PROCEDURES

Animals and Oocytes. All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University. *Xenopus laevis* frogs were purchased from a dealer and maintained in laboratory aquaria at 20 °C. Oocytes were isolated from ovaries and cultured in modified Barth's saline buffered with HEPES (MBS-H), as described previously.[35] Oocyte maturation was assessed by the appearance of a white spot at the animal pole, which indicates the occurrence of germinal vesicle breakdown (GVBD), a hallmark of the initiation of oocyte maturation.

Production of Anti-NLK1 Antibodies. cDNAs encoding an open reading frame (ORF) and a C-terminal region (amino acid residues 795−1341) of NLK1 (named NLK1full and NLK1c, respectively) were amplified by RT-PCR using primer sets, NLK1F and NLK1R for NLK1full and NLK1cF and NLK1R for NLK1c, which were designed according to a sequence, AB071285, deposited in the DNA databank (Table 1), and inserted into pENTR/D-TOPO Gateway vector (Invitrogen).

Table 1. Sequences of Oligonucleotides Used in This Study

For cDNA Construction
NLK1F: 5′-CACCATGGCAAACGTGTTGCTCTCA-3′
NLK1cF: 5′-CACCCAGAGCCCCATTCAACAG-3′
NLK1R: 5′-TCTTTCTTCCTTCTTGGAAACTTTTG-3′
NLK1ClaIF: 5′-ATCGATATGGCCTTCCCGGGCTCGGG-3′
NLK1XhoIR: 5′-CTCGAGTCTTTCTTCCTTCTTGGAAACTTT-3′
MosF: 5′- CACCATGCCTTCCCCAATCCCCGT-3′
MosR: 5′-TAGACCAGTGCCGAGAGGAG-3′
CPEB-F: 5′-GAATTCAATGGCCTTCCCACTGAAA-3′
CPEB-R: 5′-CCGCTCGAGGCTGGAGTCACGACTTTTCTG-3′
For Mutagenesis
NLK1K89M: 5′-GGCAAACGTGTTGCTCTCATGAAGATGC CGAATGTC-3′
NLK1K89Mc: 5′-GACATTCGGCATCTTCATGAGAGCAACACG TTTGCC-3′
S144A: 5′-GCCCCATGGGGAAGCCAGCCCCCTTGGGCTTTCTG-3′
S144Ac: 5′-CAGAAAGCCCAAGGGGGCTGGCTTCCCCATGG GGC-3′
S174/180A: 5′-CGATTGGACGCCCGCTCTATTTTGGATGCTC GCTCC-3′
S174/180Ac: 5′-GGAGCGAGCATCCAAAATAGAGCGGGCGTC CAATCG-3′
S184A: 5′-GGATTCTCGCTCCAGCGCCCCTTCTGACTCTGACAC-3′
S184Ac: 5′-GTGTCAGAGTCAGAAGGGGCGCTGGAGCGAGA ATCC-3′
S210A: 5′-CCTAATTTCAAGTCTGCGCATCGCTCCTCCGCTGC-3′
S210Ac: 5′-GCAGCGGAGGAGCGATGCGCAGACTTGAAATTAGG-3′
For Mos Knockdown
Control MO: 5′-CCTCTTACCTCAGTTACAATTTATA-3′
Mos MO: 5′-GCATTGCTGTGTGACTCGCTGAAAC-3′
For RNA-Ligation-Coupled RT-PCR
P1: 5′-P-GGTCACCTTGATCTGAAGC-NH_2-3′
P1′: 5′-GCTTCAGATCAAGGTGACCTTTTT-3′
Cyclin B1-1stF: 5′-CATCACTATATGGCTTACTCTGAA-3′
Cyclin B1-2ndF: 5′-GTGGCATTCCAATTGTGTATTGTT-3′
Mos-1stF: 5′-TCACTAGTAGCCAGGAGTTCAT-3′
Mos-2ndF: 5′-GTTGCATTGCTGTTTAAGTGGTAA-3′

The resulting plasmids were recombined with the destination vectors pDEST15 and pET161-DEST using the Gateway cloning system (Invitrogen) to produce proteins tagged with a glutathione-*S*-transferase (GST) at the N-terminus and with a polyhistidine (His) at the C-terminus, respectively. His-tagged NLK1full and NLK1c proteins were expressed in *Escherichia coli* and purified for use as antigens according to the method described previously.[49] NLK1full-His and NLK1c-His were injected into guinea pigs and mice, respectively, to produce antibodies. Antisera against NLK1full-His and NLK1c-His were affinity-purified with GST-NLK1full and GST-NLK1c, respectively.

Production of Flag-NLK1. To produce Flag-tagged NLK1, an ORF of NLK1 was amplified by RT-PCR using a primer set, NLK1ClaIF and NLK1XhoIR (Table 1), and ligated into pCS2+FT-N, which was produced by inserting a Flag epitope

into the *Bam*HI site of pCS2+.[50] A kinase-dead version of NLK1, in which lysine 89 was mutated to methionine (K89M), was produced by a QuickChange Site-Directed Mutagenesis Kit (Stratagene) with a primer set, NLK1K89 M and NLK1K89Mc (Table 1). Using the corresponding plasmids as templates, mRNAs encoding the wild-type or kinase-dead form of Flag-NLK1 were synthesized with an mMESSAGE mMACHINE SP6 Kit (Ambion).

Production of GST-Mos. GST-tagged Mos was produced by amplification of Mos ORF by RT-PCR using a MosF/MosR primer set (Table 1) and insertion of the ORF into pENTR/D-TOPO Gateway vector. The resulting plasmid was recombined with pDEST15 vector. GST-Mos mRNA was synthesized with an mMESSAGE mMACHINE T7 Kit (Ambion) using the cDNA in pDEST15 vector as a template.

Production of Flag-CPEB Mutants. Wild-type CPEB and six mutant forms that lack critical phosphorylation sites (6A, S138/144/184/210/248/423A; 2A, S174/180A; 6A+2A; S144A; S144/184A; S144/184/210A) were used in this study. To produce Flag-tagged CPEB, an ORF of CPEB was amplified by PCR from wild-type CPEB in pET21c[34] or 6A CPEB in pT7-GST II (a gift from Prof. Sagata, Kyushu University)[51] with a CPEB-F/CPEB-R primer set (Table 1). The resulting PCR products were ligated into pCS2+FT-N. Using 6A and wild-type CPEB cDNA in pCS2+FT-N as a template, 6A+2A and 2A CPEB was produced by a QuickChange Site-Directed Mutagenesis Kit with a primer set, S174/180A and S174/180Ac (Table 1), respectively. Similarly, S144A, S144/184A, and S144/184/210A mutants were produced with appropriate primer sets (Table 1), using wild-type CPEB cDNA in pCS2+FT-N as a template. Wild-type and mutant Flag-CPEB mRNAs were synthesized from the corresponding cDNAs in pCS2+FT-N with an mMESSAGE mMACHINE SP6 kit.

Oocyte Microinjection. To inhibit Mos synthesis, oocytes were manually isolated from ovaries with forceps and injected with 50 nL of 0.4 mg/mL antisense morpholino oligonucleotide (MO, Gene tools; Table 1). Following injection, oocytes were incubated in MBS-H overnight at 18 °C, treated with progesterone to induce maturation, and extracted for subsequent analyses as described below.

Overexpression of NLK1. For overexpression of NLK1, oocytes were injected with 50 nL of 0.1 or 1.0 mg/mL Flag-NLK1 mRNA and incubated in MBS-H for 14 h at 18 °C. After the oocytes had been treated with or without progesterone, GVBD was monitored and oocyte extracts were prepared for immunoblotting, RNA ligation-coupled RT-PCR, and NLK kinase assay at appropriate intervals. For phosphorylation assay (described below), oocytes were injected with a mixture of 0.1 mg/mL Flag-NLK1 mRNA and 0.06 mg/mL Flag-CPEB mRNA to coexpress these proteins.

Oocyte Extraction. Oocytes were washed three times with ice-cold extraction buffer (EB: 100 mM β-glycerophosphate, 1% Triton X-100, 20 mM HEPES, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol, 100 μM (*p*-amidinophenyl) methanesulfonyl fluoride, 3 μg/mL leupeptin, pH 7.5) containing RNasin Plus RNase Inhibitor (100 U/mL at the final concentration, Promega). After the final wash, EB was removed with filter paper, and 1 μL/oocyte of new EB was added. The oocytes were homogenized and centrifuged at 8000*g* for 5 min at 4 °C. The supernatant was collected and stored at −80 °C until use.

Immunoblotting. Crude oocyte extracts (1 μL, corresponding to one oocyte) were separated by SDS−PAGE, blotted onto an Immobilon membrane, and probed with anti-NLK1c, anti-Mos (a gift from Prof. Sagata), anti-MAPK 1/2 (Upstate), anti-phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling Technology), anti-Pum1N, anti-Pum2N,[36] and anti-CPEB[34] antibodies. A complex of Cyclin B1 and Cdc2 was detected by immunoblotting Suc1-precipitates from crude oocyte extracts (10 μL) with anti-Cdc2 (MC2−21)[8] and anti-Cyclin B1 (Bufo 2F5)[52] monoclonal antibodies, as described previously.[53] Monoclonal anti-Flag M2 antibody (Sigma) was used to precipitate and detect Flag-tagged proteins.

RNA Ligation-Coupled RT-PCR. For RNA ligation-coupled RT-PCR,[25,54] total RNA was incubated with 50 pmol of P1 primer (Table 1) and 4 U of T4 RNA ligase 1 (New England Biolabs) for 30 min at 37 °C and boiled for 2 min at 98 °C. The P1-ligated RNA was reverse-transcribed using 50 pmol of P1′ primer (Table 1). The resulting cDNA (5 μL) was used to examine the poly(A) length of Cyclin B1 and Mos mRNAs by two rounds of PCR using an Expand High Fidelity PCR system (Roche) with P1′ primer and gene specific first primer (Table 1) in the first PCR (15 cycles) and P1′ primer and gene specific second primer (Table 1) in the second PCR (25 cycles; 1 μL of 10-fold diluted sample of the first PCR product with distilled water was used in the second PCR).

Phosphorylation Assay. Kinase activity of NLK1 was measured by its autophosphorylation levels as follows. Immature and mature oocyte extracts were immunoprecipitated with anti-NLK1full antibody and protein A-Sepharose beads (GE Healthcare). After being washed three times with EB and once with ST buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0), the resulting immunoprecipitates were incubated in 20 μL of NLK kinase buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM ATP, 5 mCi [γ-^{32}P]ATP) for 20 min at 30 °C and then immunoblotted. The blots were subjected to autoradiography to detect the radioisotope incorporated into NLK1 proteins.

Production of NLK1 Substrates. Using *E. coli* strains carrying GST and CPEB cDNAs in pET21[34] and Pum1 and Pum2 cDNAs in pET161DEST,[36] His-tagged proteins were produced and purified for use as substrates of NLK1 in vitro. Flag-NLK1 was expressed in oocytes, immunoprecipitated by anti-Flag M2 antibody, and incubated in 20 μL of NLK kinase buffer containing 0.5 mg/mL (at the final concentration) of *E. coli*-produced His-GST, His-Pum1, His-Pum2, and His-CPEB. Following kinase reaction for 20 min at 30 °C, His-tagged proteins were immunoblotted with anti-His G-18 antibody (Santa Cruz Biotechnology) and subjected to autoradiography to examine the incorporation of ^{32}P into the substrate proteins.

Treatment of Oocytes with U0126. The MEK inhibitor U0126 (Cell Signaling Technology) was dissolved in dimethyl sulfoxide (DMSO, SIGMA) as a stock solution (50 mM) and added to MBS-H immediately before use at the final concentration of 50 μM. Oocytes were incubated for 1 h in the presence of U0126 or DMSO as a vehicle control before injection with mRNA. Treatment of the injected oocytes with U0126 or DMSO was continued until the oocytes were extracted for subsequent analyses.

RESULTS

Behaviors of NLK1 and Other Key Proteins during Oocyte Maturation. To detect *Xenopus* NLK1, we produced two antibodies, one raised against the C-terminal fragment of NLK1 (NLK1c) and the other raised against the full-length protein (NLK1full). Anti-NLK1c antibody recognized two bands with

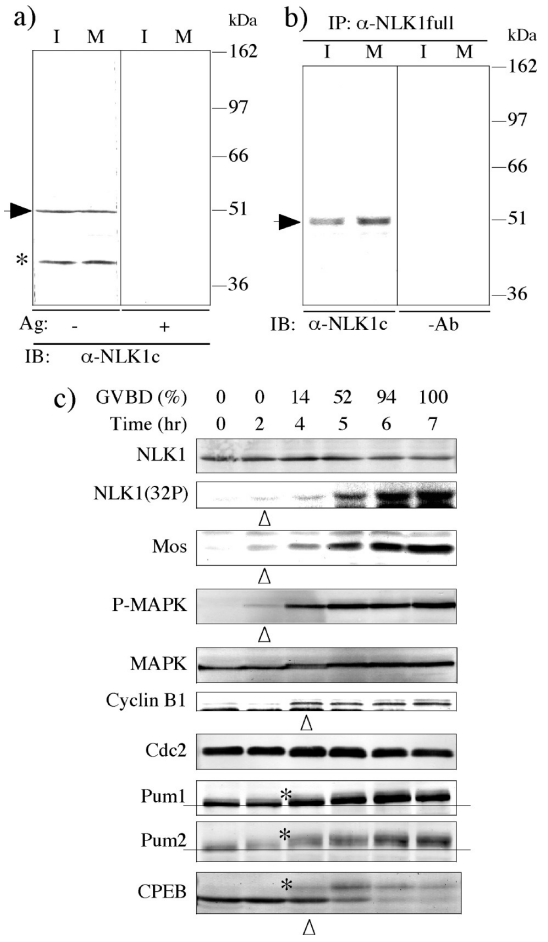

Figure 1. Behaviors of NLK1 and other key molecules during oocyte maturation: (a) Crude extracts from immature (I) and mature (M) oocytes were immunoblotted (IB) with anti-NLK1c antibody (α-NLK1c) pretreated with (+) or without (−) GST-NLK1c (Ag) to confirm the specificity of signals. An arrow indicates NLK1. An asterisk shows a 40-kDa band that is recognized by anti-NLK1c antibody but not by anti-NLK1full antibody. (b) Immature (I) and mature (M) oocyte extracts (40 μL) were immunoprecipitated (IP) with anti-NLK1full antibody, and the resulting immunoprecipitates were immunoblotted (IB) with or without (-Ab) anti-NLK1c antibody. (c) Immature oocytes were treated with progesterone and extracted at the times indicated in the figure for immunoblotting with antibodies against NLK1, Mos, phospho-MAPK (recognizing active MAPK), MAPK (recognizing both inactive and active MAPK), Pum1, Pum2, and CPEB. Suc1-precipitates from oocyte extracts were immunoblotted with antibodies against Cyclin B1 and Cdc2 to examine the presence of their complex. To measure the kinase activity (autophosphorylation activity) of NLK1, anti-NLK1full immunoprecipitates were incubated with [γ-^{32}P]ATP, blotted onto a membrane, and subjected to autoradiography to detect the incorporation of ^{32}P into NLK1 (^{32}P). The time points of the initiation of MAPK and NLK1 activation, Mos and Cyclin B1 protein synthesis, and Pum1/Pum2/CPEB phosphorylation are indicated by arrowheads. Asterisks show phosphorylated forms of Pumilio with slower electrophoretic mobility.

apparent molecular masses of 50 and 40 kDa in both immature and mature oocyte extracts, and these bands disappeared when the antibody was absorbed by recombinant NLK1c proteins prior to immunoblotting (Figure 1a). To further characterize the immunoreactive bands, we performed an immunoprecipitation assay using anti-NLK1full antibody, since anti-NLK1c antibody is unable to precipitate antigenic proteins from extracts (data not shown). The 50-kDa band was detected in the anti-NLK1full immunoprecipitates from immature and mature oocyte extracts (Figure 1b), whereas the 40-kDa band was not. According to these results and the fact that the molecular mass of NLK1 estimated from its cDNA sequence is 50 kDa, we propose that the 50-kDa band, but not the 40-kDa band, is NLK1 in *Xenopus* oocytes.

We then examined changes in protein content and kinase activity of NLK1 during oocyte maturation, in comparison with Mos and Cyclin B1 protein synthesis, p42 MAPK activation and Pum1, Pum2, and CPEB phosphorylation (Figure 1c). The protein content of NLK1 remained constant during oocyte maturation. Although NLK1 was inactive in immature oocytes, it began to be activated 2 h after progesterone treatment as demonstrated by the occurrence of its autophosphorylation, and the activity increased during the process of maturation. The NLK1 activation coincided with Mos protein synthesis and p42 MAPK activation (as revealed by appearance of the phosphorylated form of MAPK that was detected by anti-phospho-MAPK antibody). Cyclin B1 synthesis and Pum1, Pum2, and CPEB phosphorylation (phosphorylated forms of Pumilio and CPEB showing a slower electrophoretic mobility[36]) were detected 4 h after progesterone treatment, a timing apparently later than that of NLK1 activation (Figure 1c).

Dependence of NLK1 Activation on Mos but not on MAPK. Transforming growth factor β-activated kinase 1 is a MAP kinase kinase kinase and activates NLK in a Wnt signaling pathway.[55,56] Since Mos also functions as a MAP kinase kinase kinase in *Xenopus* oocytes,[12−14] it is plausible that an upstream activator of NLK1 is Mos. The coincidence between the appearance of Mos and the activation of NLK1 during oocyte maturation (Figure 1c) seems to support this notion. To verify this possibility, we examined NLK1 activation under conditions in which Mos synthesis was inhibited by MO injection during progesterone-induced oocyte maturation (Figure 2a). Injection of control MO into oocytes had no effect on Mos synthesis and NLK1 activation. Injection of Mos MO, however, suppressed Mos synthesis and NLK1 activation (Figure 2a, lane 6) and retarded the progression of maturation (Figure 2b). We also examined the dependency of NLK1 activation on Mos by overexpression of GST-Mos in oocytes by mRNA injection. GST-Mos overexpression induced NLK1 activation and oocyte maturation without progesterone (Figure 2c). These results demonstrate that Mos is both necessary and sufficient for NLK1 activation during *Xenopus* oocyte maturation, although the activation is not a prerequisite for the induction of oocyte maturation, since Mos MO-injected oocytes matured without NLK1 activation (Figure 2a, lane 6).

The finding that Mos is an upstream activator of NLK1 prompted us to test whether p42 MAPK, a kinase downstream of Mos, participates in NLK1 activation. To this end, we treated GST-Mos-injected oocytes with the MEK inhibitor U0126 to prevent MAPK activation. NLK1 was activated in the absence of active p42 MAPK in U0126-treated oocytes (Figure 2d, lanes 2 and 4), consistent with the results showing that NLK1 was not activated even in the presence of active p42 MAPK in Mos MO-injected oocytes (Figure 2a, lane 6). It is thus concluded that NLK1 is activated in a MAPK-independent manner during oocyte maturation.

Effects of NLK1 Overexpression on Progesterone-Induced Oocyte Maturation. We noticed that when wild-type, but not

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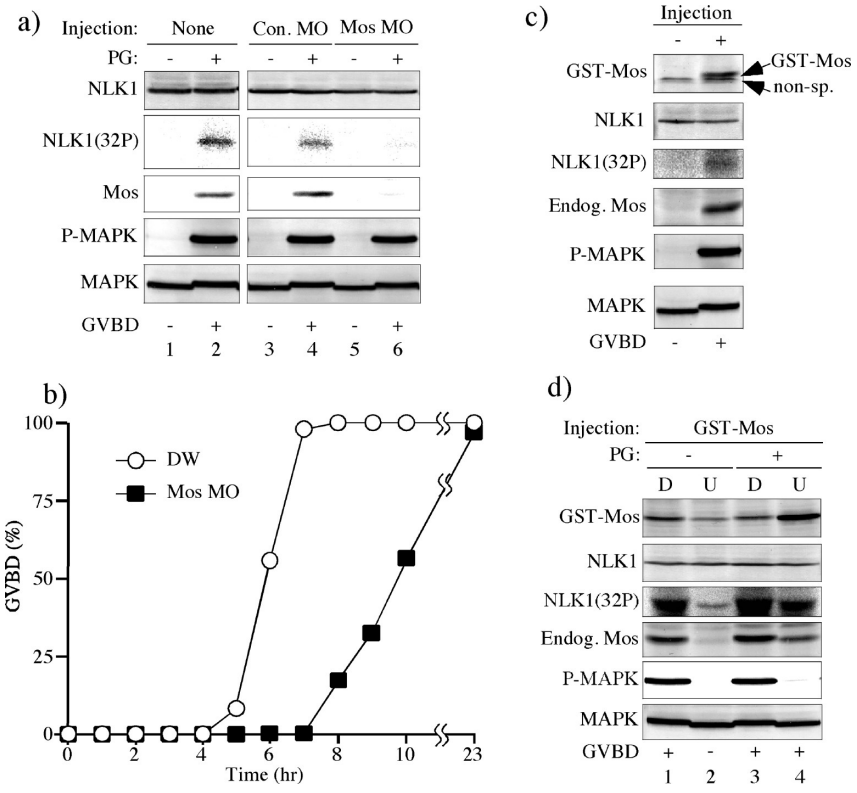

Figure 2. Involvement of Mos in NLK1 activation during oocyte maturation: (a) Mos MO-injected oocytes were incubated overnight, treated with (+) or without (−) progesterone to induce maturation, and extracted 12 h after the progesterone treatment (only the oocytes that had undergone GVBD being extracted as PG+ samples) for immunological detection of NLK1, Mos, phospho-MAPK (P-MAPK), and MAPK. Autophosphorylation of NLK1 was detected by incubating anti-NLK1 immunoprecipitates with [γ-^{32}P]ATP followed by autoradiography (^{32}P) to show the activation of NLK1. As controls, uninjected (None) and control MO-injected (Con. MO) oocytes were similarly examined. The occurrence (+) or nonoccurrence (−) of GVBD in each experiment is indicated. (b) Oocytes were injected with distilled water (DW) or 20 ng of Mos MO, incubated overnight, induced to mature with progesterone, and examined for the occurrence of GVBD at indicated times. Representative results are shown. (c) Oocytes were injected with (+) or without (−) 20 ng of GST-Mos mRNA, incubated for 12 h, and extracted for immunological detection of GST-Mos, NLK1, endogenous Mos, phospho-MAPK (P-MAPK), and MAPK. The occurrence (+) or nonoccurrence (−) of GVBD in each experiment is also indicated. A band detected just below the GST-Mos band is a nonspecific signal (nonsp.). (d) In the presence of 0.1% DMSO (D) as a vehicle control or 50 μM U0126 (U), oocytes were injected with 20 ng of GST-Mos mRNA, incubated for 2 h, treated with (+) or without (−) progesterone to induce maturation, and extracted for immunological detection of GST-Mos, NLK1, endogenous Mos, phospho-MAPK (P-MAPK), and MAPK. Activation of NLK1 was detected by its autophosphorylation (^{32}P). The occurrence (+) or nonoccurrence (−) of GVBD in each experiment is also indicated.

kinase-dead, Flag-NLK1 was expressed in immature oocytes, it is automatically activated in the absence of Mos (Figure 3a,d), probably by a positive feedback mechanism via its autophosphorylation.[57] We exploited this to examine whether artificial introduction of active NLK1 into oocytes can affect progesterone-induced maturation. Wild-type NLK1-expressed and progesterone-treated oocytes underwent GVBD more rapidly than did control oocytes injected with distilled water or mRNA encoding kinase-dead NLK1 in a dose-dependent manner (Figure 3b,c,f,g).

To gain a further insight into the roles of NLK1 in oocyte maturation, we investigated several key proteins required for oocyte maturation in oocytes injected with 5 ng of Flag-NLK1 mRNA (Figure 3d). NLK1 expression had no effect on the timing of Mos protein synthesis and MAPK activation; they were initiated 2 h after progesterone treatment in oocytes injected with wild-type Flag-NLK1 mRNA, similar to uninjected (intact) oocytes, in agreement with the notion that NLK1 is located downstream from Mos. On the other hand, expression of wild-type Flag-NLK1 accelerated Cyclin B1 protein synthesis (Figure 3d), which was accompanied by its mRNA polyadenylation (Figure 3e), in accordance with the fact that polyadenylation-mediated translational activation of Cyclin B1 mRNA induces oocyte maturation.[6,58,59] NLK1 expression not only enhanced Pum1, Pum2, and CPEB phosphorylation but also induced their phosphorylation without progesterone treatment (Figure 3d, compare the lanes at 0 h of the intact and the overexpressed oocytes), suggesting that NLK1 phosphorylates Pum1, Pum2, and CPEB during oocyte maturation. Expression of kinase-dead NLK1 had no effects on Pum1/Pum2/CPEB phosphorylation or Cyclin B1 mRNA polyadenylation (data not shown).

Induction of Oocyte Maturation by NLK1 Overexpression. Overexpression of NLK1 by injection of 5 ng of mRNA did not induce oocyte maturation by itself. When oocytes were injected with 50 ng of mRNA, however, they matured in the absence of progesterone, although they underwent GVBD much more slowly than progesterone-treated oocytes (Figure 4a). In these oocytes, MAPK was activated, Mos and Cyclin B1 proteins were synthesized in accordance with the polyA tail elongation of their mRNAs, Pum1, Pum2, and CPEB were fully phosphorylated (as judged by the extent of their electrophoretic mobility sift), and CPEB was degraded (Figure 4b,c), similar to oocytes matured by

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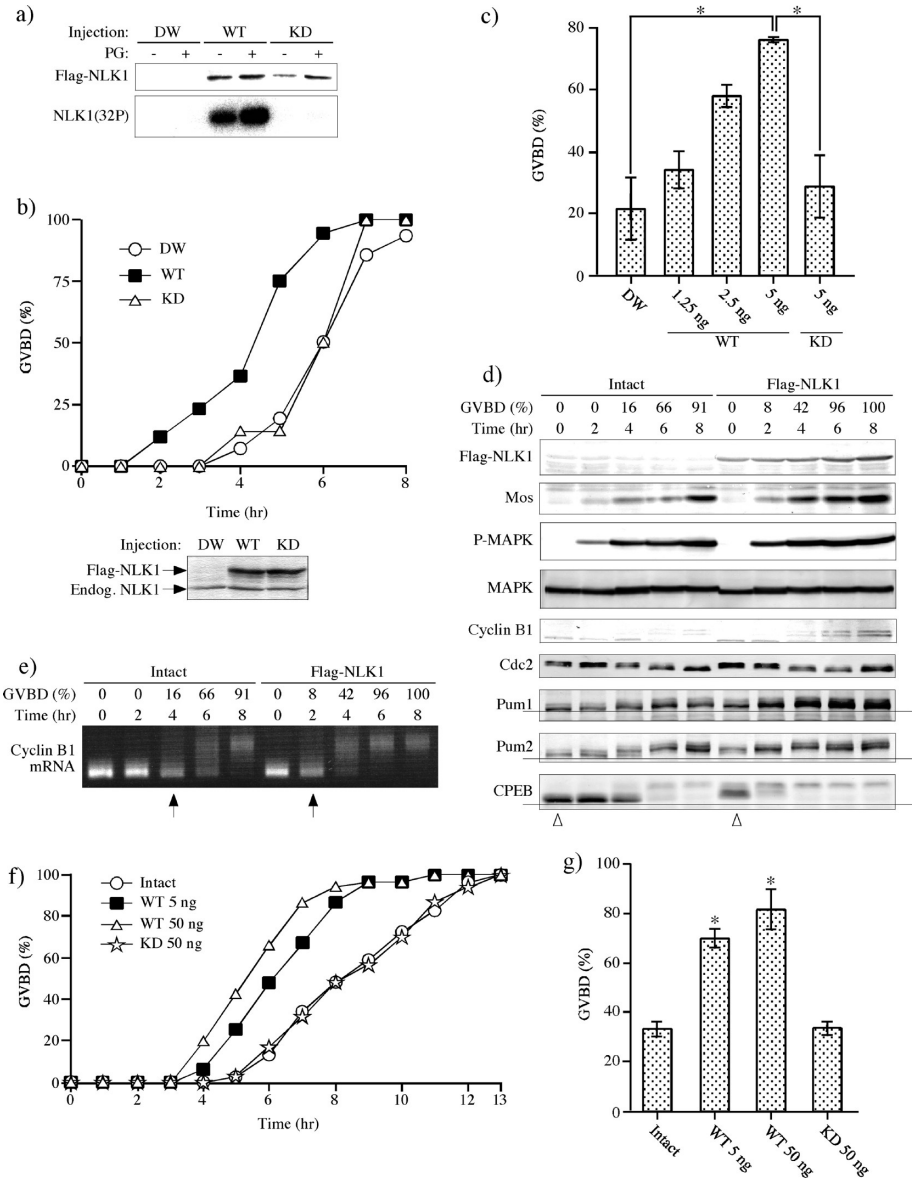

Figure 3. Acceleration of progesterone-induced oocyte maturation by Flag-NLK1: (a) Oocytes were injected with distilled water (DW) or 5 ng of mRNAs encoding wild-type (WT) or kinase-dead (KD) NLK1. The injected oocytes were incubated overnight, induced to mature with (+) or without (−) progesterone (PG), and extracted for immunoprecipitation with anti-Flag antibody followed by immunoblotting with the same antibody to detect Flag-NLK1. The blots were then subjected to autoradiography to detect active NLK1 (^{32}P). (b) Oocytes were injected with distilled water (DW) or 5 ng of mRNAs encoding wild-type (WT) or kinase-dead (KD) NLK1. The injected oocytes were incubated overnight and induced to mature with progesterone. The occurrence of GVBD was monitored at indicated times (upper panel), and the oocytes were extracted for immunoblotting with anti-NLK1c antibody (lower panel) to detect Flag-NLK1 and endogenous NLK1. Note that the protein levels of Flag-NLK1 expressed in the oocytes are noticeably higher than those of endogenous NLK1. (c) Percentage of GVBD in oocytes injected with distilled water (DW) or with the indicated amount of mRNA encoding either wild-type (WT) NLK1 or kinase-dead (KD) NLK1. Values are means ± SEM for three independent experiments. Overexpression of WT NLK1, but not that of KD NLK1, accelerated progesterone-induced GVBD significantly as shown by asterisks ($P < 0.01$, Student's *t*-test). (d) Changes in protein contents in oocytes uninjected (Intact) or injected with 5 ng of wild-type Flag-NLK1 mRNA. The oocytes were treated with progesterone and extracted for immunological detection of Flag-NLK1, Mos, phospho-MAPK (P-MAPK), MAPK, Cyclin B1, Cdc2, Pum1, Pum2, and CPEB. Note that the timing of phosphorylation of Pum1, Pum2, and CPEB (as shown by electrophoretic mobility shift) is enhanced in Flag-NLK1-overexpressed oocytes compared with that in intact oocytes. It is also notable that Pum1/Pum2/CPEB phosphorylation is induced in Flag-NLK1-overexpressed oocytes without progesterone treatment (indicated by arrowheads), although its level (at 0 h) is not comparable to that in mature oocytes (at 8 h). (e) Total RNAs were extracted from crude oocyte extracts used in (d) and subjected to RNA ligation-coupled RT-PCR to examine the state of polyadenylation of Cyclin B1 mRNA. Arrows indicate the time points at which the polyadenylation of Cyclin B1 mRNA is initiated. (f, g) Similar experiments to (c) and (d). Oocytes uninjected (Intact) or injected with the indicated amount of mRNA encoding either wild-type (WT) NLK1 or kinase-dead (KD) NLK1, incubated for 14 h, treated with progesterone and monitored the occurrence of GVBD at appropriate times in (f). Percentage of GVBD in oocytes uninjected (intact) or injected with the indicated amount of mRNA encoding either wild-type (WT) NLK1 or kinase-dead (KD) NLK1 is shown in (g). Values are means ± SEM for three independent experiments. Overexpression of WT NLK1, but not that of KD NLK1, accelerated progesterone-induced GVBD significantly as shown by asterisks ($P < 0.005$, Student's *t*-test).

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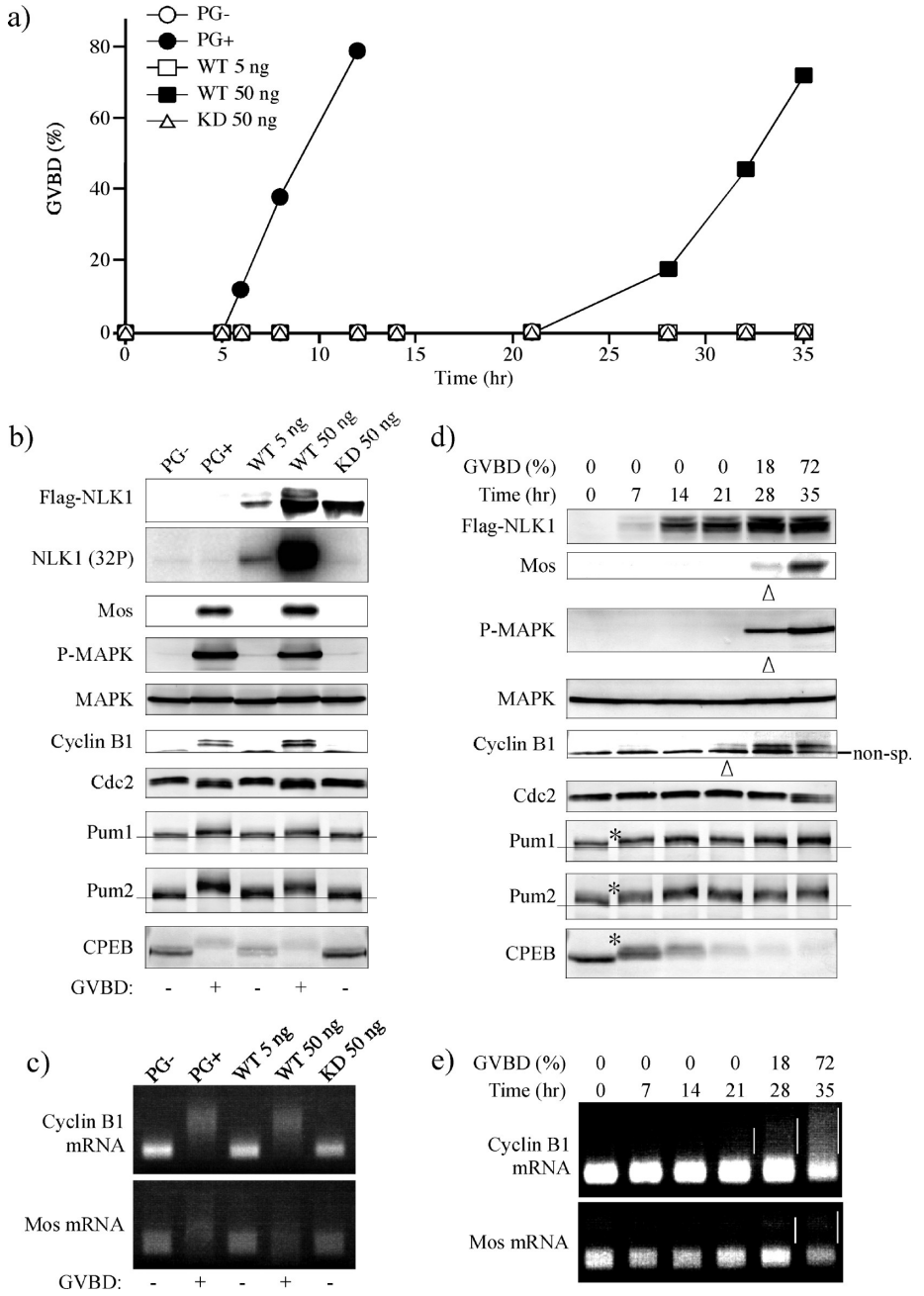

Figure 4. Induction of oocyte maturation by Flag-NLK1: (a) Oocytes were treated with (PG+) or without (PG-) progesterone or injected with 5 ng or 50 ng of mRNAs encoding wild-type (WT) or kinase-dead (KD) NLK1 and monitored the occurrence of GVBD at appropriate times. (b) PG+ and other oocytes in (a) were extracted at 12 and 35 h, respectively. The resulting extracts were used for immunological detection of Flag-NLK1, Mos, phospho-MAPK (P-MAPK), MAPK, Cyclin B1, Cdc2, Pum1, Pum2, and CPEB. To measure the kinase activity of Flag-NLK1, anti-Flag immunoprecipitates were incubated with [γ-^{32}P]ATP, blotted onto a membrane, and subjected to autoradiography to detect the incorporation of ^{32}P into Flag-NLK1 (^{32}P). (c) Total RNAs were extracted from crude oocyte extracts used in (b) and subjected to RNA ligation-coupled RT-PCR to examine the state of polyadenylation of Cyclin B1 and Mos mRNA. The occurrence (+) or nonoccurrence (−) of GVBD in each experiment is also indicated. (d) Oocytes injected with 50 ng of mRNA encoding wild-type NLK1 were collected at intervals of 7 h, extracted and immunoblotted with antibodies against Flag tag (for Flag-NLK1), Mos, phospho-MAPK (P-MAPK), MAPK, Cyclin B1, Cdc2, Pum1, Pum2, and CPEB. To measure the kinase activity of Flag-NLK1, anti-Flag immunoprecipitates were incubated with [γ-^{32}P]ATP, blotted onto a membrane, and subjected to autoradiography to detect the incorporation of ^{32}P into Flag-NLK1 (^{32}P). The time points of the initiation of MAPK activation and Mos and Cyclin B1 protein synthesis are indicated by arrowheads. Asterisks show phosphorylated forms of Pumilio and CPEB with slower electrophoretic mobility. (e) Total RNAs were isolated from crude oocyte extracts used in (d) and subjected to RNA ligation-coupled RT-PCR to examine the state of polyadenylation of Cyclin B1 and Mos mRNA. White bars indicate the polyadenylation of Cyclin B1 and Mos mRNAs.

progesterone. We then examined sequential changes of these events during NLK1-induced oocyte maturation. In the NLK1-overexpressed oocytes, the polyadenylation-mediated translational activation of Cyclin B1 mRNA preceded that of Mos

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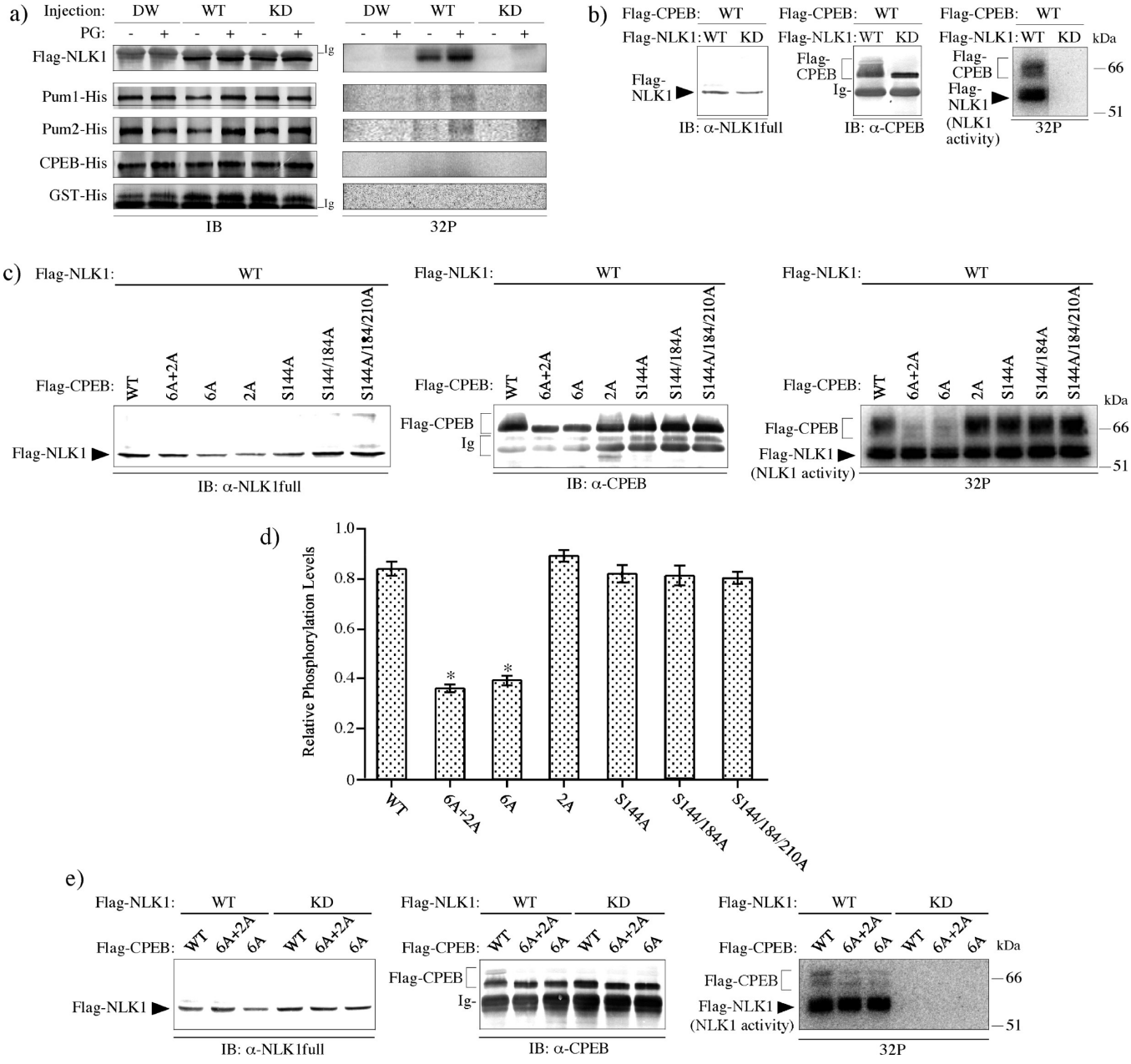

Figure 5. NLK1-catalyzed phosphorylation of Pum1, Pum2, and CPEB: (a) Oocytes were injected with distilled water (DW) or 5 ng of mRNAs encoding wild-type (WT) or kinase-dead (KD) NLK1, incubated for 14 h, treated with (+) or without (−) progesterone (PG), and extracted for immunoprecipitation with anti-Flag antibody. Following incubation of the resulting precipitates with *E. coli*-produced Pum1-His, Pum2-His, CPEB-His, and GST-His for 20 min at 30 °C in the presence of [γ-^{32}P]ATP, the samples were immunoblotted (IB) with anti-NLK1full antibody to show Flag-NLK1 or anti-His antibody to show Pum1-His, Pum2-His, CPEB-His, and GST-His. The blots were then subjected to autoradiography to detect the incorporation of ^{32}P into each protein (^{32}P). Immunoglobulins of the antibody used for immunoprecipitation are also indicated (Ig). (b) Oocytes were coinjected with 3 ng of mRNA encoding Flag-CPEB and 5 ng of mRNA encoding either the wild-type (WT) or kinase-dead (KD) form of Flag-NLK1. After incubation overnight, the oocytes were extracted and immunoprecipitated with anti-Flag antibody to precipitate Flag-CPEB and WT or KD Flag-NLK1. Following kinase reaction in the presence of [γ-^{32}P]ATP, the samples were immunoblotted (IB) with anti-NLK1full and anti-CPEB antibodies and subjected to autoradiography (^{32}P) to detect the radioisotope incorporated into Flag-CPEB and Flag-NLK1. (c) Immature oocytes were injected with mRNAs indicated in the figure. Following overnight culture, the oocytes were extracted and immunoprecipitated with anti-Flag antibody. The resulting immunoprecipitates were incubated with [γ-^{32}P]ATP to allow Flag-NLK1 to phosphorylate Flag-CPEB as well as Flag-NLK1 itself by autophosphorylation. After the phosphorylation reaction, the samples were immunoblotted (IB) with anti-NLK1full or anti-CPEB antibody to detect Flag-NLK1 or Flag-CPEB, respectively. The blots were also subjected to autoradiography (^{32}P) to detect the ^{32}P-label of Flag-CPEB and Flag-NLK1. (d) Quantification of the phosphorylation levels of each CPEB. The signal intensity of individual bands of CPEB on the kinase assay was measured by using ImageJ software. Data are shown as means ± SEM for three independent experiments using different batches of oocytes. The phosphorylation levels of 6A+2A and 6A CPEB reduced significantly as shown by asterisks ($P < 0.005$, Student's t-test). (e) Similar experiments to (c). Immature oocytes were injected with mRNAs indicated in the figure. Following overnight culture, the oocytes were extracted and subjected to phosphorylation assay.

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mRNA (Figure 4d,e), in striking contrast to the fact that Cyclin B1 mRNA activation occurs later than Mos mRNA activation in progesterone-treated oocytes (Figure 1c). This finding is consistent with the notion that the maturation of NLK1-overexpressed oocytes bypasses the Mos-mediated processes to initiate. In accordance with the accumulation of Flag-NLK1, phosphorylation levels of Pum1, Pum2, and CPEB were elevated 7−14 h after the injection of NLK1 mRNA and then CPEB was degraded, followed by polyadenylation and translational activation of Cyclin B1 mRNA (Figure 4d,e). These results suggest that NLK1 catalyzes Pum1/Pum2/CPEB phosphorylation, which leads to the translational activation of Cyclin B1 mRNA.[36,60−62]

Phosphorylation of Pum1, Pum2 and CPEB by NLK1 in Vitro. The results of NLK1 overexpression experiments (Figure 4) suggest that NLK1 is a kinase responsible for Pum1/Pum2/CPEB phosphorylation. To test this, NLK1 was expressed in oocytes and the resulting proteins were immunoprecipitated and incubated with *E. coli*-produced Pum1, Pum2, and CPEB in the presence of [γ-^{32}P]ATP (Figure 5a). Pum1 and Pum2 were phosphorylated by wild-type NLK1 but not by kinase-dead NLK1. Phosphorylation of CPEB was not detected in this experiment, probably due to incorrect folding or modification of *E. coli*-produced CPEB proteins. We then used CPEB proteins that were produced in oocytes as a substrate for NLK1. When Flag-CPEB was coexpressed with wild-type or kinase-dead NLK1 in oocytes, CPEB was phosphorylated only in the oocytes expressing wild-type NLK1 (Figure 5b), indicating that CPEB, as well as Pum1 and Pum2, is a substrate of NLK1 at least in vitro.

As a first step toward understanding NLK1-catalyzed phosphorylation sites of CPEB, we focused on the sites that have already been identified. It is known that CPEB undergoes two steps of phosphorylation;[60−62] the first phosphorylation takes place on S174 in the early phase of oocyte maturation and the second phosphorylation takes place on six serine residues (S138, 144, 184, 210, 248, and 423) in the later phase. We thus examined whether NLK1 phosphorylates three mutant forms of CPEB: 6A CPEB, in which all of the six serine residues in the second group of phosphorylation sites were changed to alanine (S138/144/184/210/248/423A); 2A CPEB, in which S174 and S180 in the first group of phosphorylation sites were changed to alanine (S174/180A); and 6A+2A CPEB, in which the two groups of phosphorylation sites were changed to alanine (S138/144/184/210/248/423A+S174/180A). Immature oocytes were injected with a mixture of mRNA encoding wild-type or kinase-dead Flag-NLK1 and that encoding wild-type, 6A+2A, 6A or 2A Flag-CPEB, and the Flag-tagged proteins were immunoprecipitated. The resulting precipitates were incubated in the presence of [γ-^{32}P]ATP to allow Flag-NLK1 to phosphorylate Flag-CPEB (Figure 5c,e).

Phosphorylation level of 6A CPEB was markedly lower than that of wild-type CPEB but similar to that of 6A+2A CPEB (Figure 5c,d). In contrast, 2A CPEB was phosphorylated at a similar level to that of wild-type CPEB (Figure 5c,d). It is therefore likely that NLK1 phosphorylates the second group of phosphorylation site, but not the first group. To further characterize NLK1-catalyzed phosphorylation sites within the S138/144/184/210/248/423 group, we used S144A, S144/184A, and S144/184/210A mutants. In comparison with wild-type CPEB or 2A CPEB, phosphorylation levels of these mutants were not significantly reduced (Figure 5c,d), suggesting that NLK1 phosphorylates S138, S248, and S423. It is also notable that although phosphorylation levels were low, 6A+2A and 6A CPEBs were evidently phosphorylated by wild-type NLK1 but not by kinase-dead NLK1 (Figure 5e), suggesting that CPEB carries NLK1-catalyzed phosphorylation sites besides the second group of phosphorylation sites.

DISCUSSION

In this study, we focused on NLK1, an atypical MAPK, during *Xenopus* oocyte maturation and obtained the following results: (1) NLK1 is expressed in immature oocytes and activated during maturation in a Mos-dependent but MAPK-independent manner, (2) overexpression of NLK1 not only accelerates progesterone-induced oocyte maturation but also induces oocyte maturation without progesterone treatment through polyadenylation-mediated translational activation of Cyclin B1 mRNA, (3) NLK1 overexpression induces and enhances phosphorylation of Pum1, Pum2, and CPEB, essential regulators for the polyadenylation-mediated translational activation of mRNAs including Cyclin B1 mRNA, and (4) NLK1 phosphorylates Pum1, Pum2, and CPEB in vitro. These findings provide the first evidence for the involvement NLK1 in oocyte maturation, in addition to its contribution to various developmental processes in vertebrates and invertebrates reported so far. Furthermore, it is suggested that NLK1, as a kinase downstream of Mos, catalyzes Pum1, Pum2, and CPEB phosphorylation to regulate the translational activation of Cyclin B1 mRNA during oocyte maturation.

The dependence of NLK1 activation on Mos and its independence of MAPK reveal the presence of at least two kinase cascades downstream of Mos in *Xenopus* oocytes, the Mos-MEK-MAPK cascade and the Mos-NLK1 cascade. The former is required for the initiation of maturation through the activation of pre-MPF[15,16] (and also the maintenance of MPF stability through the inhibition of Cyclin B proteolysis),[63,64] while the latter seems to be necessary for the normal progression of maturation through the temporal control of translation of mRNAs. In the process of transduction of progesterone signal, Mos might be an important point at which the signal is allocated to several pathways leading to the events required for initiation and progression of oocyte maturation.

Concerning the Mos-NLK1 cascade suggested in this study, it should be noted that U0126 treatment of GST-Mos-overexpressed and progesterone-treated oocytes decreased the level of NLK1 phosphorylation to some extent, in association with reduction in the level of endogenous Mos protein but that U0126 did not completely inhibit the NLK1 phosphorylation (Figure 2d, lane 4). Since U0126 completely inhibited the activation of MAPK, MEK should also be inhibited completely in these oocytes. Nevertheless, the U0126 treatment had only a limited effect on Mos-induced NLK1 activation, implying that Mos does not activate NLK1 directly but activates it through MEK-like kinase that has a lower sensitivity to U0126 than that of the genuine MEK, although we cannot exclude the possibility that the decrease in NLK1 activity is simply attributed to the reduction in endogenous Mos protein level caused by dysfunction in the feedback mechanism involving Mos/MEK/MAPK and Cdc2 that is required to sustain the Mos level during maturation.[12,65]

The timings of translational activation of dormant mRNAs stored in oocytes are precisely controlled during oocyte maturation.[23−26,30,35,58,66] A chief player for the temporal control of translational activation of dormant mRNAs during *Xenopus*

oocyte maturation is CPEB, and its phosphorylation is known to be a crucial step to trigger the translational activation of target mRNAs.[27−29] Similar to CPEB, we previously reported that Pum1 and Pum2, *trans*-acting factors controlling the translation of mRNAs, are phosphorylated during oocyte maturation, with the proposal that their phosphorylation is required for translational activation of their target mRNAs.[36] In the present study, we found that NLK1 is a kinase that catalyzes phosphorylation of Pum1, Pum2, and CPEB at least in vitro (Figure 5) and that overexpression of NLK1 in oocytes induces phosphorylation of Pum1, Pum2 and CPEB, degradation of CPEB, synthesis of Cyclin B1 protein, and oocyte maturation in the absence of progesterone (Figure 4). These findings suggest that NLK1 is involved in oocyte maturation as a kinase responsible for Pum1, Pum2, and CPEB phosphorylation, which leads to the translational activation of Cyclin B1 mRNA.

We found that NLK1 overexpression in immature oocytes promotes Pumilio and CPEB phosphorylation and oocyte maturation without progesterone treatment. However, the protein contents of NLK1 necessary for inducing oocyte maturation were extremely high compared with those of native NLK1, and the NLK1-induced maturation proceeded very slowly (to reach the maximum rate of GVBD, 12 h for the progesterone-induced maturation vs 36 h for the NLK1-induced maturation, Figure 4a). These results imply that, besides NLK1, *Xenopus* oocytes possess other kinases that catalyze Pum1, Pum2, and CPEB phosphorylation during maturation. It is known that *Xenopus* oocytes are equipped with robust mechanisms that include several functionally redundant pathways, that is, those consisting of Mos-MEK-MAPK, Raf, RINGO, and Cyclin B, to ensure the induction of oocyte maturation even under conditions in which one of these pathways is accidentally blocked.[10,67] We confirmed the robustness by demonstrating that p42 MAPK is activated in Mos MO-injected oocytes (Figure 2a) and that these oocytes mature, though taking a longer time than intact oocytes (Figure 2b). A possible explanation for these phenomena is that p42 MAPK is activated through a positive feedback loop that is driven by Cdc2 activated in maturing oocytes via a cascade consisting of either Raf,[68,69] RINGO[10] or Cyclin B1[67] other than the Mos-MEK-MAPK cascade. The existence of plural kinases responsible for Pum1, Pum2, and CPEB probably reflects the robustness of mechanisms for inducing *Xenopus* oocyte maturation.

Our findings suggest that NLK1 is a kinase responsible for Pumilio and CPEB phosphorylation. Nevertheless, it should be noted that there is an apparent time lag between NLK1 activation and Pumilio and CPEB phosphorylation (Figure 1c). In addition, we previously showed that Pum1 and Pum2 are not phosphorylated at the same timing but that Pum2 phosphorylation precedes Pum1 phosphorylation in most cases, although the timings vary in oocyte batches.[36] Therefore, *Xenopus* oocytes must carry mechanisms that provide the cue for active NLK1 to phosphorylate Pumilio and CPEB at definite timings. A next target of our research is to understand these mechanisms. The biological significance of Pumilio phosphorylation also remains to be elucidated, although we proposed that Pum1 phosphorylation induces a conformational change in the complex consisting of Pum1 and CPEB that targets CPEB for dissociation and degradation, the process required for translational activation of Cyclin B1 mRNA.[36]

To analyze the functions of NLK1 in *Xenopus* oocyte maturation more closely, we tried to knockdown NLK1 in oocytes by using the corresponding MO and antisense mRNA. They did not work, however, probably because NLK1 is slowly turned over in oocytes. We then attempted to repress NLK1 activity by injection of anti-NLK antibodies into oocytes, but neither anti-NLK1c antibody nor anti-NLK1full antibody showed an apparent effect on oocyte maturation, probably due to the failure of antibodies to inactivate NLK1. (Indeed, anti-NLK1full immunoprecipitates contain active NLK1 and anti-NLK1c antibody cannot recognize native NLK1 as shown in this study.) Furthermore, we found that injection into oocytes of 50 ng of kinase-dead NLK1 mRNA had no effect on oocyte maturation, indicating that kinase-dead NLK1 does not operate in a dominant negative fashion (data not shown). Therefore, we need to obtain tools that can inhibit the activity or activation of NLK1 to elucidate the functions of NLK1 during oocyte maturation.

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ABBREVIATIONS USED

CPE, cytoplasmic polyadenylation element; CPEB, CPE-binding protein; DMSO, dimethyl sulfoxide; EB, extraction buffer; GST, glutathione-*S*-transferase; GVBD, germinal vesicle breakdown; MAPK, mitogen-activated protein kinase; MBE, Musashi-binding element; MBS-H, modified Barth's saline buffered with HEPES; MO, morpholino oligonucleotide; MPF, maturation-promoting factor; NLK, Nemo-like kinase; ORF, open reading frame; PBE, Pumilio-binding element; Pum1, Pumilio1; Pum2, Pumilio2; TCS, translational control sequence; UTR, untranslated region

REFERENCES

(1) Nagahama, Y., and Yamashita, M. (2008) Regulation of oocyte maturation in fish. *Dev. Growth Differ. 50* (Suppl 1), S195–219.

(2) Dunphy, W. G., Brizuela, L., Beach, D., and Newport, J. (1988) The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell 54*, 423–431.

(3) Gautier, J., Norbury, C., Lohka, M., Nurse, P., and Maller, J. (1988) Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene cdc2. *Cell 54*, 433–439.

(4) Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T., and Maller, J. L. (1990) Cyclin is a component of maturation-promoting factor from *Xenopus*. *Cell 60*, 487–494.

(5) Gautier, J., and Maller, J. L. (1991) Cyclin B in *Xenopus* oocytes: implications for the mechanism of pre-MPF activation. *EMBO J. 10*, 177–182.

(6) Kobayashi, H., Minshull, J., Ford, C., Golsteyn, R., Poon, R., and Hunt, T. (1991) On the synthesis and destruction of A- and B-type

cyclins during oogenesis and meiotic maturation in *Xenopus laevis*. *J. Cell Biol. 114*, 755–765.

(7) Minshull, J., Murray, A., Colman, A., and Hunt, T. (1991) *Xenopus* oocyte maturation does not require new cyclin synthesis. *J. Cell Biol. 114*, 767–772.

(8) Tanaka, T., and Yamashita, M. (1995) Pre-MPF is absent in immature oocytes of fishes and amphibians except *Xenopus*. *Dev. Growth Differ. 37*, 387–393.

(9) Yamashita, M. (2000) Toward modeling of a general mechanism of MPF formation during oocyte maturation in vertebrates. *Zool. Sci. 17*, 841–851.

(10) Nebreda, A. R., and Ferby, I. (2000) Regulation of the meiotic cell cycle in oocytes. *Curr. Opin. Cell Biol. 12*, 666–675.

(11) Yamashita, M., Mita, K., Yoshida, N., and Kondo, T. (2000) Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects. *Prog. Cell Cycle Res. 4*, 115–129.

(12) Nebreda, A. R., and Hunt, T. (1993) The *c-mos* proto-oncogene protein kinase turns on and maintains the activity of MAP kinase, but not MPF, in cell-free extracts of *Xenopus* oocytes and eggs. *EMBO J. 12*, 1979–1986.

(13) Posada, J., Yew, N., Ahn, N. G., Vande Woude, G. F., and Cooper, J. A. (1993) Mos stimulates MAP kinase in *Xenopus* oocytes and activates a MAP kinase kinase *in vitro*. *Mol. Cell. Biol. 13*, 2546–2553.

(14) Shibuya, E. K., and Ruderman, J. V. (1993) Mos induces the *in vitro* activation of mitogen-activated protein kinases in lysates of frog oocytes and mammalian somatic cells. *Mol. Biol. Cell 4*, 781–790.

(15) Mueller, P. R., Coleman, T. R., Kumagai, A., and Dunphy, W. G. (1995) Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science 270*, 86–90.

(16) Palmer, A., Gavin, A. C., and Nebreda, A. R. (1998) A link between MAP kinase and p34(cdc2)/cyclin B during oocyte maturation: p90(rsk) phosphorylates and inactivates the p34(cdc2) inhibitory kinase Myt1. *EMBO J. 17*, 5037–5047.

(17) Bagowski, C. P., Xiong, W., and Ferrell, J. E., Jr. (2001) c-Jun N-terminal kinase activation in *Xenopus laevis* eggs and embryos. A possible non-genomic role for the JNK signaling pathway. *J. Biol. Chem. 276*, 1459–1465.

(18) Perdiguero, E., Pillaire, M. J., Bodart, J. F., Hennersdorf, F., Frodin, M., Duesbery, N. S., Alonso, G., and Nebreda, A. R. (2003) Xp38gamma/SAPK3 promotes meiotic G(2)/M transition in *Xenopus* oocytes and activates Cdc25C. *EMBO J. 22*, 5746–5756.

(19) Liang, C. G., Su, Y. Q., Fan, H. Y., Schatten, H., and Sun, Q. Y. (2007) Mechanisms regulating oocyte meiotic resumption: roles of mitogen-activated protein kinase. *Mol. Endocrinol. 21*, 2037–2055.

(20) Belloc, E., and Mendez, R. (2008) A deadenylation negative feedback mechanism governs meiotic metaphase arrest. *Nature 452*, 1017–1021.

(21) Radford, H. E., Meijer, H. A., and de Moor, C. H. (2008) Translational control by cytoplasmic polyadenylation in *Xenopus* oocytes. *Biochim. Biophys. Acta 1779*, 217–229.

(22) Stebbins-Boaz, B., Hake, L. E., and Richter, J. D. (1996) CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and *c-mos* mRNAs and is necessary for oocyte maturation in *Xenopus*. *EMBO J. 15*, 2582–2592.

(23) Sheets, M. D., Fox, C. A., Hunt, T., Vande Woude, G., and Wickens, M. (1994) The 3′-untranslated regions of *c-mos* and cyclin mRNAs stimulate translation by regulating cytoplasmic polyadenylation. *Genes Dev. 8*, 926–938.

(24) Ballantyne, S., Daniel, D. L., Jr., and Wickens, M. (1997) A dependent pathway of cytoplasmic polyadenylation reactions linked to cell cycle control by *c-mos* and CDK1 activation. *Mol. Biol. Cell 8*, 1633–1648.

(25) Charlesworth, A., Cox, L. L., and MacNicol, A. M. (2004) Cytoplasmic polyadenylation element (CPE)- and CPE-binding protein (CPEB)-independent mechanisms regulate early class maternal mRNA translational activation in *Xenopus* oocytes. *J. Biol. Chem. 279*, 17650–17659.

(26) MacNicol, M. C., and MacNicol, A. M. (2010) Developmental timing of mRNA translation--integration of distinct regulatory elements. *Mol. Reprod. Dev. 77*, 662–669.

(27) Hake, L. E., and Richter, J. D. (1994) CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell 79*, 617–627.

(28) Mendez, R., and Richter, J. D. (2001) Translational control by CPEB: a means to the end. *Nat. Rev. Mol. Cell Biol. 2*, 521–529.

(29) Richter, J. D. (2007) CPEB: a life in translation. *Trends Biochem. Sci. 32*, 279–285.

(30) Arumugam, K., Wang, Y., Hardy, L. L., MacNicol, M. C., and MacNicol, A. M. (2010) Enforcing temporal control of maternal mRNA translation during oocyte cell-cycle progression. *EMBO J. 29*, 387–397.

(31) Charlesworth, A., Ridge, J. A., King, L. A., MacNicol, M. C., and MacNicol, A. M. (2002) A novel regulatory element determines the timing of Mos mRNA translation during *Xenopus* oocyte maturation. *EMBO J. 21*, 2798–2806.

(32) Charlesworth, A., Wilczynska, A., Thampi, P., Cox, L. L., and MacNicol, A. M. (2006) Musashi regulates the temporal order of mRNA translation during *Xenopus* oocyte maturation. *EMBO J. 25*, 2792–2801.

(33) Cao, Q., Padmanabhan, K., and Richter, J. D. (2010) Pumilio 2 controls translation by competing with eIF4E for 7-methyl guanosine cap recognition. *RNA 16*, 221–227.

(34) Nakahata, S., Katsu, Y., Mita, K., Inoue, K., Nagahama, Y., and Yamashita, M. (2001) Biochemical identification of *Xenopus* Pumilio as a sequence-specific cyclin B1 mRNA-binding protein that physically interacts with a Nanos homolog, Xcat-2, and a cytoplasmic polyadenylation element-binding protein. *J. Biol. Chem. 276*, 20945–20953.

(35) Nakahata, S., Kotani, T., Mita, K., Kawasaki, T., Katsu, Y., Nagahama, Y., and Yamashita, M. (2003) Involvement of *Xenopus* Pumilio in the translational regulation that is specific to cyclin B1 mRNA during oocyte maturation. *Mech. Dev. 120*, 865–880.

(36) Ota, R., Kotani, T., and Yamashita, M. (2011) Biochemical characterization of Pumilio1 and Pumilio2 in *Xenopus* oocytes. *J. Biol. Chem. 286*, 2853–2863.

(37) Padmanabhan, K., and Richter, J. D. (2006) Regulated Pumilio-2 binding controls RINGO/Spy mRNA translation and CPEB activation. *Genes Dev. 20*, 199–209.

(38) Wang, Y. Y., Charlesworth, A., Byrd, S. M., Gregerson, R., MacNicol, M. C., and MacNicol, A. M. (2008) A novel mRNA 3′ untranslated region translational control sequence regulates *Xenopus* Wee1 mRNA translation. *Dev. Biol. 317*, 454–466.

(39) Pique, M., Lopez, J. M., Foissac, S., Guigo, R., and Mendez, R. (2008) A combinatorial code for CPE-mediated translational control. *Cell 132*, 434–448.

(40) Brott, B. K., Pinsky, B. A., and Erikson, R. L. (1998) Nlk is a murine protein kinase related to Erk/MAP kinases and localized in the nucleus. *Proc. Natl. Acad. Sci. U. S. A. 95*, 963–968.

(41) Choi, K. W., and Benzer, S. (1994) Rotation of photoreceptor clusters in the developing *Drosophila* eye requires the nemo gene. *Cell 78*, 125–136.

(42) Meneghini, M. D., Ishitani, T., Carter, J. C., Hisamoto, N., Ninomiya-Tsuji, J., Thorpe, C. J., Hamill, D. R., Matsumoto, K., and Bowerman, B. (1999) MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in *Caenorhabditis elegans*. *Nature 399*, 793–797.

(43) Ishitani, T., Hirao, T., Suzuki, M., Isoda, M., Ishitani, S., Harigaya, K., Kitagawa, M., Matsumoto, K., and Itoh, M. (2010) Nemo-like kinase suppresses Notch signalling by interfering with formation of the Notch active transcriptional complex. *Nat. Cell Biol. 12*, 278–285.

(44) Thorpe, C. J., and Moon, R. T. (2004) nemo-like kinase is an essential co-activator of Wnt signaling during early zebrafish development. *Development 131*, 2899–2909.

(45) Hyodo-Miura, J., Urushiyama, S., Nagai, S., Nishita, M., Ueno, N., and Shibuya, H. (2002) Involvement of NLK and Sox11 in neural induction in *Xenopus* development. *Genes Cells 7*, 487–496.

(46) Ohnishi, E., Goto, T., Sato, A., Kim, M. S., Iemura, S., Ishitani, T., Natsume, T., Ohnishi, J., and Shibuya, H. (2010) Nemo-like kinase, an essential effector of anterior formation, functions downstream of p38 mitogen-activated protein kinase. *Mol. Cell. Biol. 30*, 675–683.

(47) Kortenjann, M., Nehls, M., Smith, A. J., Carsetti, R., Schuler, J., Kohler, G., and Boehm, T. (2001) Abnormal bone marrow stroma in mice deficient for nemo-like kinase, Nlk. *Eur. J. Immunol. 31*, 3580–3587.

(48) Jung, K. H., Kim, J. K., Noh, J. H., Eun, J. W., Bae, H. J., Xie, H. J., Ahn, Y. M., Park, W. S., Lee, J. Y., and Nam, S. W. (2010) Targeted disruption of Nemo-like kinase inhibits tumor cell growth by simultaneous suppression of cyclin D1 and CDK2 in human hepatocellular carcinoma. *J. Cell. Biochem. 110*, 687–696.

(49) Ota, R., Suwa, K., Kotani, T., Mita, K., and Yamashita, M. (2008) Possible involvement of phosphatidylinositol 3-kinase, but not protein kinase B or glycogen synthase kinase 3beta, in progesterone-induced oocyte maturation in the Japanese brown frog, *Rana japonica*. *Zool. Sci. 25*, 773–781.

(50) Kotani, T., and Kawakami, K. (2008) *misty somites*, a maternal effect gene identified by transposon-mediated insertional mutagenesis in zebrafish that is essential for the somite boundary maintenance. *Dev. Biol. 316*, 383–396.

(51) Setoyama, D., Yamashita, M., and Sagata, N. (2007) Mechanism of degradation of CPEB during *Xenopus* oocyte maturation. *Proc. Natl. Acad. Sci. U. S. A. 104*, 18001–18006.

(52) Sakamoto, I., Takahara, K., Yamashita, M., and Iwao, Y. (1998) Changes in cyclin B during oocyte maturation and early embryonic cell cycle in the newt, *Cynops pyrrhogaster*: requirement of germinal vesicle for MPF activation. *Dev. Biol. 195*, 60–69.

(53) Yamashita, M., Yoshikuni, M., Hirai, T., Fukada, S., and Nagahama, Y. (1991) A monoclonal antibody against the PSTAIR sequence of p34cdc2, catalytic subunit of maturation-promoting factor and key regulator of the cell cycle. *Dev. Growth Differ. 33*, 617–624.

(54) Rassa, J. C., Wilson, G. M., Brewer, G. A., and Parks, G. D. (2000) Spacing constraints on reinitiation of paramyxovirus transcription: the gene end U tract acts as a spacer to separate gene end from gene start sites. *Virology 274*, 438–449.

(55) Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Meneghini, M., Barker, N., Waterman, M., Bowerman, B., Clevers, H., Shibuya, H., and Matsumoto, K. (1999) The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. *Nature 399*, 798–802.

(56) Ohkawara, B., Shirakabe, K., Hyodo-Miura, J., Matsuo, R., Ueno, N., Matsumoto, K., and Shibuya, H. (2004) Role of the TAK1-NLK-STAT3 pathway in TGF-beta-mediated mesoderm induction. *Genes Dev. 18*, 381–386.

(57) Ishitani, S., Inaba, K., Matsumoto, K., and Ishitani, T. (2011) Homodimerization of Nemo-like kinase is essential for activation and nuclear localization. *Mol. Biol. Cell 22*, 266–277.

(58) de Moor, C. H., and Richter, J. D. (1997) The Mos pathway regulates cytoplasmic polyadenylation in *Xenopus* oocytes. *Mol. Cell. Biol. 17*, 6419–6426.

(59) Hochegger, H., Klotzbucher, A., Kirk, J., Howell, M., le Guellec, K., Fletcher, K., Duncan, T., Sohail, M., and Hunt, T. (2001) New B-type cyclin synthesis is required between meiosis I and II during *Xenopus* oocyte maturation. *Development 128*, 3795–3807.

(60) Mendez, R., Barnard, D., and Richter, J. D. (2002) Differential mRNA translation and meiotic progression require Cdc2-mediated CPEB destruction. *EMBO J. 21*, 1833–1844.

(61) Mendez, R., Hake, L. E., Andresson, T., Littlepage, L. E., Ruderman, J. V., and Richter, J. D. (2000) Phosphorylation of CPE binding factor by Eg2 regulates translation of *c-mos* mRNA. *Nature 404*, 302–307.

(62) Mendez, R., Murthy, K. G., Ryan, K., Manley, J. L., and Richter, J. D. (2000) Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. *Mol. Cell 6*, 1253–1259.

(63) Inoue, D., Ohe, M., Kanemori, Y., Nobui, T., and Sagata, N. (2007) A direct link of the Mos-MAPK pathway to Erp1/Emi2 in meiotic arrest of *Xenopus laevis* eggs. *Nature 446*, 1100–1104.

(64) Nishiyama, T., Ohsumi, K., and Kishimoto, T. (2007) Phosphorylation of Erp1 by p90rsk is required for cytostatic factor arrest in *Xenopus laevis* eggs. *Nature 446*, 1096–1099.

(65) Matten, W. T., Copeland, T. D., Ahn, N. G., and Vande Woude, G. F. (1996) Positive feedback between MAP kinase and Mos during *Xenopus* oocyte maturation. *Dev. Biol. 179*, 485–492.

(66) Charlesworth, A., Welk, J., and MacNicol, A. M. (2000) The temporal control of Wee1 mRNA translation during *Xenopus* oocyte maturation is regulated by cytoplasmic polyadenylation elements within the 3′-untranslated region. *Dev. Biol. 227*, 706–719.

(67) Haccard, O., and Jessus, C. (2006) Oocyte maturation, Mos and cyclins--a matter of synthesis: two functionally redundant ways to induce meiotic maturation. *Cell Cycle 5*, 1152–1159.

(68) Dupre, A., Jessus, C., Ozon, R., and Haccard, O. (2002) Mos is not required for the initiation of meiotic maturation in *Xenopus* oocytes. *EMBO J. 21*, 4026–4036.

(69) Galaktionov, K., Jessus, C., and Beach, D. (1995) Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. *Genes Dev. 9*, 1046–1058.