Mixed-lineage kinase 3 regulates B-Raf through maintenance of the B-Raf/Raf-1 complex and inhibition by the NF2 tumor suppressor protein

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The Ras → Raf → MEK1/2 → extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway couples mitogenic signals to cell proliferation. B-Raf and Raf-1 function within an oligomer wherein they are regulated in part by mutual transactivation. The MAPK kinase kinase (MAP3K) mixed-lineage kinase 3 (MLK3) is required for mitogen activation of B-Raf and cell proliferation. Here we show that the kinase activity of MLK3 is not required for support of B-Raf activation. Instead, MLK3 is a component of the B-Raf/Raf-1 complex and is required for maintenance of the integrity of this complex. We show that the activation of ERK and the proliferation of human schwannoma cells bearing a loss-of-function mutation in the *neurofibromatosis 2* (*NF2*) gene require MLK3. We find that merlin, the product of *NF2*, blunts the activation of both ERK and c-Jun N-terminal kinase (JNK). Finally, we demonstrate that merlin and MLK3 can interact *in situ* and that merlin can disrupt the interactions between B-Raf and Raf-1 or those between MLK3 and either B-Raf or Raf-1. Thus, MLK3 is part of a multiprotein complex and is required for ERK activation. The levels of this complex may be negatively regulated by merlin.

extracellular signal-regulated kinase | mitogen-activated protein kinase/merlin

Mitogen-activated protein kinase (MAPK) kinase kinase (MAP3K) → MAPK/extracellular signal-related kinase (ERK) kinase (MEK) → MAPK modules are evolutionarily conserved signaling networks that regulate numerous processes crucial to cell proliferation, survival, inflammation, and metabolism (1). The regulation of MAP3Ks is still poorly understood, especially with regard to how proximal elements that recruit different MAPKs integrate these pathways to produce an appropriate response. Mixed-lineage kinase 3 (MLK3) is one of a family of MAP3Ks, the MLKs, originally identified as specific regulators of the c-Jun N-terminal kinase (JNK) group of MAPKs (1, 2). MLK3 has been linked to TNF activation of JNK (3, 4), and, indeed, targeted disruption of *mlk3* in mice impairs TNF recruitment of JNK (4). Other MAPK pathways are unaffected in *mlk3*$^{-/-}$ mice (4). In contrast, we found that RNA interference (RNAi) silencing of *mlk3* revealed, in multiple, diverse cell types, a broader function for MLK3. Thus, our results indicate that MLK3 is required for activation of ERK, JNK, and p38 MAPKs by mitogenic and proinflammatory stimuli (3). The reasons for the discrepancy between our findings and those for the *mlk3*$^{-/-}$ mice are unclear and may reflect a compensatory redundancy among the different MLKs expressed in these mice, a phenomenon that presumably did not occur in our cultured cell models. Nevertheless, our findings point to a signal-integrating role for MLK3.

Of particular interest, we uncovered a potential non-MAP3K role for MLK3 (3). B-Raf and the related Raf-1 are major MAP3Ks coupling Ras to ERKs 1 and 2 (ERK1/2) and MAPK/ERK kinases 1 and 2 (MEK1/2) (5). We observed that MLK3 was necessary for the signal-dependent phosphorylation of B-Raf at Thr-599 and Ser-602 (3). This phosphorylation, which is not catalyzed directly by MLK3, is Ras-dependent and is absolutely required for B-Raf activation (6, 7). We wished to establish whether the kinase activity of MLK3 was at all required for support of B-Raf and ERK activation and whether there were mechanisms by which MLK3 regulation of ERK might be negatively regulated.

Results and Discussion

Consistent with the observation that MLK3 is required for B-Raf Thr-599/Ser-602 phosphorylation, we observe that silencing of human embryonic kidney (HEK) 293 cell MLK3 completely abrogates the mitogen activation of B-Raf kinase activity assayed *in vitro* by using the substrate MEK1 (Fig. 1*A*).

In contrast to our results (3), CEP1347, a small molecule inhibitor that blocks the activity of all MLKs (8), fails, under conditions of complete inhibition of MLK3, to blunt Ras-dependent cell proliferation (9). CEP1347 is an ATP competitor (8); therefore, its effects cannot reveal situations in which the MLK3 polypeptide is required independently of its catalytic activity. Accordingly, we wished to determine whether the kinase activity of MLK3 is required for mitogen recruitment of B-Raf. HEK 293 cells were a convenient model system for investigation of MLK3 function insofar as we could achieve nearly complete silencing of detectable endogenous MLK3 [>90% oligofection efficiency with small interfering RNA (siRNA)-based RNAi]. In addition, we could transfect these cells at high efficiency with plasmids encoding recombinant MLK3. Thus, we could exploit these cells in "rescue" experiments to determine whether the kinase activity of MLK3 is important to B-Raf activation.

Accordingly, we asked whether ectopic overexpression of recombinant kinase-inactive human MLK3 (at levels sufficient to overcome the effects of RNAi) could complement silencing of endogenous HEK 293 cell *mlk3* and restore mitogen activation of B-Raf and ERK. To silence HEK 293 cell MLK3, specific

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Abbreviations: MAPK, mitogen-activated protein kinase; MAP3K, MAPK kinase kinase; MLK, mixed-lineage kinase; JNK, c-Jun N-terminal kinase; RNAi, RNA interference; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; siRNA, small interfering RNA; HA, hemagglutinin; WCE, whole-cell extract.

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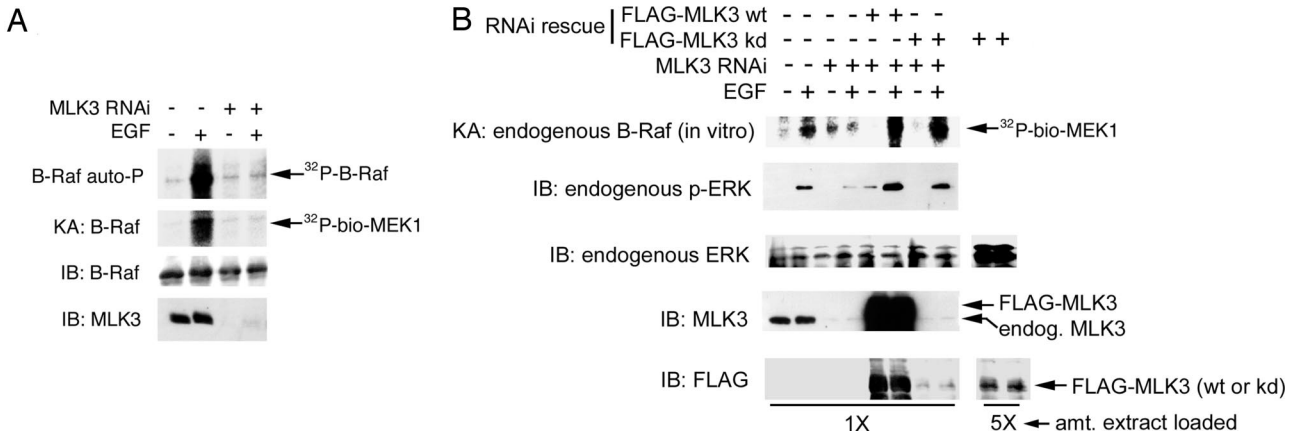

Fig. 1. MLK3 regulates B-Raf in a kinase-independent manner. (*A*) Silencing of MLK3 blunts mitogen activation of the kinase activity of B-Raf. HEK 293 cells were oligofected with human-specific MLK3 siRNA as indicated. Cells then were treated with EGF as indicated. Endogenous B-Raf was immunoprecipitated and assayed *in vitro* for autophosphorylation or, by using biotinylated MEK1 (bio-MEK1) as a substrate, for MAP3K activity. Extracts also were probed with anti-B-Raf or anti-MLK3 as indicated. auto-P, autophosphorylation; KA, kinase assay; IB, immunoblot. (*B*) MLK3 RNAi can be rescued with either WT or kinase-dead MLK3. HEK 293 cells were treated with MLK3 siRNA as indicated. Cells were then transfected with empty vector or WT or kinase-dead (K144R) FLAG-MLK3 cDNA as indicated (FLAG-MLK3 wt or FLAG-MLK3 kd, respectively). Cells were then treated with EGF as indicated. Extracts were prepared, and B-Raf was immunoprecipitated and assayed *in vitro* as in *A*. In parallel, 20 μg of cell extract protein (1×) was probed with phospho-ERK Ab (p-ERK), anti-MLK3 Ab, or anti-FLAG Ab (to detect the transfected MLK3 construct). Because the kinase-dead MLK3 consistently expressed more poorly than WT, a sample of this same extract containing 100 μg of protein (5×), five times the protein loaded for the other immunoblots, is also presented to document the expression of the kinase-dead MLK3.

siRNAs were used. Cells then were transfected with either WT or kinase-dead (K144R) human MLK3. From Fig. 1*B*, it is evident that both WT and kinase-inactive MLK3 can rescue silenced MLK3 and restore both EGF activation of ERK *in situ* and activation of B-Raf, assayed *in vitro*. It is worth noting that the K144R MLK3 expresses much more poorly than WT MLK3 (even in the absence of siRNAs) and does not restore overall MLK3 levels to endogenous levels. Although we cannot quantitatively compare the relative effects of WT and K144R MLK3 on restoring the ERK activation lost upon MLK3 RNAi, what little K144R MLK3 is expressed does completely rescue the effect on ERK activation of MLK3 RNAi. Thus, MLK3 is required for B-Raf activation through a mechanism distinct from its catalytic properties. This finding is consistent with the observation that CEP1347 inhibition of MLK3 kinase activity does not block Ras activation of cell proliferation (9). This result is also consistent with the observation that CEP1347 can impair JNK activation by numerous upstream stimuli (8). In this instance, MLK3 is likely acting as a direct MAP3K, phosphorylating the JNK-specific MEKs MKK4 and MKK7.

The lack of a catalytic role for MLK3 in the regulation of B-Raf prompted us to investigate whether MLK3 might exist as part of a multiprotein signaling complex, the integrity of which is necessary for ERK activation. Activation of both B-Raf and Raf-1 commences upon the binding of GTP-Ras. This binding triggers a series of phosphorylation events culminating in B-Raf/Raf-1 activation. Of particular note, both B-Raf and Raf-1 undergo Ras-dependent regulatory phosphorylation at a highly conserved region within the kinase domain activation loop (residues Thr-599/Ser-602 for B-Raf and Thr-491/Ser-494 for

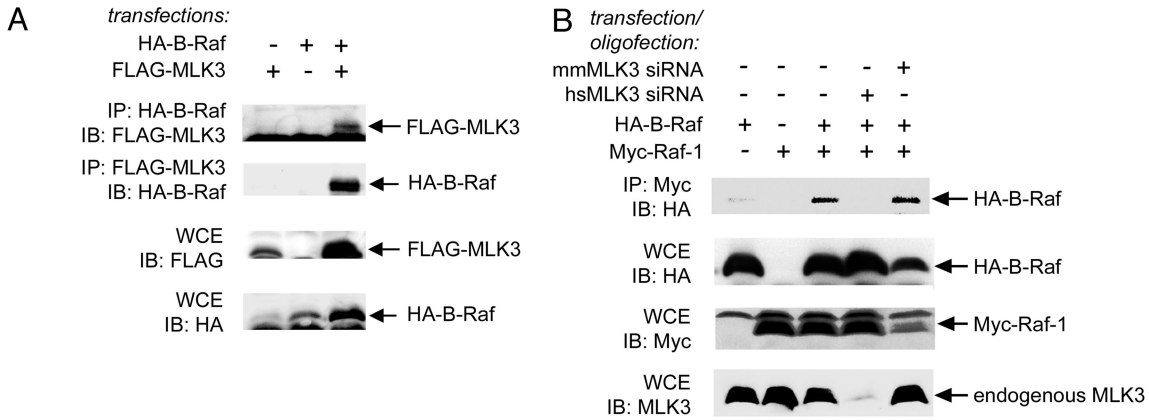

Fig. 2. MLK3 and B-Raf interact *in situ*, as do B-Raf and Raf-1. MLK3 is required for the B-Raf/Raf-1 interaction, suggesting the existence of a MLK3/B-Raf/Raf-1 oligomer. IP, immunoprecipitate; IB, immunoblot. (*A*) *In situ* MLK3/B-Raf interaction. HEK 293 cells were transfected with HA-tagged B-Raf or FLAG-tagged MLK3 as indicated. To document the MLK3/B-Raf interaction, FLAG-MLK3 immunoprecipitates were subjected to anti-HA immunoblotting, and anti-HA immunoprecipitates were subjected to anti-FLAG immunoblotting, as indicated. Whole-cell extracts (WCE) were immunoblotted with anti-FLAG or anti-HA to monitor expression of transfected constructs. (*B*) MLK3 is required for maintenance of the B-Raf/Raf-1 complex. HEK 293 cells were oligofected with either human- or mouse-specific MLK3 siRNA (hsMLK3 or mmMLK3, respectively). Cells then were transfected with HA-B-Raf and Myc-Raf-1 as indicated. Myc-Raf-1 was immunoprecipitated and immunoblotted with anti-HA to detect associated B-Raf. WCE were immunoblotted, as indicated, with anti-HA, anti-Myc, or anti-MLK3 to monitor expression of the Raf constructs and MLK3 RNAi.

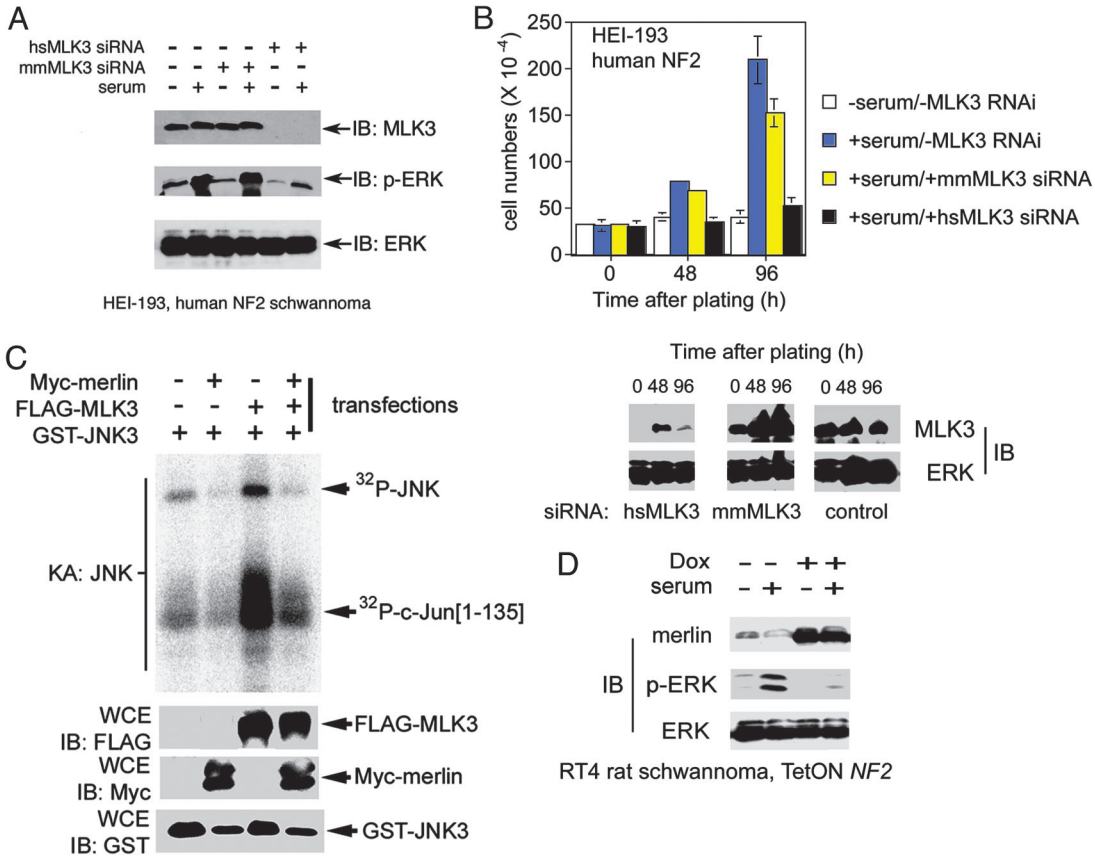

Fig. 3. MLK3 is required for ERK activation and the proliferation of NF2 tumor cells. Merlin suppresses MLK3 activation of JNK and mitogen activation of ERK. IB, immunoblot. (*A*) ERK activation in HEI 193 human NF2 schwannoma cells requires MLK3. Human HEI 193 cells were treated with human- or mouse-specific MLK3 siRNA (hsMLK3 or mmMLK3, respectively). Cells were serum-starved (0.5% serum) and then treated with 20% FCS. WCE were immunoblotted, as indicated, with anti-phospho-ERK (p-ERK) and total ERK (to monitor ERK activation), as well as anti-MLK3. (*B*) MLK3 is required for HEI 193 cell proliferation. HEI 193 cells were treated with hsMLK3 or mmMLK3 and serum-starved as in *A*. Cells (2.5×10^4) were plated onto triplicate wells and treated with vehicle or serum (20%) as indicated. (*Upper*) Cell proliferation was quantitated, by hemocytometer, at the indicated times (bars indicate mean ± SD, $n = 3$). (*Lower*) In parallel, cell extracts were probed with anti-MLK3 to monitor MLK3 RNAi. For each immunoblot (either anti-MLK3 or anti-ERK), samples from the different sets of cells were run on the same gel. Different cell sample immunoblots are shown separately to improve legibility. (*C*) Merlin suppresses MLK3 activation of coexpressed JNK. HEK 293 cells were transfected with Myc-merlin, FLAG-MLK3, or GST-JNK as indicated. WCE were probed with the cognate Abs as indicated. JNK was isolated on glutathione agarose and assayed *in vitro* for phosphorylation of c-Jun (1–135). Autophosphorylation of the GST-JNK, as well as phosphorylation of the c-Jun, is apparent in the figure (^{32}P-JNK or c-Jun, respectively). KA, kinase assay. (*D*) Merlin suppresses mitogen activation of ERK. RT4 NF2.17 rat schwannoma cells expressing a TetON *NF2* cDNA construct were treated with doxycycline (Dox) to induce merlin expression. Cells then were serum-starved and treated with serum as in *B*. WCE were assayed for *in situ* ERK activation as in *A*.

Raf-1) (5–7, 10). The molecular mechanisms controlling this phosphorylation remain unclear. Although MLK3 is required for B-Raf phosphorylation at Thr-599/Ser-602, MLK3 does not itself phosphorylate these residues, nor is MLK3 required for Raf-1 phosphorylation at Thr-491/Ser-494 (3). Disruption of either *B-raf* or *raf-1* impairs activation of the remaining Raf gene, suggesting that Raf activation might include transactivation. Oligomerization is important to the activation of Raf family kinases (10–15). Consistent with this finding, recent studies have shown that B-Raf and Raf-1 exist as a complex in cells and that, within this context, B-Raf can activate Raf-1 in trans (16). Of particular interest, several oncogenic mutations of B-Raf do not increase B-Raf kinase activity *per se*. Instead, these mutants enable a constitutive transactivation of Raf-1 that is associated with B-Raf. Indeed, the signaling capacity of these B-Raf mutants is severely impaired upon silencing of *raf-1* (16).

Might the function of MLK3 be to maintain the integrity of the B-Raf/Raf-1 complex? We have previously observed that endogenous MLK3 and B-Raf interact *in situ* (3). Consistent with this result, we find that recombinant MLK3 and B-Raf interact *in situ* and can be coimmunoprecipitated (see Figs. 2*A* and 4*C*). As with the endogenous proteins, the levels of the recombinant B-Raf/MLK3 complex do not change with mitogen stimulation (data not shown). In addition, recombinant MLK3 and Raf-1 interact *in situ* (see Fig. 4*C*).

We also find that recombinant B-Raf and Raf-1, expressed in HEK 293 cells, interact *in situ* and can be coimmunoprecipitated (Fig. 2*B*). Treatment with human-specific MLK3 siRNA completely silences endogenous HEK 293 cell *mlk3*. An analogous murine MLK3 siRNA is without effect. Silencing of *mlk3* totally abolishes the observed *in situ* interaction between B-Raf and Raf-1 (Fig. 2*B*). Together, the results shown in Figs. 1 and 2 support the idea that MLK3, B-Raf, and Raf-1 exist as a complex in cells and that the integrity of this complex requires the presence of the MLK3 polypeptide. Moreover, inasmuch as B-Raf can transactivate Raf-1 (and possibly vice versa) (10–12, 16), it is reasonable to propose that the activation of the Raf kinases by mitogens requires that they be in the MLK3/B-Raf/Raf-1 complex. The existence of this complex may enable the steps involved in Raf protein transactivation.

How might the levels of the MLK3/B-Raf/Raf-1 complex be regulated? As noted above, we did not observe mitogen-

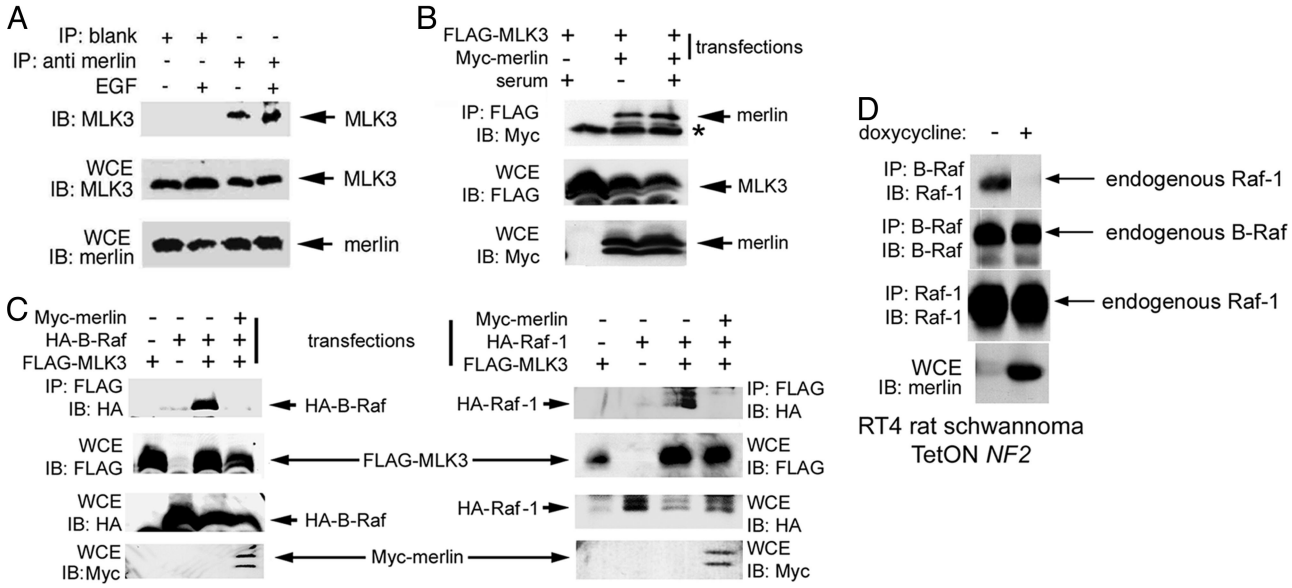

Fig. 4. Merlin associates with MLK3 *in situ* and can blunt the MLK3/B-Raf interaction. (*A*) Association of endogenous MLK3 and merlin. F4328 $NF2^{+/+}$, $p53^{-/-}$ osteosarcoma cells were treated with vehicle or EGF. Endogenous merlin was immunoprecipitated as indicated, and the immunoprecipitate (IP) was probed on immunoblots (IB) with anti-MLK3 to detect associated MLK3. WCE also were subjected to immunoblotting with anti-MLK3 or anti-merlin as indicated. (*B*) Association of recombinant merlin and MLK3. HEK 293 cells were transfected with FLAG-MLK3 and Myc-merlin as indicated. FLAG-MLK3 was immunoprecipitated and probed on immunoblots with anti-Myc to detect associated merlin. WCE also were probed with the cognate epitope Abs. The asterisk indicates a nonspecific band, probably the IgG heavy chain. (*C*) Expression of merlin blocks the association of coexpressed B-Raf and MLK3 or Raf-1 and MLK3. (*Left*) HEK 293 cells were transfected with HA-B-Raf, FLAG-MLK3, or Myc-merlin as indicated. Recombinant MLK3 was immunoprecipitated with anti-FLAG, and the immunoprecipitate was probed with anti-HA to detect associated B-Raf. WCE also were probed in parallel with the cognate epitope Abs. (*Right*) The same as in *Left*, except that HA-Raf-1 was transfected instead of HA-B-Raf. (*D*) Induction of ectopic merlin expression disrupts the integrity of B-Raf/Raf-1 complexes. RT4 NF2.17 cells were treated with doxycycline as indicated. B-Raf or Raf-1 then was immunoprecipitated, and the immunoprecipitates were subjected to SDS/PAGE and immunoblotting with the indicated Abs. In addition, to monitor merlin induction, WCE were subjected to SDS/PAGE and immunoblotting with anti-merlin.

dependent changes in the level of this complex. We have found that the proliferation of several Ras-dependent tumor cell lines requires MLK3. This finding includes tumors manifesting loss-of-function mutations in the *NF1* and *NF2* genes (3). *NF2* encodes merlin, an ezrin/radixin/moesin (ERM) family protein implicated in the regulation of the actin cytoskeleton (17, 18). Gene disruption and transfection studies have indicated that merlin can suppress the activation of the JNK group of MAPKs by upstream stimuli. In contrast, parallel studies showed that ERK activation, in cells not subjected to serum starvation and subsequent serum readdition, seemed not to be inhibited by merlin (19). On the other hand, merlin can provoke the proteasomal degradation of the platelet-derived growth factor (PDGF) receptor, a potent upstream activator of the ERKs (20). We wished to evaluate further the role of MLK3 and the ERKs in NF2 tumor cell proliferation and to determine whether indeed merlin could exert an impact on ERK activation.

HEI 193 is a human schwannoma cell line established from a patient bearing a loss-of-function splice-site mutation in *NF2* (20, 21). These cells exhibit a high basal ERK activity stimulated further by serum (Fig. 3*A*). Use of a human-specific (but not a mouse-specific) siRNA to silence *mlk3* in these cells suppresses both the basal and stimulated phosphorylation of ERK at the activating phosphoacceptor sites (Fig. 3*A*). The proliferation of HEI 193 cells is serum-dependent; however, silencing of *mlk3* profoundly blocks proliferation of these cells (Fig. 3*B*). Thus, in NF2 tumor cells, ERK activation and proliferation require MLK3, a finding consistent with our previous results (3) for $NF2^{-/-}$ osteosarcoma cells.

Merlin also can strikingly inhibit MLK3-dependent JNK activation, a result that fits with previous observations indicating that merlin can blunt the activation of JNK by extracellular stimuli (19). Thus, coexpression of merlin with MLK3 and JNK completely inhibits MLK3 activation of recombinant JNK (Fig. 3*C*). Inasmuch as MLK3 is a direct MKK4/7 kinase and is required in several cell types for JNK activation by mitogens and cytokines (3), the ability of merlin to suppress MLK3 recruitment of JNK may explain, in part, the mechanism by which merlin reduces JNK activation.

To assess the ability of merlin to blunt ERK activation, we exploited RT4 NF2.17 cells, a rat schwannoma cell line in which merlin can be expressed from a stably transfected ectopic, doxycycline-inducible (TetON) construct (22). In the absence of merlin induction, ERK is readily activated (phosphorylated at the regulatory phosphoacceptor sites) in response to serum. Treatment with doxycycline to induce the ectopic merlin construct resulted in nearly complete suppression of ERK activation (Fig. 3*D*). Thus, merlin not only can inhibit JNK activation, but it also can reduce serum-stimulated ERK activation.

Given the observation that merlin can disrupt ERK activation and the findings that MLK3 and B-Raf exist in a multiprotein complex and that MLK3 is required for mitogen activation of B-Raf, we wondered whether merlin could disrupt MLK3 signaling functions, perhaps through protein–protein interactions. F4328 is an $NF2^{+/+}$, $p53^{-/-}$ osteosarcoma cell line (23). These cells were serum-starved and then treated with EGF. Endogenous merlin was immunoprecipitated from these cells and subjected to immunoblotting with anti-MLK3. As is evident from Fig. 4*A*, endogenous MLK3 and merlin associate *in situ*. In addition, EGF pretreatment does not substantially alter the amount of MLK3 recovered in anti-merlin immunoprecipitates. Similarly, recombinant merlin and MLK3 interact *in situ* and can be coimmunoprecipitated from lysates of transfected HEK 293 cells. Moreover, as with the F4328 cells, treatment of the HEK 293 cells with serum does not affect the MLK3/merlin interaction (Fig. 4*B*). Thus, as was shown for the MLK3/B-Raf/Raf-1 complex, the MLK3/merlin complex seems to be refractory to extracellular stimuli.

From Figs. 1 and 2, it is evident that MLK3, independent of its kinase activity, is necessary for ERK activation, likely by supporting

the maintenance of the B-Raf/Raf-1 complex shown (16) to enable B-Raf/Raf-1 transactivation. From Fig. 3*D*, we see that merlin can disrupt ERK activation by mitogen. Expression of merlin also can disrupt the interactions between coexpressed MLK3 and B-Raf and MLK3 and Raf-1, essentially eliminating all B-Raf or Raf-1 recovered in MLK3 immunoprecipitates (Fig. 4*C*).

B-Raf can activate Raf-1 in trans, and the integrity of the B-Raf/Raf-1 complex is important to Raf signaling (16). We used RT4 NF2.17 cells to determine whether doxycycline induction of ectopic *NF2* expression could disrupt the endogenous B-Raf/Raf-1 complex. From Fig. 4*D*, it is clear that induction of merlin completely abolished the detectable interaction between endogenous B-Raf and Raf-1. Thus, by disrupting the binding of MLK3 to B-Raf or Raf-1, as well as the binding of B-Raf to Raf-1, it is conceivable that merlin could affect the levels of MLK3/B-Raf/Raf-1 complex in cells, thereby reducing signaling through B-RAF and Raf-1.

Insofar as MLK3/B-Raf/Raf-1 and merlin/MLK3 complex levels do not change with mitogen stimulation, how might merlin function to regulate ERK activation in cells? Although acute stimuli do not dynamically affect merlin levels (24), it is possible that the static expression level of merlin in a given tissue or cell type, relative to MLK3 levels, could, by modulating the level of the MLK3/B-Raf/Raf-1 complex, determine the intensity and/or duration of ERK activation. Indeed, weak or transient ERK activation is insufficient to trigger cell proliferation (25). Moreover, loss of merlin expression or function, consequent to mutations in *NF2*, is associated with proliferation, and we find, in at least two merlin loss-of-function models (including cells derived from an NF2 patient), that this proliferation requires MLK3. Many loss-of-function mutations in merlin are associated with the production of unstable merlin polypeptides (17). It is possible that pathophysiologically reduced merlin protein levels or mutant merlin proteins with reduced MLK3 binding capacity could foster not only elevated JNK activation but also increased levels of MLK3/B-Raf/Raf-1 complexes and ERK activation.

Although we can detect interactions between MLK3 and B-Raf and Raf-1, as well as interactions between B-Raf and Raf-1 and between merlin and MLK3, and whereas the levels of MLK3/B-Raf and MLK3/merlin interactions apparently do not change with mitogen, the precise molecular compositions of these complexes remain unknown. It is possible that mitogens trigger the binding/dissociation of other polypeptides within these complexes. Accordingly, in addition to merlin-mediated changes in the tonic levels of MLK3/B-Raf as well as B-Raf/Raf-1, extracellular stimuli and tumor suppressor proteins may affect the dynamic nature of multiprotein signaling complexes that contain MLK3 and B-Raf and Raf-1.

In conclusion, our results indicate that MLK3 is a signal-integrating kinase with both conventional MAP3K catalytic activity (contributing to JNK activation) and noncatalytic functions that serve to recruit the Raf/ERK pathway and that signaling through MLK3 can be negatively regulated by the tumor suppressor merlin.

Materials and Methods

Cell Lines, Treatments, and Proliferation Assays. HEK 293 cells were cultured in DMEM supplemented with 10% FBS. HEI 193 human NF2 schwannoma, inducible TetON RT4 NF2.17 rat schwannoma, and F4328 ($NF2^{+/+}$, $p53^{-/-}$) mouse osteosarcoma cells were cultured in DMEM supplemented with 15% FBS. For some experiments, HEK 293, HEI 193, and F4328 $NF2^{+/+}$ cells were serum-starved (0.5% FBS, 24 h) and treated with vehicle, EGF (50 ng/ml) or 20% FBS, for 10 min. RT4 NF2.17 cells were serum-starved (as described above), treated with vehicle or 1 μg/ml doxycycline (Calbiochem) to induce merlin protein expression, and then stimulated with vehicle or 20% FBS for 10 min. HEI 193 proliferation assays were performed as described in ref. 3.

Plasmids and Constructs. The following vectors were used: pCMV5-FLAG, pCMV5-Myc, human pCMV5-FLAG-MLK3 WT, pCDNA3.1-FLAG-K144R-MLK3 kinase-dead, influenza hemagglutinin (HA)-tagged human pMT3-B-Raf, human pMT3-HA-Raf-1, human pMT2-Myc-Raf-1, human pCMV5-Myc-merlin, and pEBG (GST-tagged) JNK3.

Transfections. HEK 293 cells were cultured in 10-cm dishes and transfected with 2–5 μg of plasmid DNA by using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Transfected DNA levels were balanced with empty plasmid. Cells were harvested 16–20 h after transfection. In some cases, cells were serum-starved and stimulated with EGF before harvesting.

Oligofections/Transfections. MLK3 siRNA oligofections and siRNA oligonucleotide sequences are described in ref. 3. Briefly, cells were seeded in 6-cm culture dishes, and at ≈70% confluence they were oligofected. For contemporaneous introduction of siRNAs and cDNA plasmids, Lipofectamine 2000 was used; for situations in which only siRNAs were introduced, we used Oligofectamine. Both agents were from Invitrogen. For complementation assays, we oligofected cells with 5 μg of MLK3 siRNA oligonucleotide duplex per dish; MLK3 siRNA was oligofected alone or together with 0.3 μg of pCMV5-FLAG-MLK3 or 2.0 μg of pCMV5-FLAG-K144R kinase-dead MLK3 plasmid DNA. For B-Raf/Raf-1 coimmunoprecipitation assays, cells were seeded in 10-cm culture dishes and oligofected with 15 μg of MLK3 siRNA per dish together with 2 μg of HA-B-Raf and 5 μg of Myc-Raf-1 plasmid DNA. Cells were harvested after 16–20 h. All conventional transfections used Lipofectamine 2000. To assay merlin/MLK3 interactions and the effects of merlin on MLK3/B-Raf or MLK3/Raf-1 interactions, 10-cm dishes of HEK 293 cells were transfected with 2 μg of pCMV5-Myc-merlin, 5 μg of pCMV5-FLAG-MLK3, and/or 5 μg of pMT3-HA-B-Raf or pMT3-HA-Raf-1 as indicated in Fig. 4. For experiments examining JNK activity, 10-cm dishes of HEK 293 cells were transfected with 1 μg of pEBG-JNK3 (GST-tagged), 5 μg of pCMV5-FLAG-MLK3, and 2 μg of pCMV5-Myc-merlin.

Coimmunoprecipitations. Lysis and immunoprecipitations were performed as described in ref. 3 by using anti-FLAG (Kodak), anti-GST (Upstate Biotechnology, Lake Placid, NY), anti-HA, or anti-Myc antibodies (Abs). Merlin Ab used for coimmunoprecipitation of endogenous merlin and MLK3 was from Santa Cruz Biotechnology.

Immunoblotting. Proteins were resolved by 15% SDS/PAGE, transferred to poly(vinylidene difluoride) (PVDF) membranes and detected with anti-FLAG, anti-GST, anti-HA, or anti-Myc Abs. Activation-state phospho-ERK (Thr-202/Tyr-204) Ab was from Cell Signaling Technology (Beverly, MA). B-Raf, MLK3, ERK, and merlin Abs were from Santa Cruz Biotechnology.

Kinase Assays. For JNK assays, transfections were performed as described above. GST-JNK was purified on glutathione agarose beads. JNK activity was assayed by using GST-c-Jun (1–135) as described in ref. 3. For assay of endogenous B-Raf kinase activity, B-Raf was immunoprecipitated from HEK 293 cells by using anti-B-Raf Ab (Santa Cruz Biotechnology) and assayed by using biotinylated MEK1 as substrate (3).

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