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Cross-talk between JNK/SAPK and ERK/MAPK Pathways

SUSTAINED ACTIVATION OF JNK BLOCKS ERK ACTIVATION BY MITOGENIC FACTORS*

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Mixed lineage kinases (MLKs) are a family of serine/threonine kinases that function in the SAPK signaling cascade. MLKs activate JNK/SAPK *in vivo* by directly phosphorylating and activating the JNK kinase SEK-1 (MKK4 and -7). Importantly, the MLK member MLK3/SPRK has been shown recently to be a direct target of ceramide and tumor necrosis factor-α (TNF-α) and to mediate the TNF-α and ceramide-induced JNK activation in Jurkat cells. Here we report that MLK3 can phosphorylate and activate MEK-1 directly *in vitro* and also can induce MEK phosphorylation on its activation sites *in vivo* in COS-7 cells. Surprisingly, this induction of MEK phosphorylation does not result in ERK activation *in vivo*. Rather, in cells expressing active MLK3, ERK becomes resistant to activation by growth factors and mitogens. This restriction in ERK activation requires MLK3 kinase activity, is independent of Raf activation, and is reversed by JNK pathway inhibition either at the level of SEK-1, JNK, or Jun. These results demonstrate that sustained JNK activation uncouples ERK activation from MEK in a manner requiring Jun-mediated gene transcription. This in turn points to the existence of a negative cross-talk relationship between the stress-activated JNK pathway and the mitogen-activated ERK pathway. Thus, our findings imply that some of the biological functions of JNK activators, such as TNF-α and ceramide, may be attributed to their ability to block cell responses to growth and survival factors acting through the ERK/MAPK pathway.

Mixed lineage kinases (MLKs)[1] were identified a decade ago as dual specificity kinases, *i.e.* kinases with both serine/threonine and tyrosine kinase activity (1–4). Although the tyrosine kinase activity of MLKs has not yet been reported, MLKs contain the tyrosine kinase signature motif, in addition to the

serine/threonine kinase motif (for review see Ref. 5). Soon after their identification, several groups (6–9) independently demonstrated that MLK family members can directly phosphorylate and activate the JNK upstream kinase SEK-1 *in vitro* and induce JNK activation *in vivo*. These findings classified MLK family members as mitogen-activated protein kinase kinase kinases (MAPKKKs) in the stress-activated signaling cascade (for review see Refs. 5 and 10). The validity of this classification is strongly substantiated by the recent identification of scaffold proteins that bind several members of the stress-activated protein kinase cascade (*i.e.* MLK, SEK-1, and JNK) and augment the signaling through the cascade (11–13). The identification of an upstream activator(s) of MLK family members turned out, however, to be a much more complicated task. Besides the signature kinase motif, MLK members contain a Cdc42/Rac-interactive binding domain, the presence of which pointed to the possibility that Cdc42 and Rac may be upstream regulators of MLKs (14, 15). Additional work is required to determine more conclusively the role of Cdc42 or Rac in MLK activation.

A breakthrough in understanding MLK activation comes from two recent studies (16, 17) that used *Drosophila* MLK as a model. Together, these studies provide genetic and biochemical evidence positioning MLK as a critical JNK activator and define TNF-α and ceramide as potent natural activators of MLKs. These findings are in agreement with other studies (18–20) demonstrating a critical role of MLKs in JNK-mediated neuronal apoptosis.

Besides SEK-1 (MKK4 and -7), no other MLK targets have been defined clearly (5). Few reports indicate that MLK members can activate the ERK/MAPK pathway, possibly through MEK-1 activation, and suggest an oncogenic potential of MLK (21). However, the details of MEK activation and the oncogenic potential have not been determined.

The various MAPK cascades (*e.g.* ERK1/2, JNK, p38, and ERK5) are often portrayed in the literature as linear cascades, and indications for cross-talk between the various cascades are limited (5, 10). In this respect, the present study examines the consequences of MLK3 overexpression on the ERK/MAPK pathway and its subsequent response to mitogenic stimuli. We find that MLK3 can phosphorylate and activate MEK-1 both *in vitro* and *in vivo*. MEK activation *in vivo*, however, is uncoupled from ERK activation. Moreover, in cells expressing active MLK3, ERK becomes resistant to activation by mitogens. This restriction in ERK activation involves the SEK-1-JNK-Jun cascade, as demonstrated by the ability of specific inhibitors of the pathway to reverse the blockage of ERK activation. Our results demonstrate negative cross-talk between the stress-activated MLK-SEK-JNK-Jun pathway and the ERK/MAPK pathway, and suggest that sustained activation of the JNK pathway can

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[1] The abbreviations used are: MLKs, mixed lineage kinases; JNK, c-Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor; MEK, MAPK/ERK kinase; PVDF, polyvinylidene difluoride; HA, hemagglutinin; GST, glutathione *S*-transferase; MAPKKKs, mitogen-activated protein kinase kinase kinases; MBP, myelin basic protein; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate.
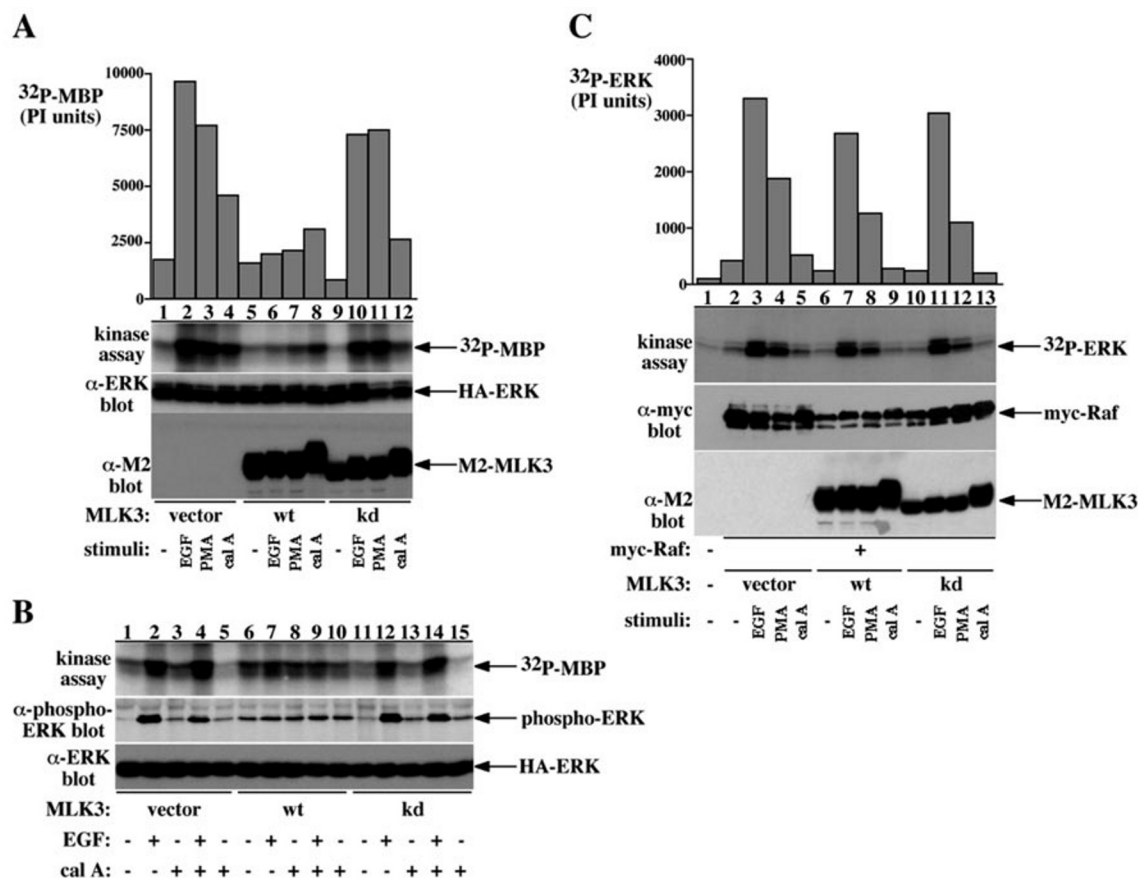
FIG. 1. **Expression of active MLK3 attenuates ERK activation *in vivo* downstream of Raf.** *A*, COS-7 cells in 10-cm plates were co-transfected with 1.5 µg of pMT2 vector expressing HA-ERK-1 and either 3.5 µg of empty pRK-5 vector (*lanes 1–4*), pRK-5 vector expressing wild-type MLK3 (*wt, lanes 5–8*), or pRK-5 vector expressing kinase-dead MLK3 (*kd, lanes 9–12*). Twenty four hours after transfection, cells were deprived of serum for 18 h and were treated with either vehicle (−, *lanes 1, 5,* and *9*), 100 ng/ml EGF (*EGF, lanes 2, 6,* and *10*), 200 nM PMA (*PMA, lanes 3, 7,* and *11*), or 150 nM calyculin A (*cal A, lanes 4, 8,* and *12*) for 10 min. Cells were lysed, and ERK kinase activity in the HA immunoprecipitates was assayed as detailed under "Experimental Procedures." Presented are an autoradiogram and a bar graph showing ³²P incorporation into MBP, a Western blot showing HA-ERK recovery, and an M2-FLAG immunoblot showing M2-MLK3 expression in total cell extracts. *B*, COS-7 cells transfected as in *A*, were deprived of serum for 18 h and treated with either vehicle (*lanes 1, 6,* and *11*), 100 ng/ml EGF (*lanes 2, 7,* and *12*), or 150 nM calyculin A (*lanes 3, 8,* and *13*) for 10 min, or with 150 nM calyculin A for 10 min followed by 100 nM EGF for 10 min (*lanes 4, 9,* and *14*) or with 150 nM calyculin A for 20 min (*lanes 5, 10,* and *15*). HA-ERK kinase activity was determined as in *A*, and the level of ERK phosphorylation at its activation sites was determined by immunoblotting with phosphospecific antibodies for ERK activation sites (Cell Signaling Technology). Presented are an autoradiogram showing ³²P incorporation into MBP (*top panel*), a Western blot showing HA-ERK phosphorylation at its activation sites (*middle panel*), and a Western blot showing HA-ERK recovery (*bottom panel*). *C*, COS-7 cell were co-transfected with 1.5 µg of pMT2 vector expressing myc-Raf-1 and either 3.5 µg of empty pRK-5 vector, pRK-5-MLK3, or pRK-5-MLK3 (kd), as indicated. Cells were stimulated as in *A*, and Raf kinase activity in Myc-immunoprecipitates was assayed as detailed under "Experimental Procedures." Presented are an autoradiogram and a bar graph showing ³²P incorporation in ERK, a Western blot showing myc-Raf recovery, and an M2-FLAG immunoblot showing M2-MLK3 expression in total cell extracts.

result in the attenuation of the mitogen-activated ERK pathway.

EXPERIMENTAL PROCEDURES

cDNA Constructs, Antibodies, and Kinase Inhibitors—M2-FLAG-MLK3 and M2-FLAG-kinase-dead MLK3 (K144A) were in the pRK-5 mammalian expression vector (for construction details see Ref. 3)). myc-Raf-1 and HA-ERK-1 were in the pMT2 mammalian expression vector (6, 22). Wild-type c-Jun and the dominant negative c-Jun variants TAM-67 (deletion mutant lacking the transactivating domain) and DBM-3 (variant containing mutations in the DNA binding domain) were in pCMV vector (23–25). Wild-type EE epitope-tagged MEK-1 and constitutively active MEK-1 (S218D/S222D) were in the pCDNAI vector (26). Constitutively active c-Raf (GST-Bxb-Raf) and dominant negative HA-SEK-1-AL (S220A and T224L) were in the pEBG vector (6, 27, 28).

Phosphospecific antibodies for the active forms of ERK, MEK, and c-Jun and corresponding antibodies for the non-phosphorylated forms were from Cell Signaling Technology (Beverly, MA). Antibodies against HA, Myc, and the EE epitope were produced from 12CA5, 9E10, and EE hybridoma cell lines, respectively. Anti-M2-FLAG epitope antibody was purchased from Sigma. JNK inhibitors, JNK inhibitor I (a cell-permeable peptide inhibitor), and JNK inhibitor II (an ATP competitive cell-permeable inhibitor) were from Calbiochem. The MLK inhibitor CEP-

11004 was a kind gift from Cephalon.

Cell Culture and Transfection—COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For transient expression of proteins, cells were transfected using LipofectAMINE (Invitrogen) according to the manufacturer's instructions (detailed in the figure legends). Details for cell stimulation and treatment with inhibitors are provided in the figure legends.

Cell Extraction and Protein Purification—Cells were lysed for 30 min in ice-cold extraction buffer containing 50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 2 mM Na₃VO₄, 50 mM β-glycerophosphate, and a protease inhibitor mixture (Amersham Biosciences). For immunoprecipitation, cleared cell lysates were incubated at 4 °C for 90 min with the appropriate antibody precoupled to protein A/G-agarose beads (Santa Cruz Biotechnology). The beads were washed twice with extraction buffer, twice with extraction buffer containing 0.5 M LiCl, and twice with kinase assay buffer (40 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 5 mM MgCl₂, and 2 mM dithiothreitol). Proteins were eluted directly in SDS sample buffer for Western blot analysis or were assayed for kinase activity, as indicated in the figure legend.

Kinase Assays—Raf kinase activity was assayed as described previously (22). Briefly, following Myc immunoprecipitation, myc-Raf containing beads were incubated for 20 min at 30 °C in kinase assay buffer
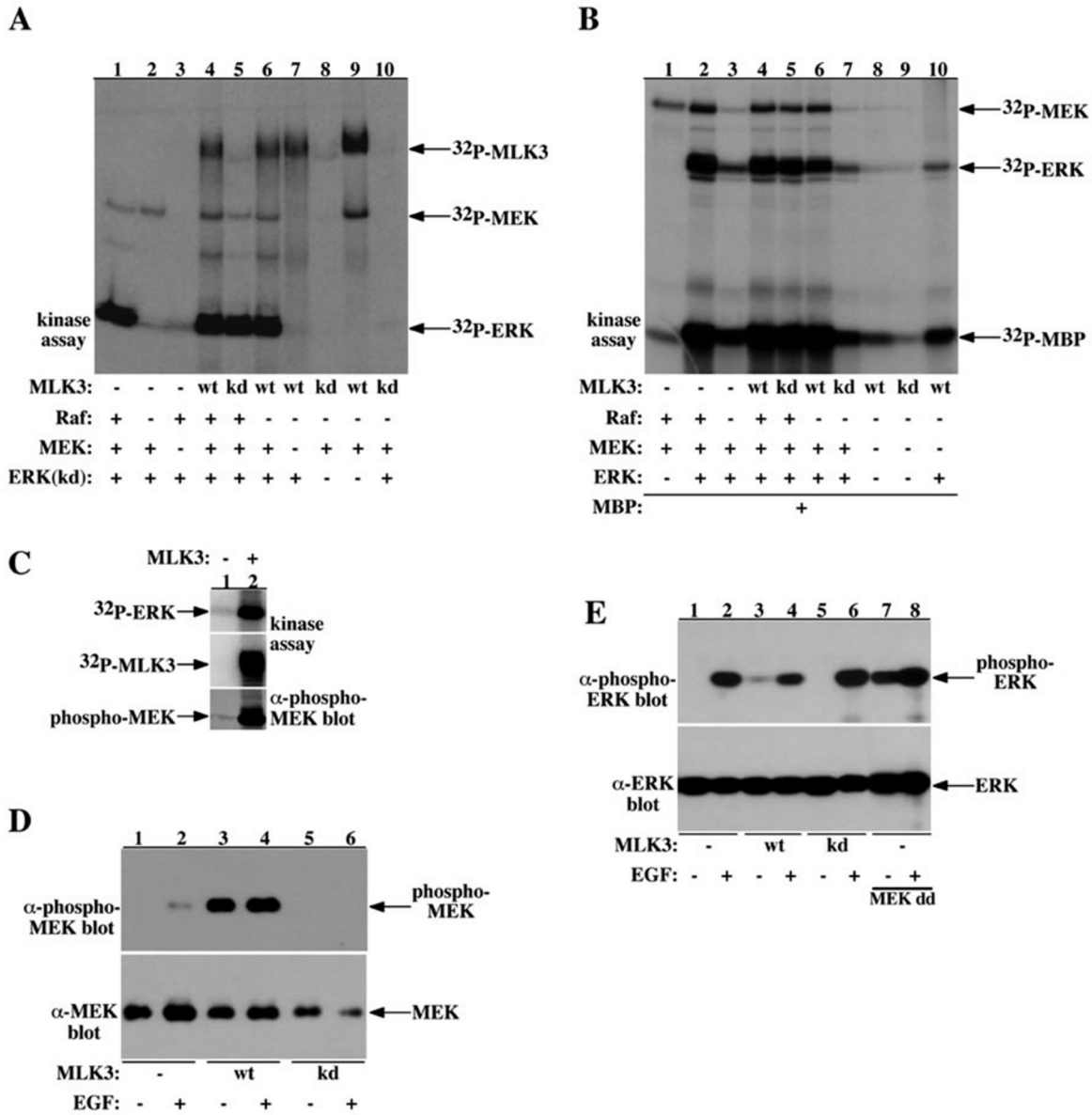
FIG. 2. **MLK3 does not inhibit the Raf-MEK-ERK-MBP phosphorylation cascade *in vitro*, rather MLK3 activates MEK *in vitro* and induces MEK phosphorylation at its activation sites *in vivo*.** *A*, 50 ng of active Raf-1, produced in sf9 cells by co-infection with active Ras and Src, was incubated in kinase assay buffer at 30 °C with either vehicle, 1 μg of wild-type, or kinase-dead MLK3, as indicated. Following a 15-min incubation, vehicle, 500 ng of recombinant GST-MEK-1, and 2 μg of kinase-dead recombinant GST-ERK-1 (*kd*) were added at the indicated combinations, and the incubation was continued for additional 15 min. The protein samples were separated on 8.5% SDS-PAGE and analyzed by autoradiography. Note that the samples in *lanes 2* and *6–10* do not contain Raf. *B*, the experiment was conducted as in *A*, except that recombinant wild-type GST-ERK-1 was used instead of kinase-dead ERK-1. MBP was added after the second 15-min incubation, and the samples were incubated for an additional 10 min. Proteins were separated using 12% SDS-PAGE and analyzed by autoradiography. *C*, 2 μg of recombinant GST-MEK-1 was incubated in kinase buffer with vehicle or 1 μg of MLK3 for 15 min, followed by addition of recombinant kinase-dead GST-ERK-1. After an additional 15 min of incubation, protein samples were resolved on 8.5% SDS-PAGE, transferred to PVDF membranes, and analyzed for kinase activity by autoradiography (*top* and *middle panel*) and immunoblotting with phospho-MEK antibody (*bottom panel*). *D*, COS-7 cells were transfected with 1.5 μg of pCDNAI vector expressing EE-tagged MEK-1 and either 3.5 μg of empty pRK-5 vector, pRK-5-MLK3, or pRK-5-MLK3 (kd). Cells were treated with 100 ng/ml EGF and lysed, and the EE-MEK was immunoprecipitated. Phosphorylation of EE-MEK was determined by immunoblotting with MEK activation site phosphospecific antibodies (*top panel*), and MEK recovery was determined by MEK immunoblotting (*bottom panel*). *E*, COS-7 cells were co-transfected with 1.5 μg of pMT2-HA-ERK-1 and either 3.5 μg of empty pRK-5 vector or pRK-5-MLK3, pRK-5-MLK3 (kd), or pCDNAI vector expressing active MEK-1 (MEK-1-DD). ERK-1 phosphorylation in HA immunoprecipitates was determined by immunoblotting with phosphospecific ERK antibodies (*top panel*), and HA-ERK recovery was determined by immunoblotting with ERK-1 antibody (*bottom panel*).

(100 μl final volume) supplemented with 100 μM ATP, 10 μCi of [γ-^{32}P]ATP, and 0.3 μg of prokaryotic recombinant GST-MEK-1. Two micrograms of prokaryotic recombinant kinase inactive ERK (K52R) were added, and the samples were incubated for an additional 30 min. Samples were separated on 10% SDS-PAGE and transferred to PVDF membranes. ^{32}P incorporation into ERK was quantified by Phosphor-Imager (PI) analysis (Storm, Amersham Biosciences). Data are presented PI × 10^3. myc-Raf recovery was determined by Myc immunoblotting. For assessment of ERK kinase activity, following HA

immunoprecipitation, HA-ERK containing beads were incubated for 10 min at 30 °C in kinase assay buffer (50 μl final volume) supplemented with 100 μM ATP, 10 μCi of [γ-^{32}P]ATP, and 10 μg of myelin basic protein (MBP, Sigma). Samples were separated on 12% SDS-PAGE and transferred to PVDF membranes. ^{32}P incorporation into MBP was quantified by PhosphorImager analysis (data are presented PI unit × 10^3). ERK recovery was determined by immunoblotting with anti-HA or anti-ERK antibodies, as detailed in the figure legends. To assess MLK3 kinase activity and to determine the effect of MLK3 on the Raf-MEK-
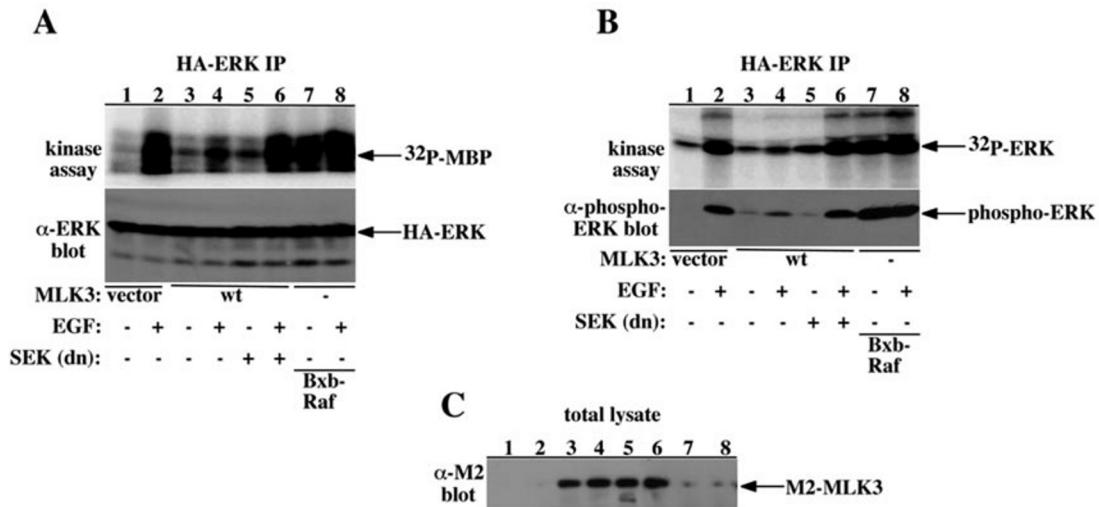
FIG. 3. **Dominant negative SEK-1 reverses the effect of MLK3 on ERK activation.** COS-7 cells were transfected with 1.5 μg of pMT2-HA-ERK-1 vector together with 1.5 μg of pRK-5 empty vector and 2 μg of pEBG empty vector (*lanes 1* and *2*), 1.5 μg of pRK-5-MLK3 and 2 μg of pEBG empty vector (*lanes 3* and *4*), 1.5 μg of pRK-5-MLK3 and 2 μg of pEBG-SEK-1-AL (*dn, lanes 5* and *6*), or 3.5 μg of pEBG-GST-Bxb-Raf (*lanes 7* and *8*). Twenty four hours after transfection, cells were deprived of serum for 18 h and treated with vehicle or 100 ng/ml EGF as indicated. *A*, HA-ERK kinase activity was determined using MBP as the substrate, as in Fig. 1*A*. Presented are an autoradiogram showing MBP phosphorylation (*top panel*) and an ERK immunoblot showing HA-ERK recovery (*bottom panel*). *B*, HA-ERK activation in same samples was determined by assaying HA-ERK autophosphorylation *in vitro* (*top panel*) and HA-ERK phosphorylation at its activation sites *in vivo* (*bottom panel*). *C*, M2-FLAG-MLK3 expression in total cell lysates was determined by M2 immunoblotting. *IP*, immunoprecipitation.

ERK-MBP cascade *in vitro*, the combination of proteins indicated in the figure legends was incubated for the indicated times at 30 °C in kinase assay buffer (100 μl final volume) supplemented with 100 μM ATP and 10 μCi of [γ-^{32}P]ATP. Samples were separated on 8.5% SDS-PAGE; samples containing MBP were separated on 12% SDS-PAGE. Separated proteins were transferred to PVDF membranes. Kinase activity was analyzed by autoradiography and PhosphorImaging. Phosphospecific antibodies recognizing the activated forms of ERK, MEK, and c-Jun were used for determining *in vivo* activation levels of ERK, MEK, and Jun, respectively.

<div style="text-align:center">RESULTS</div>

Expression of Active MLK3 in COS-7 Cells Attenuates ERK Activation by Growth Factors and Mitogens without Affecting Raf Activation—MLK3 functions as a MAPKKK in the stress-activated JNK pathway; however, a few reports suggest that MLK3 may also affect the ERK/MAPK pathway (21, 29, 30). To determine the effect of MLK3 on the ERK/MAPK pathway, ERK-1 was co-expressed together with wild-type MLK3 or a kinase-dead MLK3 mutant, MLK3 (K144A), and the basal and mitogen-stimulated ERK-1 kinase activity was examined (Fig. 1, *A* and *B*). In serum-deprived COS-7 cells, expression of active MLK3 results in a slight, reproducible activation of basal ERK kinase activity and ERK phosphorylation (Fig. 1*B*, compare *lane 1* with *6*, Fig. 2*E*, compare *lane 1* with *3,* and Fig. 3, compare *lane 1* with *3*). This activation depends on MLK3 kinase activity, because it is not observed in cells expressing the kinase-dead MLK3 mutant. In contrast to basal ERK activation, MLK3 expression almost completely attenuates ERK activation in response to EGF and PMA (Fig. 1*A*, compare *lanes 2* and *3* with *lanes 6* and *7* and Fig. 1*B*, compare *lane 2* with *7*). This attenuation depends on MLK3 kinase activity, because ERK activation is not affected in cells expressing the kinase-dead MLK3 mutant (Fig. 1, *A* and *B*). Treatment of cells with the phosphatase inhibitor calyculin A fails to reverse the MLK3 effect on ERK activation (Fig. 1, *A* and *B*), indicating that activation of phosphatases sensitive to calyculin A were not responsible for the observed inhibition of ERK activation.

To examine whether the inhibition of ERK activation was due to an inhibition of Raf activation, Myc-tagged c-Raf-1 was co-expressed with the two MLK3 variants, and the kinase activity of Raf was measured in a coupled kinase assay (Fig.

1*C*). Expression of active or inactive MLK3 does not affect basal or mitogen-stimulated Raf kinase activity, suggesting that expression of active MLK3 uncouples the activation of ERK from Raf.

MLK3 Does Not Inhibit the Raf-MEK-ERK-MBP Phosphorylation Cascade in Vitro, Rather MLK3 Functions as a Potent MEK Kinase, Both in Vitro and in Vivo—To examine whether MLK3 directly inhibits the kinase activities of Raf, MEK, or ERK, we tested the effect of MLK3 on the Raf-MEK-ERK phosphorylation cascade *in vitro* (Fig. 2, *A* and *B*). We find that under *in vitro* conditions, MLK3 does not affect the ability of Raf to phosphorylate MEK-1 or to activate MEK-1, and it does not affect the ability of MEK-1 to phosphorylate ERK-1 (Fig. 2*A*). In addition, MLK3 does not affect the ability of MEK-1 to activate ERK-1, and it does not affect ERK-1 kinase activity (Fig. 2*B*).

In contrast to the inhibitory effect of MLK3 on the ERK pathway *in vivo*, our experiments show that *in vitro*, MLK3 phosphorylates MEK-1 and activates its kinase activity as potently as Raf (Fig. 2, *A* and *B*). These findings demonstrate that MLK3 can function as a MAPKKK also in the ERK/MAPK cascade. Because MEK-1-phosphospecific antibodies that recognize the doubly phosphorylated MEK-1 react with MEK-1 phosphorylated by MLK3 (Fig. 2*C*), we infer that MEK-1 sites phosphorylated by MLK3 are similar to the sites phosphorylated by Raf.

MLK3 also induces MEK-1 phosphorylation *in vivo*, in a manner depending on its kinase activity (Fig. 2*D*); however, this does not translate to ERK activation (Fig. 2*E*). These results indicate that, in cells expressing active MLK3, there is an uncoupling of ERK and MEK kinase activities.

Dominant Negative SEK-1 Reverses the Effect of MLK3 on ERK Activation—Because MLK3 apparently phosphorylates both MEK-1 and SEK-1, we wanted to determine which of these two MLK3-associated activities is responsible for inhibition of ERK-1 activation. To exclude the possibility that strong activation of MEK-1 and/or ERK-1 results in a feedback that blocks ERK-1 activation by mitogens, we examined the effects of the active forms of MEK-1 (MEK-1-DD, Fig. 2*E*, *lanes 7* and *8*) and Raf-1 (Bxb-Raf, Fig. 3, *lanes 7* and *8*) on ERK-1 activity.
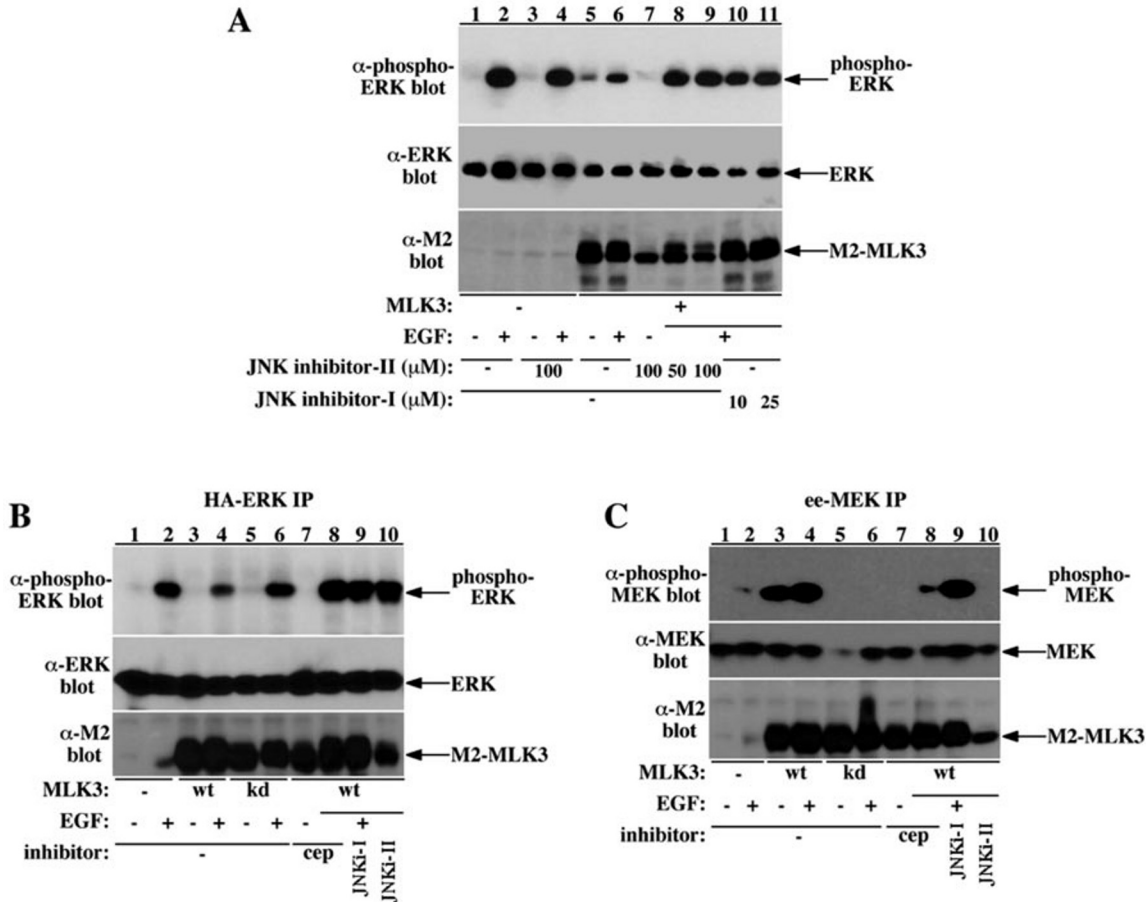
Fig. 4. **MLK3 and JNK kinase activities are required for MLK3-induced ERK inhibition.** *A*, COS-7 cells were co-transfected with 1.5 μg of pMT2-HA-ERK-1 vector and either 3.5 μg of pRK-5 empty vector (*lanes 1–4*) or pRK-5-MLK3 (*lanes 5–11*). Twenty four hours after transfection, cells were deprived of serum for 18 h in the presence or absence of the indicated concentrations of JNK inhibitors I and II, as indicated. Cells were treated with 100 ng/ml EGF for 10 min, as indicated, and HA-ERK was immunoprecipitated. ERK activation in the immunoprecipitates was determined by phospho-ERK immunoblotting (*top panel*); HA-ERK recovery was determined by ERK immunoblotting (*middle panel*), and M2-FLAG-MLK3 expression in total cell lysates was determined by M2-FLAG immunoblotting (*bottom panel*, note that MLK-3 expressed in COS-7 cells appears as a double band in which the top band is the phosphorylated, active form of MLK3). *B* and *C*, COS-7 cells were co-transfected with 1.5 μg of pMT2-HA-ERK-1 (*B*) or 1.5 μg of pCDNI-EE-MEK-1 (*C*) and either 3.5 μg of pRK-5-wild-type or kinase-dead MLK3, as indicated. Cells were treated as in *A* with either 25 μM JNK inhibitor I (*JNKi-I, lane 9*), 100 μM JNK inhibitor II (*JNKi-II, lane 10*), or 500 nM CEP-11004 (*cep, lanes 7* and *8*), and then stimulated with 100 ng/ml EGF for 10 min. Phosphorylation of HA-ERK in HA-immunoprecipitates (*B*) and phosphorylation of MEK in EE-immunoprecipitates (*C*) was determined by immunoblotting with phospho-ERK and phospho-MEK, respectively (*top panel*). Recovery of HA-ERK and EE-MEK was determined by immunoblotting with ERK and MEK, respectively (*middle panel*). M2-FLAG-MLK3 expression in total cell lysates was determined by M2-FLAG immunoblotting (*bottom panel*).

These experiments demonstrate that a constitutive activation of the Raf-MEK-ERK pathway does not result, by itself, in ERK inhibition. To examine the possibility that MLK3 attenuates ERK activation through activation of SEK-1, we co-expressed dominant negative SEK-1 (SEK-1-AL) and MLK3, and we assayed ERK activation induced by EGF (Fig. 3). Co-expression with SEK-1-AL completely reverses the effect of MLK3 on ERK activation (Fig. 3, *A* and *B*, compare *lanes 3* and *4* with *lanes 5* and *6*). This reversal occurs without affecting MLK3 expression levels (Fig. 3*C*). Although this reversal is most likely due to inhibition of endogenous SEK-1 activity, it is important to note that SEK-1-AL may also inhibit MLK3 by out-competing other substrates. This possibility is addressed below by using inhibitors for SEK-1 effectors.

JNK Inhibitors and the MLK3 Inhibitor CEP-11004 Reverse the Effect of MLK3 on ERK Activation—To determine which of the components of the JNK pathway are required for the MLK3-induced ERK inhibition, and to exclude the possibility that the SEK-1 dominant negative acts by merely inhibiting the MLK3 kinase activity, we tested the effect of two reportedly specific JNK inhibitors on the ability of MLK3 to inhibit ERK activation (Fig. 4, *A* and *B*). Incubation of MLK3-expressing

COS-7 cells with two distinct JNK inhibitors (JNK inhibitor I, a cell-permeable peptide inhibitor, and JNK inhibitor II, an ATP competitive cell-permeable inhibitor) for 24 h prior to mitogenic stimulation completely reverses MLK3-induced ERK inhibition (Fig. 4*A,* compare *lanes 5* and *6* with *lanes 7–11*; Fig. 4*B*, compare *lanes 3* and *4* with *lanes 9* and *10*). This reversal happens without affecting basal ERK activity or the magnitude of ERK activation by EGF in cells that do not express MLK3 (Fig. 4*A,* compare *lanes 1* and *2* with *lanes 3* and *4*).

To support the notion that MLK3 kinase activity is required for MLK3-induced ERK inhibition, the MLK-specific inhibitor CEP-11004 (17, 31) was used to block MLK3 kinase activity (Fig. 4*B*). CEP-11004 completely reverses MLK3-induced ERK inhibition, without affecting basal ERK activity (Fig. 4*B*, compare *lanes 3* and *4* with *lanes 7* and *8*).

In parallel experiments, the effects of the JNK inhibitors and CEP-11004 were examined on the ability of MLK3 to induce MEK phosphorylation (Fig. 4*C*). Because JNK peptide inhibitor I reverses ERK inhibition without affecting the ability of MLK3 to induce MEK-1 phosphorylation (Fig. 4, *B* and *C, lane 9*), we conclude that the inhibition in ERK activation is independent of MEK activation but requires JNK activity. In addition, these
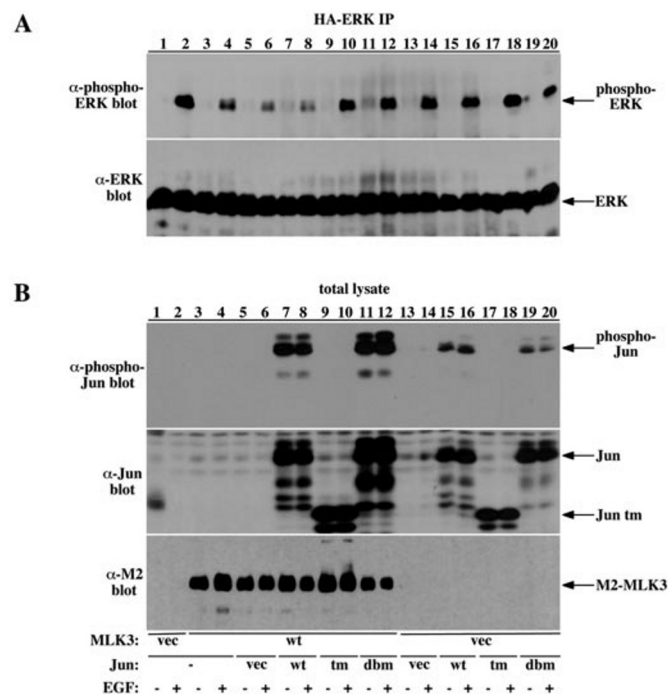
FIG. 5. **Jun transcriptional activity is required for MLK3-induced ERK inhibition.** COS-7 cells were transfected with 1.5 μg of pMT2-HA-ERK-1 vector together with 1.5 μg of empty pRK-5 vector (*lanes 1*, *2*, and *13–20*) or 1.5 μg of pRK-5-M2-FLAG-MLK3 (*lanes 3–12*) and 2 μg of either pCMV empty vector (*lanes 5*, *6*, *13*, and *14*), pCMV-wild type c-Jun (*lanes 7*, *8*, *15*, and *16*), pCMV-Jun-TAM-67 (*tm*, *lanes 9*, *10*, *17*, and *18*), or pCMV-Jun-DBM-3 (*dbm*, *lanes 11*, *12*, *19*, and *20*). *A*, ERK phosphorylation in HA-ERK immunoprecipitates (*IP*) was determined by phospho-ERK immunoblotting (*top panel*), and HA-ERK recovery was determined by ERK immunoblotting (*bottom panel*). *B*, Jun phosphorylation at its activation sites was determined by phospho-Jun immunoblotting of total cell lysates (*top panel*); Jun expression was determined by Jun immunoblotting (*middle panel*), and M2-FLAG-MLK3 expression was determined by M2-FLAG immunoblotting (*bottom panel*). *vec*, vector.

results demonstrate that MLK3-induced MEK phosphorylation is independent of the ability of MLK3 to activate JNK. MLK3-induced MEK phosphorylation, however, requires MLK3 kinase activity, because CEP-11004 blocks MLK3-induced MEK phosphorylation (Fig. 4*C*, *lane 8*). It is important to note that JNK inhibitor II appears not to specifically target JNK, because it inhibits both MLK3 autophosphorylation (Fig. 4*A*, compare *lanes 5* and *6* with *lanes 7–9*) and MLK3-induced MEK phosphorylation (Fig. 4*C*, compare *lanes 3* and *4* with *lanes 7* and *8*).

c-Jun-mediated Gene Transcription Is Required for the Ability of MLK3 to Block ERK Activation—To examine whether JNK directly affects ERK activation or whether the MLK3-induced ERK inhibition requires c-Jun-mediated gene transcription, we co-expressed dominant negative forms of c-Jun with MLK3 and tested their effect on the MLK3-induced ERK inhibition (Fig. 5). Two forms of dominant negative c-Jun (23–25), one lacking the amino-terminal transactivation domain (TAM-67) and the second impaired in its DNA binding (DBM-3), completely reversed MLK3-induced ERK inhibition (Fig. 5*A*, compare *lanes 3–6* with *lanes 9–12*). This reversal happens without affecting MLK3 expression (Fig. 5*B*, *bottom panel*) or MLK3-induced Jun phosphorylation (Fig. 5*B*, *top panel*). In contrast, expression of wild-type c-Jun fails to reverse ERK inhibition and perhaps, if at all, enhances the ability of MLK3 to block ERK activation (Fig. 5*A*, compare *lanes 3* and *4* with *7* and *8*). These results demonstrate that c-Jun-mediated gene transcription is critical for the ability of MLK3 to attenuate
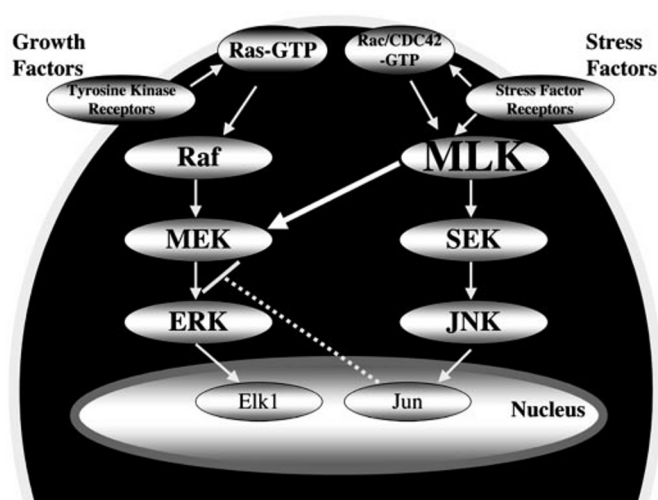

FIG. 6. **A model describing the dual potential of MLK3 in regulating the ERK/MAPK pathway.** Sustained activation of MLK3 negatively regulates the ERK/MAPK pathway by uncoupling ERK activation from MEK via activation of Jun transcriptional activity. In addition, MLK3 has the potential to positively regulate the ERK/MAPK pathway by directly phosphorylating and activating MEK.

ERK activation in response to EGF. Notably, expression of active MLK3 results in a significant increase of c-Jun phosphorylation on its activation sites (Fig. 5*B*, compare *lanes 7, 8, 11,* and *12* with *lanes 15, 16, 19,* and *20*). This increased phosphorylation is observed on both wild-type and DBM-3 c-Jun but not on TAM-67 c-Jun lacking the transactivation amino-terminal part (Fig. 5*B*). Together, the results presented in Fig. 5 demonstrate that, even under conditions in which MLK3 is active and is able to activate the SEK-1-JNK-c-Jun pathway, inhibition of c-Jun reverses MLK3-induced MEK-ERK uncoupling. This indicates that MLK3 restricts ERK activation at a point downstream of c-Jun.

DISCUSSION

Cellular response to environmental changes may be affected by variations in cell type, development, and extracellular conditions. The mechanisms underlying differential cellular responses are only partially understood and can be explained in certain cases by differential gene and protein expression patterns. The complex relationships between varying extracellular conditions and cellular response to a distinct stimulus only recently began to be elucidated at the mechanistic level, especially following the introduction of high throughput technologies in genomics and proteomics. A key question that remains unresolved is how a relatively small number of seemingly linear, intracellular signaling pathways can mediate a large variety of responses to a large variety of stimuli. Our results demonstrating a cross-talk relationship between the stress-activated MLK-SEK-1-stress-activated protein kinase/JNK-Jun pathway and the growth factor/MAPK/ERK pathway (Fig. 6) offer one more answer to this key question. Our results suggest that cells exposed to factors that induce sustained activation of MLK3 and the JNK-Jun pathway will be less responsive to growth factor-induced ERK activation. The recent finding that TNF-α and ceramide activate MLK3 (17), together with the results presented in this paper, suggest that prolonged exposure to TNF-α or to other factors that result in ceramide generation, may render cells less responsive to growth factor-induced ERK activation. This in turn can result in altered cell cycle control, differentiation or cell growth, all cellular responses involving the ERK/MAPK pathway.

Our results demonstrating that MLK3 phosphorylates and activates MEK suggest that under certain physiological condi-

tions MLK3 may function as an activator of the ERK/MAPK pathway, serving as an alternative path to the Raf-mediated pathway (Fig. 6). In addition, these findings imply that short and long phase MLK3 activation may result in a different cellular response; whereas a short phase activation may lead to ERK activation, a long phase activation results in an opposite response. Also, although ERK activation may be mediated directly by MLK3, ERK inhibition is mediated indirectly and involves the ability of MLK3 to activate the JNK-Jun pathway and requires Jun-mediated gene transcription. These implications point to a mechanism in which treatment with the same agonist may result in a different cellular outcome, depending on the duration of the treatment.

The MLK family of serine/threonine kinases consists of more than nine related members that may have isoform-specific functions (5). Knowledge of their physiological function comes mainly from biochemical studies that classify family members as MAPKKK in the stress-activated JNK pathway and from recent genetic studies in *Drosophila*, which confirm the biochemical studies and position the *Drosophila* single MLK isoform (d-MLK/slipper) upstream of JNK (16, 17). The biological function of MLKs in mammalian systems may be, however, more complicated than the one identified in *Drosophila*. For example, in mammals, MLK isoforms are differentially expressed in different tissues and have some variability in their substrate specificity. In addition, differences in association with scaffold proteins have been reported (5). Our findings that MLK3 can activate both SEK-1 and MEK-1 make this picture even more complex. For example, hematopoietic cells that express high levels of MLK3 may respond to cytokines that induce ceramide formation, such as TNF-α, by ERK activation. On the other hand, cells that express little or no MLK3 would be unable to activate ERK under the same conditions. In addition, TNF-α may have a different effect on the same hematopoietic cell, depending on the duration of exposure.

Our finding that the MLK3-induced attenuation of ERK activation depends on c-Jun-mediated gene transcription is in agreement with recent findings showing that stable cell lines expressing the constitutively active form of Jun (v-Jun) exhibit attenuated ERK activation (32). In another model, activation of p38 by arsenite attenuates MEK activation by activating protein phosphatase 1 and 2A (33).

The ability of MLK3 to activate the ERK/MAPK pathway and to induce a transformed phenotype in NIH 3T3 cells has also been reported (21). This work suggested that this transformation is MEK-dependent; however, the role of JNK activation was not examined. Thus, it is possible that the transformed phenotype was a result of Jun activation, but the activity of the MEK-ERK pathway is required for cell survival and normal growth.

An important question that remains unanswered is: What mediates the uncoupling of ERK from MEK? Obvious candidates are phosphatases, which can negatively regulate ERK activation. However, we were unable to detect elevated expression of MKP1–3 (potential ERK specific phosphatases) in cells expressing active MLK3 (data not shown). More detailed study will be required to determine whether elevated expression or activity of phosphatases underlies the observed MEK-ERK uncoupling.

An additional key question remaining to be resolved is: What is the physiological role of the ability of MLK3 to activate MEK? This question can be addressed by developing MLK forms differentially impaired in their ability to activate MEK and SEK or by examining the effects of Raf- and MLK-specific inhibitors on ERK activation under various physiological conditions in different cell types.

In summary, the results in this paper propose a new role for MLK family members in the regulation of the ERK/MAPK pathway both negative and positive. These two opposite effects are mediated by different functions of MLK3: 1) positive regulation through MLK3-mediated phosphorylation and activation of MEK, and (2) negative regulation through MLK3-induced activation of the SEK-1-JNK-Jun pathway that requires Jun-mediated gene transcription. The physiological consequences of the dual MLK3 potential remain to be uncovered.

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