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RESEARCH ARTICLE

LATS1 Regulates Mixed-Lineage Kinase 3 (MLK3) Subcellular Localization and MLK3-Mediated Invasion in Ovarian Epithelial Cells

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ABSTRACT Mixed-lineage kinase 3 (MLK3) activates mammalian mitogen-activated protein kinase (MAPK) signaling pathways in response to cytokines and stress stimuli. MLK3 is important for proliferation, migration, and invasion of different types of human tumor cells. We observed that endogenous MLK3 was localized to both the cytoplasm and the nucleus in immortalized ovarian epithelial (T80) and ovarian cancer cells, and mutation of arginines 474 and 475 within a putative MLK3 nuclear localization sequence (NLS) resulted in exclusion of MLK3 from the nucleus. The large tumor suppressor (LATS) Ser/Thr kinase regulates cell proliferation, morphology, apoptosis, and mitotic exit in response to cell-cell contact. RNA interference (RNAi)-mediated knockdown of LATS1 increased nuclear, endogenous MLK3 in T80 cells. LATS1 phosphorylated MLK3 on Thr477, which is within the putative NLS, and LATS1 expression enhanced the association between MLK3 and the adapter protein 14-3-3ζ. Thr477 is essential for MLK3–14-3-3ζ association and MLK3 retention in the cytoplasm, and a T477A MLK3 mutant had predominantly nuclear localization and significantly increased invasiveness of SKOV3 ovarian cancer cells. This study identified a novel link between the MAPK and Hippo/LATS1 signaling pathways. Our results reveal LATS1 as a novel regulator of MLK3 that controls MLK3 nuclear/cytoplasmic localization and MLK3-dependent ovarian cancer cell invasion.

KEYWORDS MLK3, LATS1, 14-3-3, ovarian cancer, invasion, LATS, mitogen-activated protein kinases, ovarian

Mammalian mitogen-activated protein kinase (MAPK) signal transduction pathways mediate cellular responses to extracellular stimuli through the coordinated phosphorylation and activation of three MAPK enzymes within a phosphorelay signaling module (1). Extracellular stimulus-dependent activation of cell surface receptors leads to the activation of MAPK kinase kinases (MAP3Ks), which phosphorylate and activate MAPK kinases (MAP2Ks), which, in turn, phosphorylate and activate MAPKs. Activated MAPKs phosphorylate substrates in the cytoplasm and also translocate into the nucleus to phosphorylate transcription factors and elicit specific cellular responses (1). Mixed-lineage kinases (MLKs) are a family of serine and threonine MAP3Ks which consist of three subfamilies, including MLKs, the dual leucine zipper kinases (DLKs), and the zipper sterile α-motif kinases (ZAKs) (2). MLK3, a member of the MLK subfamily, activates Jun N-terminal protein kinase (JNK) and p38 pathways and can promote extracellular signal-regulated kinase (ERK) activation through mechanisms that are dependent or independent of MLK3 kinase activity (2–6).

MLK3 plays a crucial role in invasion, migration, and proliferation in different tumor cell types (5, 7–14). Furthermore, MLK3 mutations have been identified in mismatch repair-deficient gastrointestinal tumors. A P252H mutation enhanced MLK3 signaling and promoted tumor formation in mice, and it was suggested that this mutation

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affected MLK3 scaffolding function rather than its kinase activity (15, 16). High levels of MLK3 protein have been identified in ovarian, breast, and pancreatic cancers (9, 12, 13). In addition, MLK3 is required for ovarian cancer cell invasion and matrix metalloproteinase 2 (MMP-2) and MMP-9 activities and for breast cancer MDA-MB-231 xenograft tumor growth and metastasis (9, 17). Collectively, these findings underscore the importance of MLK3 and its regulation in tumor development and cancer progression.

The Rho GTPase Cdc42 is one of the few signaling proteins that have been identified as upstream activators of MLK3 (18, 19). Cdc42-dependent activation of MLK3 occurs via its interaction with the MLK3 CRIB motif (20). Negative regulators of MLK3 kinase activity include MLK4β and the tumor suppressor Merlin (NF2), which inhibits Cdc42-MLK3 binding (10, 21). Merlin is also a key regulator of Hippo/MST signaling and functions to coordinate the activation of large tumor suppressor (LATS) kinases (22).

LATS1/2 Ser/Thr kinases are highly conserved members of the AGC family of kinases that regulate cell proliferation, morphology, apoptosis, and mitotic exit (23, 24). LATS1 is also a key member of the Hippo/MST signaling pathway that regulates organ size. In Hippo/MST signaling, activation of LATS1 involves MOB1-mediated autophosphorylation and phosphorylation of hydrophobic C-terminal residues by MST family kinases (25). LATS1 phosphorylates the transcriptional coactivator YAP1, which results in 14-3-3 binding and retention of YAP1 in the cytoplasm. Cytoplasmic YAP1 is eventually targeted for degradation after further phosphorylation and ubiquitination (26). The Hippo/YAP pathway regulates tissue proliferation and apoptosis in response to external developmental stimuli, such as cell-cell contact and density (27). Loss of function of either LATS1 or LATS2 has been observed in different types of tumors, including those associated with leukemia and ovarian, prostate, breast, lung, and esophageal cancers (28, 29). Silencing LATS1/2 reduces p53 phosphorylation and increases cell migration and, in turn, breast cancer progression (30). LATS1-null mice are prone to soft tissue sarcomas and ovarian tumors, and loss of LATS2 leads to loss of contact inhibition (31, 32). High levels of LATS1 have recently been associated with better prognosis in ovarian cancer patients, and LATS1 overexpression suppresses tumorigenesis (29, 33).

This study identified LATS1 as an upstream regulator of MLK3, uncovering a novel link between the MLK3/MAPK and Hippo/MST signaling pathways. LATS1 phosphorylates MLK3 and promotes MLK3 binding to 14-3-3ζ and MLK3 localization in the cytoplasm. Confluent ovarian cancer SKOV3 cells, which have low levels of activated LATS1, have predominantly nuclear MLK3, and heightened amounts of MLK3 in the nucleus correlate with increased invasiveness of ovarian cancer cells.

RESULTS

MLK3 subcellular localization in immortalized ovarian epithelial and ovarian cancer cells. Results from previous studies indicate that the outcome of MLK3 signaling varies depending on the cellular context (8, 34–38). The subcellular localization of MLK3 is also critical for spatiotemporal dynamics of MLK3 signaling; however, the localization of MLK3 in different cell types is not well understood. We analyzed MLK3 subcellular localization by indirect immunofluorescence staining of endogenous MLK3 in ovarian cancer SKOV3 and TOV112D cells and in immortalized ovarian epithelial T29 and T80 cells (Fig. 1A). MLK3 localization was both nuclear and cytoplasmic in all cell lines tested; however, nuclear MLK3 was significantly higher in the ovarian cancer SKOV3 and TOV112D cells than in T29 and T80 cells. Similar results were obtained by immunoblotting of MLK3 in nuclear and cytoplasmic fractions from T80, T29, SKOV3, and TOV112D cells. Immunoblotting of α-tubulin and histone H3 proteins verified the purity of the nuclear and cytoplasmic fractions (Fig. 1B). The antibody specificity for endogenous MLK3 was verified by RNA interference (RNAi)-mediated knockdown of MLK3 in T80 and SKOV3 cells (Fig. 1C, left) with two double-stranded small interfering RNA (siRNA) oligonucleotides for different MLK3 target sequences, followed by immunofluorescence staining of endogenous MLK3. Endogenous MLK3 protein was substantially reduced in T80 and SKOV3 cells transfected with MLK3 siRNA1 or −2, as determined by immunoblotting of whole-cell extracts (Fig. 1C, right).

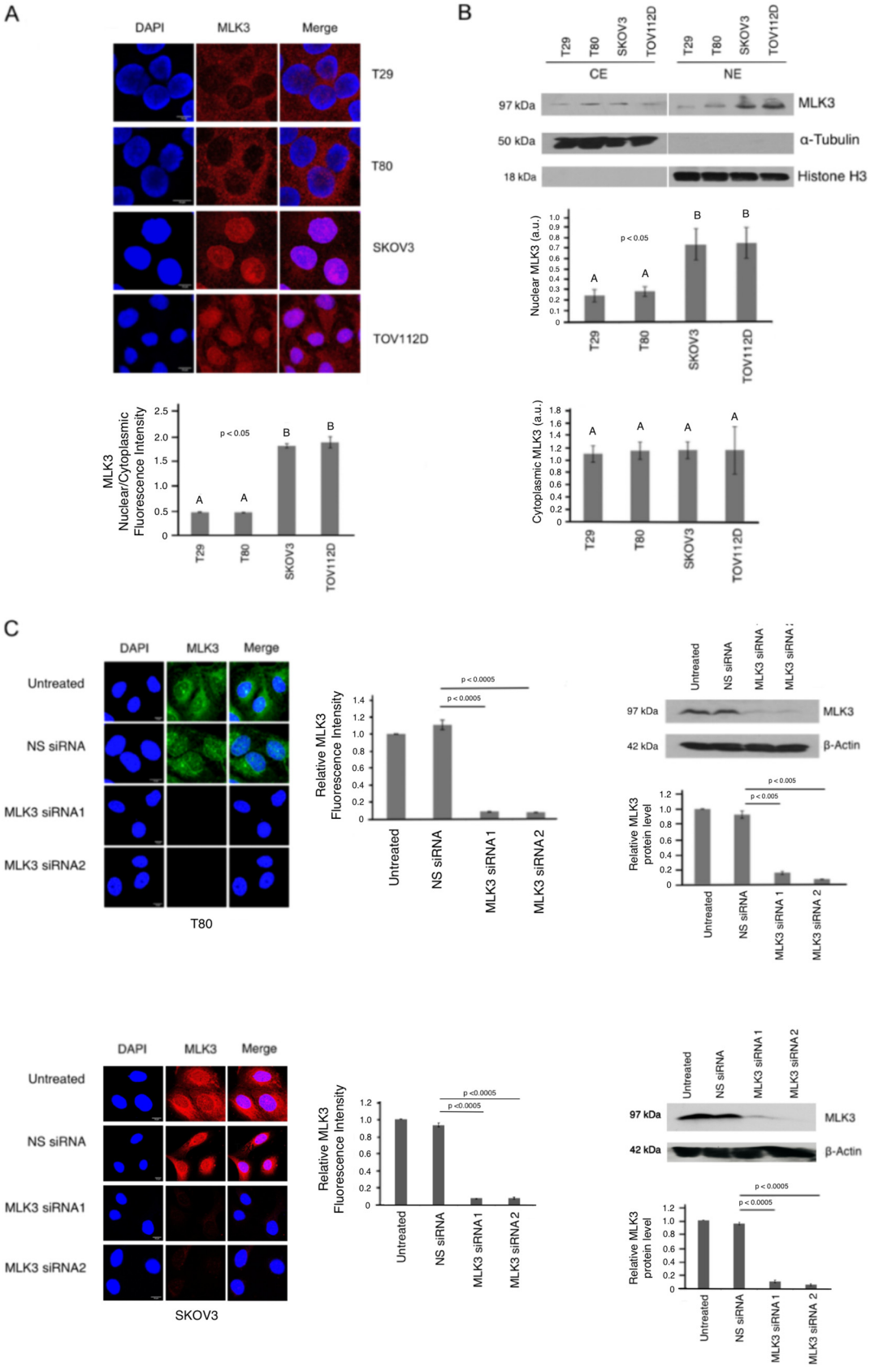

FIG 1 MLK3 subcellular localization in immortalized ovarian epithelial and ovarian cancer cells. (A) Immunofluorescence staining of endogenous MLK3 (red) was performed in T29, T80, SKOV3, and TOV112D cells. Cells were counterstained with

(Continued on next page)

LATS1 activity in ovarian epithelial cells. High expression of LATS1 is associated with a better prognosis in patients with ovarian serous carcinoma (29). In addition, phosphorylation on Thr1079 is essential for LATS1 activity (24). To investigate LATS1 expression and activation in ovarian (normal and cancer) epithelial cells, LATS1 total protein and phosphorylated, activated LATS1 (p-LATS1; phosphorylated on Thr1079) were analyzed by immunoblotting of T29, T80, SKOV3, and TOV112D whole-cell extracts (confluent at the time of harvest). LATS1 protein was detected in all cell lines tested; however, activated p-LATS1 was detected only in the normal T29 and T80 cell lines and not in the ovarian cancer SKOV3 or TOV112D cell lines (Fig. 2A). These results indicate that SKOV3 and TOV112D ovarian cancer cell lines have minimal functional, activated LATS1. We investigated whether reduced LATS1 activation was due to reduced levels of activated MST1/2 and observed that neither normal nor ovarian cancer cells had detectable levels of activated, phosphorylated MST1/2 (p-MST1/2) (Fig. 2). LATS1 can be activated independently of MST-mediated phosphorylation by MAP4K 4/6/7 kinases, and our results suggest that LATS1 activation in these cells is dependent, at least in part, on a kinase(s) other than MST1/2 (39–41).

Cell-cell contact is a major inducer of LATS1 kinase activity (26). To establish whether LATS1 activation is induced by cell confluence in T80 cells, immunoblotting was performed to assess the amounts of p-LATS1, p-Yap, and p-MST1/2 in T80 cells that were cultured at low and high confluence (Fig. 2B, left). The amounts of p-LATS1 and p-Yap were significantly larger in confluent T80 cells than in cells at low confluence, which indicates that high cell confluence stimulates LATS1 pathway activation in T80 cells (Fig. 2B, right). There was no detectable level of p-MST1/2 in T80 cells at low or high confluence.

LATS1 interacts with and phosphorylates MLK3. We hypothesized that LATS1 may be an upstream regulator of MLK3. To explore this possibility, we analyzed whether LATS1 and MLK3 proteins are associated by coimmunoprecipitation experiments. HEK293 cells were transiently transfected with pCMV vector and FLAG-LATS1, endogenous MLK3 was immunoprecipitated, and the immunoprecipitates were immunoblotted with FLAG antibody to detect associated FLAG-LATS1. For control immunoprecipitations, mouse IgG was used as the immunoprecipitating antibody. Cell lysates were immunoblotted with FLAG, MLK3, and β-actin antibodies. The results show that endogenous MLK3 and FLAG-LATS1 are associated in HEK293 cells (Fig. 3A). Furthermore, in an *in vitro* binding assay, recombinant FLAG-LATS1 was pulled down with recombinant His-MLK3, which indicates that LATS1 directly binds to MLK3 (Fig. 3B). To investigate the subcellular localization of LATS1 and MLK3, FLAG-LATS1 was ectopically expressed in T80 and SKOV3 cells and immunofluorescence staining was performed to detect FLAG-LATS1 and endogenous MLK3. FLAG-LATS1 and endogenous MLK3 were found to have similar cytoplasmic localization patterns (Fig. 3C).

The observation that LATS1 bound to MLK3 prompted us to examine whether LATS1 could directly phosphorylate MLK3. The LATS/NDR kinase consensus phosphorylation sequence is HX(R/H/K)XXS/T, and MLK3 amino acids (aa) 470 to 478 (HVRRRRGTF) have similarity to this sequence (42). To investigate whether T477 of MLK3 is phosphorylated by LATS1 *in vitro*, pCMV vector, FLAG-MLK3(KD) which is kinase dead (KD) with a K144R mutation, and FLAG-MLK3 (KD/T477A) were expressed in HEK293 cells, immunoprecipitated, and used as substrates in an *in vitro* LATS1 kinase

FIG 1 Legend (Continued)
DAPI (blue). The ratio of nuclear to cytoplasmic MLK3 fluorescence intensity was quantitated. Data are means with SEM ($n = 3$) using Kruskal-Wallis ANOVA (bottom). (B) Cell fractionation of confluent T29, T80, SKOV3, and TOV112D cells was performed and cytoplasmic and nuclear extracts were immunoblotted for endogenous MLK3, α-tubulin, and histone H3 (upper portion). MLK3 protein in the cytoplasmic and nuclear fractions was quantified. Data are means with SEM ($n = 3$) using Kruskal-Wallis ANOVA (lower portion). a.u., arbitrary units. (C) Immunofluorescence staining of MLK3 in T80 (green) or SKOV3 (red) cells transfected with MLK3 or nonspecific (NS) siRNA (left portions). Cells were counterstained with DAPI (blue). Relative MLK3 fluorescence intensity was quantified and MLK3 knockdown was confirmed by immunoblotting cell extracts with MLK3 and β-actin antibodies (middle and right portions). Data are means $\pm$ SEM ($n = 3$); Student's *t* test *P* value is indicated.

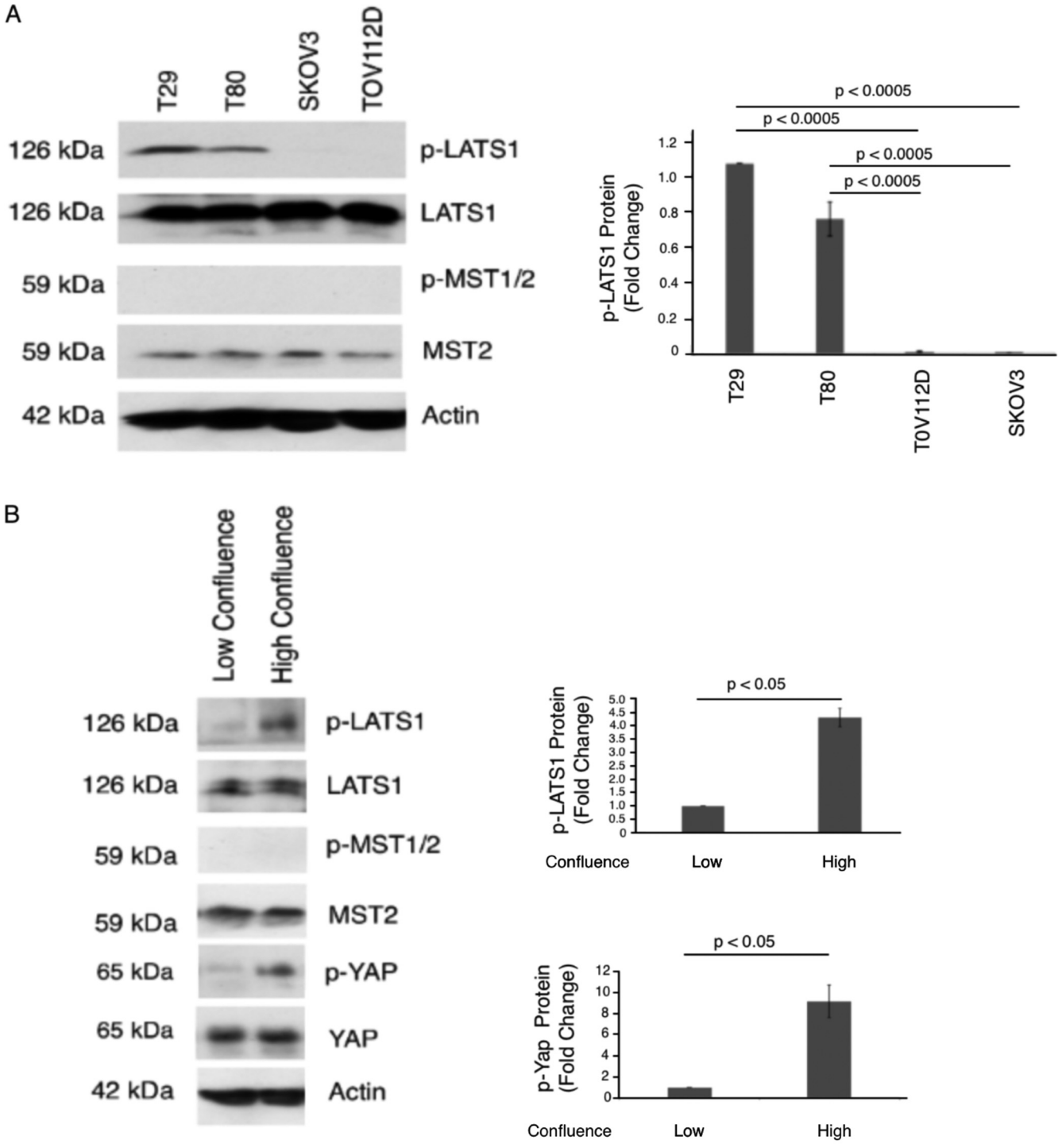

FIG 2 LATS1 activity in ovarian epithelial cells. (A) T29, T80, SKOV3, and TOV112D cell lines were cultured to approximately 95% confluence and whole-cell extracts were immunoblotted with p-LATS1, LATS1, p-MST1/2, MST2, and β-actin antibodies (left). Data are means ± SEM ($n = 3$); Student's t test P value is indicated (right). (B) T80 cells were cultured to low (30%) and high (95%) confluence. Cell extracts were immunoblotted with p-LATS1, total LATS1, p-MST1/2, MST2, p-YAP, YAP, and β-actin antibodies (left). Data are means ± SEM ($n = 3$); Student's t test P value is indicated (right).

assay. LATS1 phosphorylated FLAG-MLK3 (KD) to a significantly greater extent than FLAG-MLK3(KD/T477A), which indicates that LATS1 phosphorylates MLK3 *in vitro*, and T477 is a major residue that is phosphorylated by LATS1 (Fig. 3D).

LATS1 enhances the interaction between MLK3 and 14-3-3ζ. In Hippo/MST signaling, LATS1 phosphorylation of YAP1 enables 14-3-3 binding and cytoplasmic retention of YAP1. Thus, 14-3-3 and LATS1 regulate YAP1-mediated transcription of

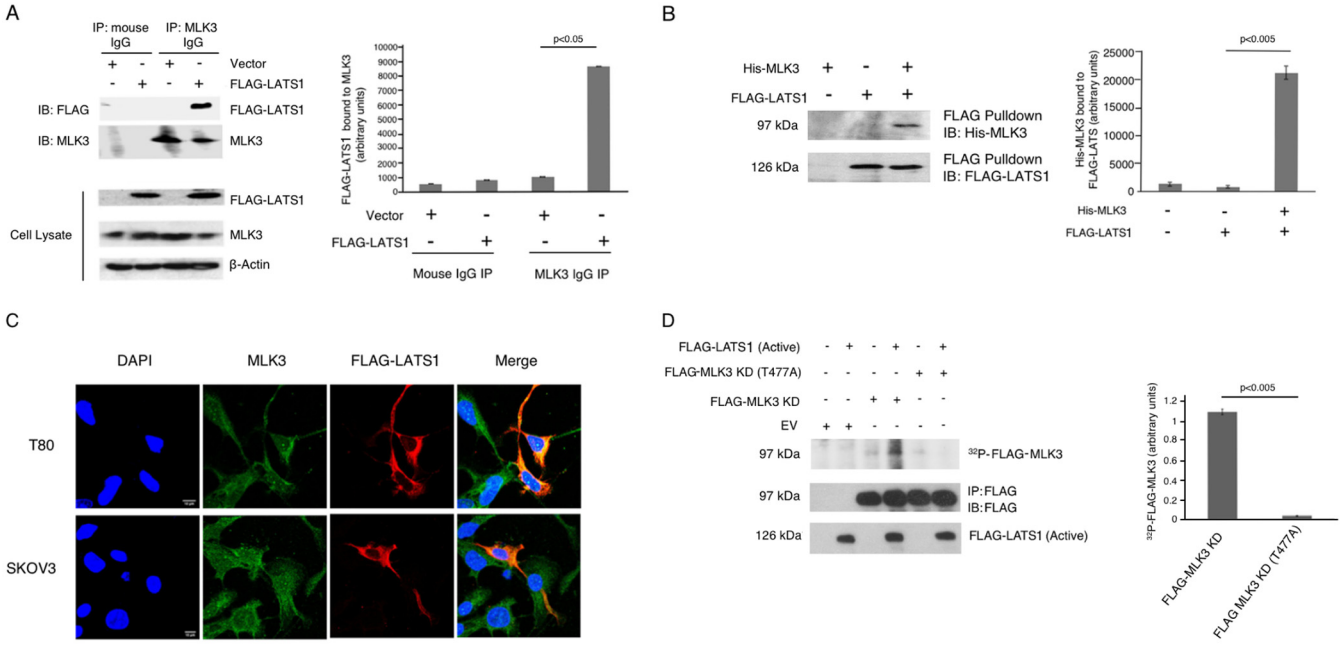

FIG 3 LATS1 interacts with and phosphorylates MLK3. (A) FLAG-LATS1 or pCMV vector were ectopically expressed in HEK293 cells, and MLK3 and IgG mouse immunoprecipitates (IP) were immunoblotted with FLAG and MLK3 antibodies. Cell extracts were immunoblotted with FLAG, MLK3, and β-actin antibodies (left). FLAG-LATS1 in MLK3 IP was quantified. Data are means ± SD ($n = 3$); Student's t test P value is indicated (right). (B) Purified recombinant FLAG-LATS1 and His-MLK3 proteins were combined in an *in vitro* binding assay. FLAG-LATS1 pulldowns were immunoblotted with His and FLAG antibodies (left). His-MLK3 in FLAG pulldown was quantified. Data are means ± SD ($n = 3$); Student's t test P value is indicated (right). (C) FLAG-LATS1 was overexpressed in T80 and SKOV3 cells. Immunofluorescence staining was performed for FLAG-LATS1 (red) and MLK3 (green), and cells were counterstained with DAPI (blue). (D) Autoradiography and Western blotting of FLAG-LATS1 kinase assay with empty vector (EV), FLAG-MLK3(KD), and FLAG-MLK3(KD/T477A) as substrates (left). ^{32}P-FLAG-MLK3 levels were quantified, and data are means ± SEM ($n = 3$); Student's t test, $P < 0.005$ (right).

proproliferative genes. The activities of other MAP3Ks, such as A-Raf, B-Raf, and C-Raf, are regulated by binding to 14-3-3 proteins (43, 44); hence, we postulated that LATS phosphorylation of T477 may create a binding site for 14-3-3, and MLK3–14-3-3 binding could promote retention of MLK3 in the cytoplasm. To test this, HEK293 cells were transfected with combinations of pCMV vector, glutathione *S*-transferase (GST)–14-3-3ζ, FLAG–wild-type MLK [MLK3(WT)], and FLAG-LATS1. GST pulldowns were immunoblotted with FLAG and 14-3-3ζ antibodies, and cell lysates were immunoblotted with FLAG and β-actin antibodies (Fig. 4A). FLAG-MLK3 was pulled down with GST–14-3-3ζ, and cells with FLAG-LATS1 overexpression had a significantly larger amount of FLAG-MLK3(WT) associated with GST–14-3-3ζ (Fig. 4A). Furthermore, in HEK293 cells expressing GST–14-3-3ζ and FLAG-MLK3(WT), LATS1 siRNA knockdown significantly reduced the amount of FLAG-MLK3(WT) associated with GST–14-3-3ζ (Fig. 4B). These results are consistent with the hypothesis that LATS1 enhances MLK3–14-3-3ζ binding. To verify that the endogenous LATS1, MLK3, and 14-3-3ζ proteins are associated in T80 cells, MLK3 immunoprecipitates from T80 cell extracts were immunoblotted with endogenous LATS1 and 14-3-3ζ antibodies. Our results indicate that endogenous MLK3 is associated with endogenous LATS1 and 14-3-3ζ proteins in T80 cells (Fig. 4C). To establish that LATS1 levels affect the endogenous MLK3/14-3-3 interaction in ovarian cells, T80 cells were transfected with either nonspecific (NS) or LATS1 siRNA, and endogenous MLK3 (or mouse IgG control) was immunoprecipitated. The endogenous MLK3/14-3-3 interaction was completely abrogated in cells expressing LATS1 siRNA, which indicates that LATS1 is essential for the interaction between endogenous MLK3 and 14-3-3 (Fig. 4D).

LATS1 regulates MLK3 subcellular localization. Our results indicate that LATS1 enhances the MLK3–14-3-3ζ association. To investigate whether LATS1 also affects MLK3 subcellular localization, immunofluorescence staining of endogenous MLK3 was performed in T80 cells with siRNA-mediated LATS1 knockdown. LATS1 siRNA knockdown

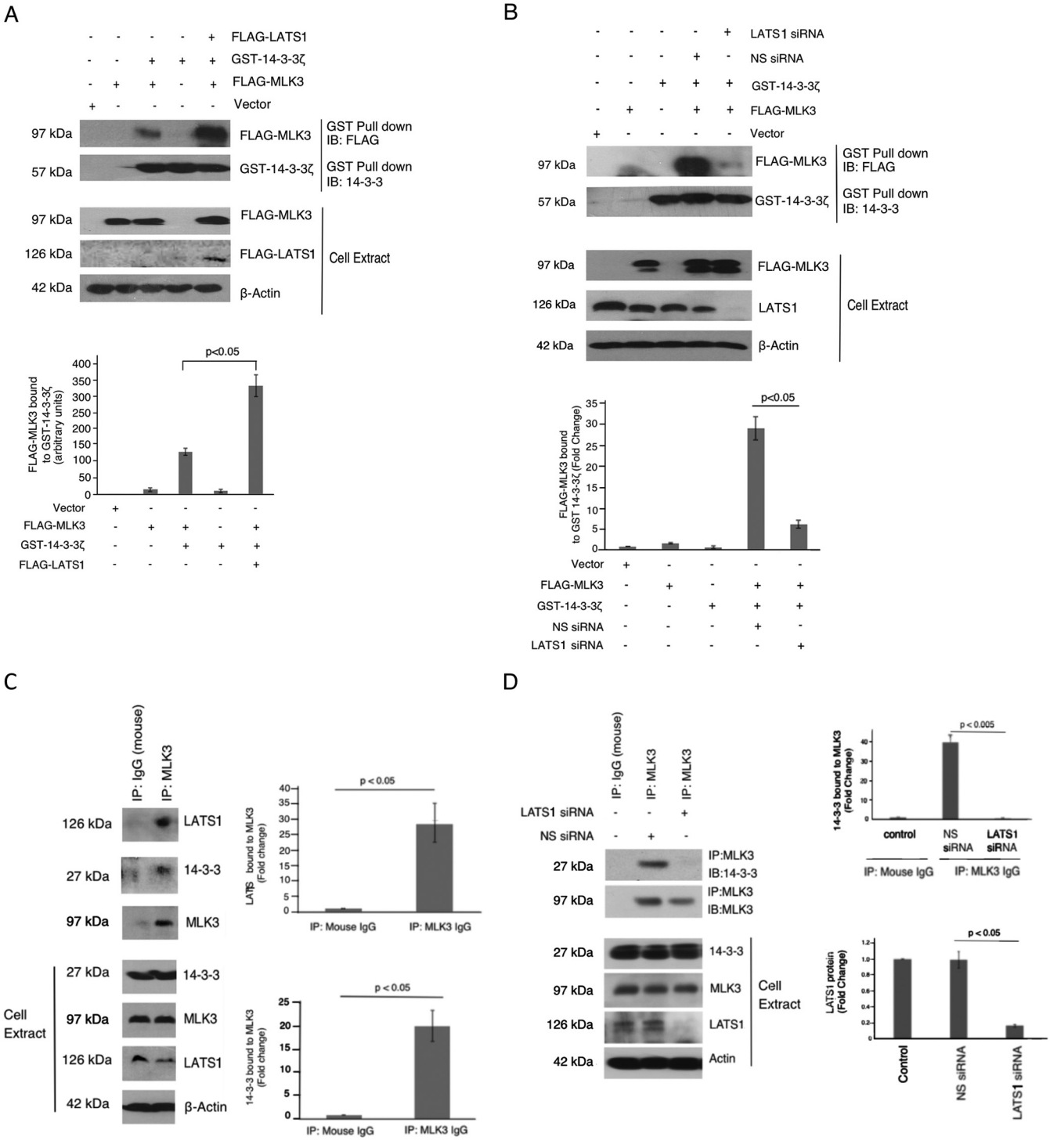

FIG 4 LATS1 enhances the interaction between MLK3 and 14-3-3ζ. (A) GST pulldowns from HEK293 cells expressing pCMV vector, FLAG-MLK3, GST–14-3-3ζ, and FLAG-LATS1 were immunoblotted with FLAG and 14-3-3ζ antibodies. Cell extracts were immunoblotted with FLAG and β-actin antibodies (top). FLAG-MLK3 in the GST pulldown was quantitated. Data are means with SEM ($n = 3$); Student's *t* test *P* value is indicated (bottom). (B) HEK293 cells were transfected with nonspecific (NS) or LATS1 siRNA. GST pulldowns from cells expressing pCMV vector, FLAG-MLK3, and GST–14-3-3ζ were immunoblotted with FLAG and 14-3-3ζ antibodies. Cell extracts were immunoblotted with FLAG, LATS1, and β-actin antibodies (top). FLAG-MLK3 in the GST pulldowns was quantified. Data are means $\pm$ SEM ($n = 3$); Student's *t* test *P* value is indicated (bottom). (C) Endogenous MLK3 and mouse IgG immunoprecipitates were immunoblotted with LATS1, MLK3, and 14-3-3ζ antibodies. Whole-cell extracts were immunoblotted with 14-3-3ζ, LATS1, MLK3, and β-actin antibodies (left). LATS1 and 14-3-3ζ in MLK3 IP were quantitated. Data are means $\pm$ SD ($n = 3$); Student's *t* test *P* value is indicated (right). (D) T80 cells were transfected with NS or LATS1 siRNA, and endogenous MLK3 was immunoprecipitated. Immunoprecipitates were immunoblotted with 14-3-3ζ and MLK3 antibodies. Cell extracts were immunoblotted with 14-3-3ζ, MLK3, LATS1, and β-actin antibodies (left). The amounts of coimmunoprecipitated 14-3-3ζ in the MLK3 IP and LATS1 in the cell extracts were quantified. Data are means $\pm$ SEM ($n = 3$); Student's *t* test *P* value is indicated (right).

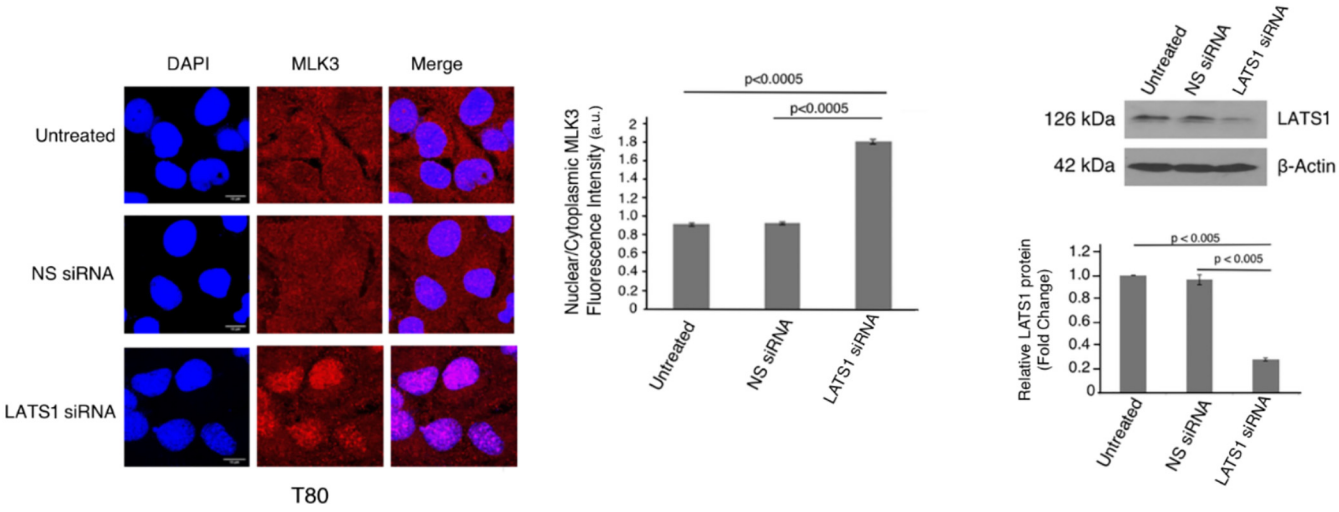

FIG 5 LATS1 regulates MLK3 subcellular localization. T80 cells were transfected with NS siRNA or LATS1 siRNA, and immunofluorescence staining was performed for endogenous MLK3 (red). Cells were counterstained with DAPI (blue) (left). Cell extracts were immunoblotted with LATS1 and β-actin antibodies (right). Relative LATS1 protein in extracts and nuclear/cytoplasmic MLK3 signal were quantified. Data are means ± SEM ($n = 3$); Student's t test P values are indicated.

cells had significantly higher nuclear MLK3 than cells transfected with NS siRNA, which suggests that LATS1 regulates the nuclear/cytoplasmic localization of MLK3 (Fig. 5).

T477, R474, and R475 are critical for MLK3 subcellular localization. LATS1 can phosphorylate MLK3 and affect MLK3 binding to 14-3-3ζ. We observed that T477 is located within a putative nuclear localization signal (NLS), and we postulated that LATS1 phosphorylation of T477 may regulate MLK3 nuclear/cytoplasmic localization (Fig. 6A). Hence, FLAG-MLK3(WT), FLAG-MLK3(T477A), FLAG-MLK3(R474A/R475A), and FLAG-MLK3 (T477E) were ectopically expressed in T80 cells and immunofluorescence was performed to visualize FLAG-MLK3 localization. FLAG-MLK3(WT) had both nuclear and cytoplasmic localization, FLAG-MLK3(R474A/R475A) was almost exclusively cytoplasmic, MLK3 (T477A) was predominantly localized in the nucleus, and the phosphomimetic mutant MLK3(T477E) was localized almost exclusively in the cytoplasm (Fig. 6B). These results suggest that the putative MLK3 NLS is a functional nuclear targeting sequence and that phosphorylation of T477 within this sequence is required for cytoplasmic retention of MLK3. These findings are also consistent with FLAG-MLK3 (WT, R474A/R475A, and T477A) localization in T80 nuclear and cytoplasmic extracts (Fig. 6C). To study the effect of T477A and T477E mutants on the MLK3/14-3-3ζ interaction, HEK293 cells were transfected with combinations of pCMV vector, FLAG-MLK3(WT), FLAG-MLK3(T477A), FLAG-MLK3(T477E), and GST–14-3-3ζ. GST pulldowns were immunoblotted with FLAG antibody to assess MLK3–14-3-3ζ binding. FLAG-MLK3(T477A) did not bind to GST–14-3-3ζ, while FLAG-MLK3(T477E) had significantly greater binding to GST–14-3-3ζ than FLAG-MLK3(WT) (Fig. 6D and E). These results indicate that phosphorylation of T477 of MLK3 is essential for the MLK3/14-3-3ζ interaction.

Nuclear MLK3 promotes ovarian cancer cell invasion. MLK3 promotes activation of ERK signaling and matrix metalloproteinases 2 and 9 and is required for ovarian cancer cell invasion (9). The specific functions of the pool of MLK3 within the nucleus remain unclear. To investigate the effect of nuclear MLK3 on ovarian cancer cell invasion, pCMV vector, FLAG-MLK3(WT), FLAG-MLK3(NLS), and FLAG-MLK3(T477A) were ectopically expressed in SKOV3 cells and invasion was measured by Transwell assays with Matrigel. Overexpression of FLAG-MLK3(WT) resulted in a significant increase in invasion in comparison to that in vector-transfected cells; however, cells expressing FLAG-MLK3(T477A), which is predominantly localized in the nucleus, had an approximately 7-fold increase in invasion in comparison to that of FLAG-MLK3(WT)-transfected cells (Fig. 7). Cells expressing the FLAG-MLK3(R474A/R475A) NLS mutant, which is predominantly localized in the cytoplasm, had significantly less invasion than cells that

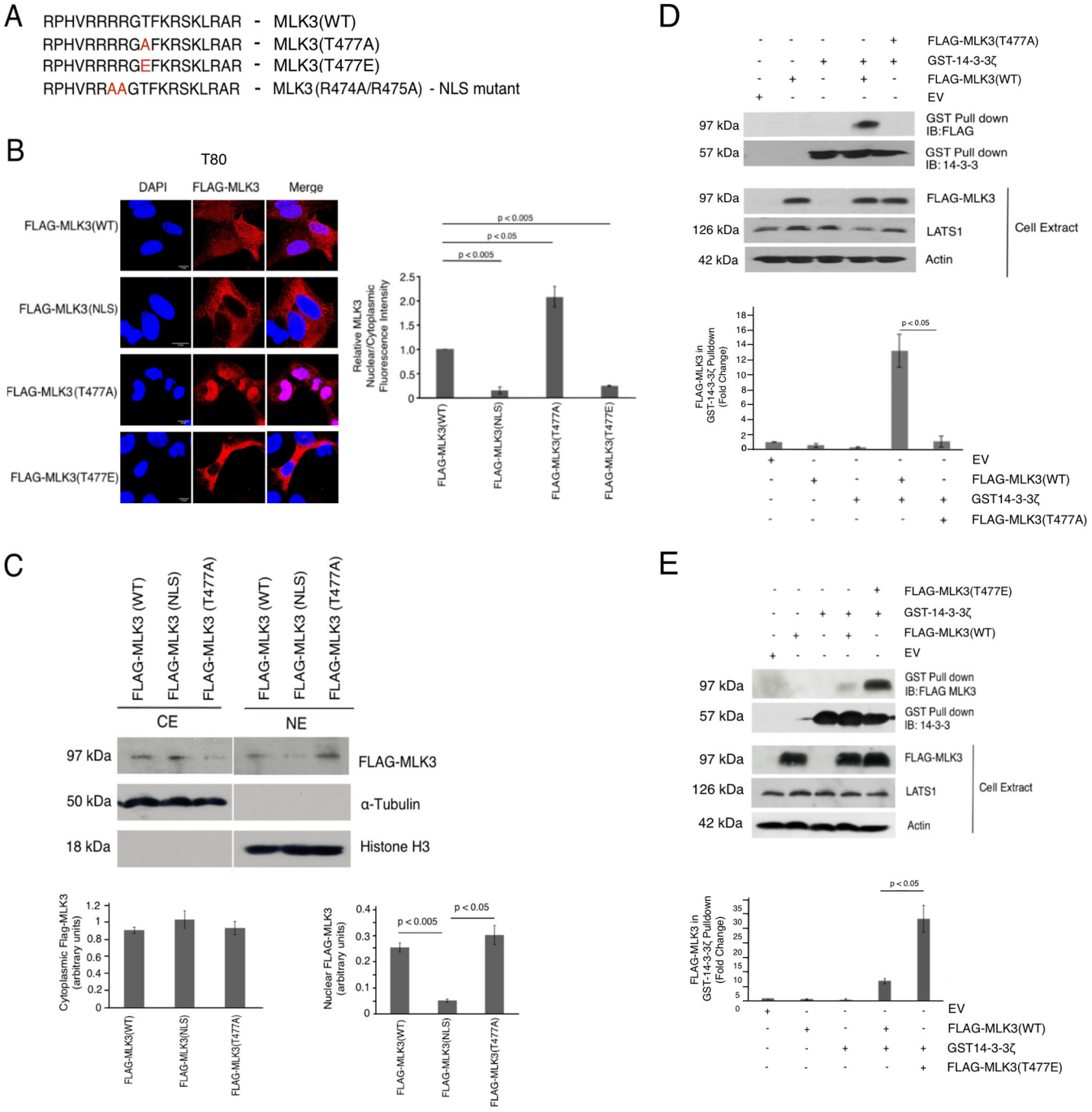

FIG 6 T477, R474, and R475 are critical for MLK3 subcellular localization. (A) Putative nuclear localization sequence on MLKs showing T477A, T477E, and R474A/R475A mutations. (B) T80 cells were transfected with FLAG-MLK3(WT), FLAG-MLK3(R474A/R475A), FLAG-MLK3(T477A), or FLAG-MLK3(T477E), and immunofluorescence staining was performed for FLAG-MLK3 (red). The nuclei were stained with DAPI, and nuclear FLAG-MLK3 was quantified. Data are means ± SEM ($n = 3$); Student's t test P values are indicated. (C) Nuclear and cytoplasmic extracts from cells expressing FLAG-MLK3(WT), FLAG-MLK3(R474A/R475A), or FLAG-MLK3(T477A) were immunoblotted with FLAG, α-tubulin, and histone H3 antibodies. Nuclear and cytoplasmic FLAG-MLK3 were quantified. Data are means ± SEM ($n = 3$); Student's t test P values are indicated. (D) GST pulldowns from HEK293 cells expressing pCMV vector, FLAG-MLK3 (WT or T477A), and GST–14-3-3ζ were immunoblotted with FLAG and 14-3-3ζ antibodies (top). Cell extracts were immunoblotted with LATS1, FLAG, and β-actin antibodies. FLAG-MLK3 in GST–14-3-3ζ pulldown was quantified (bottom). Data are means ± SEM ($n = 3$); Student's t test P value is indicated. (E) GST pulldowns from HEK293 cells expressing pCMV vector, FLAG-MLK3 (WT or T477E), and GST–14-3-3ζ were immunoblotted with FLAG and 14-3-3ζ antibodies (top). Cell extracts were immunoblotted with LATS1, FLAG, and β-actin antibodies. FLAG-MLK3 in GST–14-3-3ζ pulldown was quantified (bottom). Data are means ± SEM ($n = 3$); Student's t test P value is indicated.

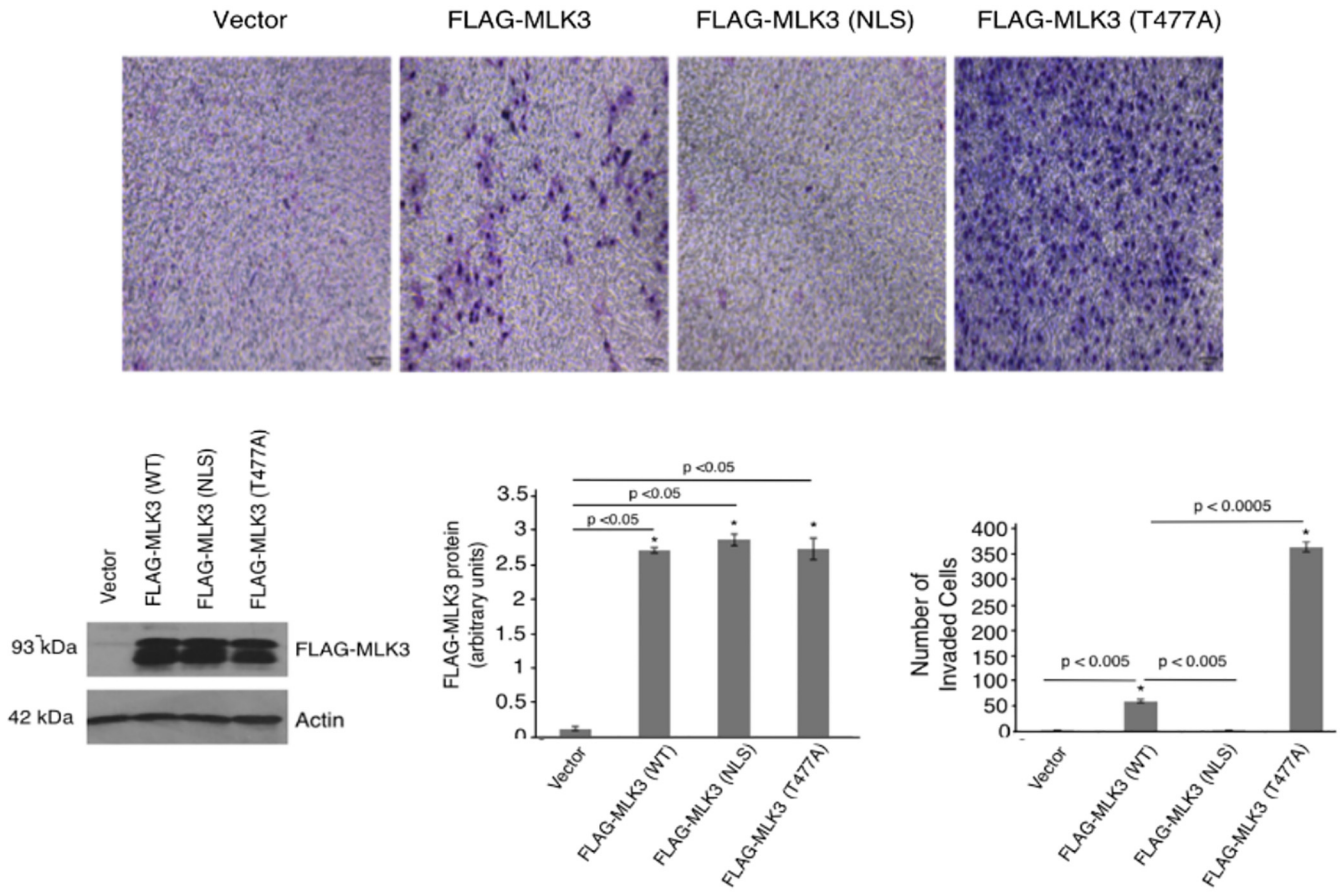

FIG 7 Nuclear MLK3 promotes ovarian cancer invasion. SKOV3 cells were transfected with EV, FLAG-MLK3(WT), FLAG-MLK3(R474A/R475A), or FLAG-MLK3 (T477A), and cell invasion was assessed using Transwells with Matrigel (top). The cells that invaded the Matrigel were stained and counted. Cell extracts were immunoblotted with FLAG and β-actin antibodies, and FLAG-MLK3 protein was quantified. Data are means ± SEM ($n = 3$); Student's t test P value is indicated (bottom).

expressed FLAG-MLK3(WT) or FLAG-MLK3-T477A (Fig. 7). These results suggest that heightened amounts of nuclear MLK3 may drive ovarian cancer cell invasion.

DISCUSSION

The critical role of MLK3 in invasion and migration of melanoma, breast, ovarian, and gastric cancers has been established; however, the underlying molecular mechanisms that control invasion in these cancer cells remain ill defined (8, 14, 21, 38). Our results reveal that LATS1 is a novel regulator of MLK3, and we propose a model wherein LATS1 regulates MLK3 subcellular localization by promoting MLK3–14-3-3ζ association and MLK3 cytoplasmic retention; in ovarian cancer cells, the absence of activated LATS1 results in enhanced MLK3 nuclear localization, which drives cell invasion (Fig. 8).

Confluent T80 cells have activated LATS1 and both nuclear and cytoplasmic MLK3, whereas confluent SKOV3 and TOV112D cells have minimal activated LATS1 and predominantly nuclear MLK3. These results suggest an inverse correlation between the amount of active LATS1 and the amount of nuclear MLK3 in ovarian epithelial cells. Furthermore, our results indicate that R474 and R475 are part of a functional NLS, and LATS1 phosphorylation of T477 within this NLS is critical for MLK3–14-3-3ζ association. We propose that MLK3 nucleocytoplasmic localization is regulated by LATS1 phosphorylation of T477, which facilitates MLK3–14-3-3ζ binding and MLK3 cytoplasmic retention. This mechanism is similar to LATS1-dependent regulation of the Yap oncoprotein, where LATS1 phosphorylation YAP leads to YAP–14-3-3 binding and cytoplasmic retention of YAP (26, 42). It is not known how 14-3-3ζ interaction with MLK3 might affect

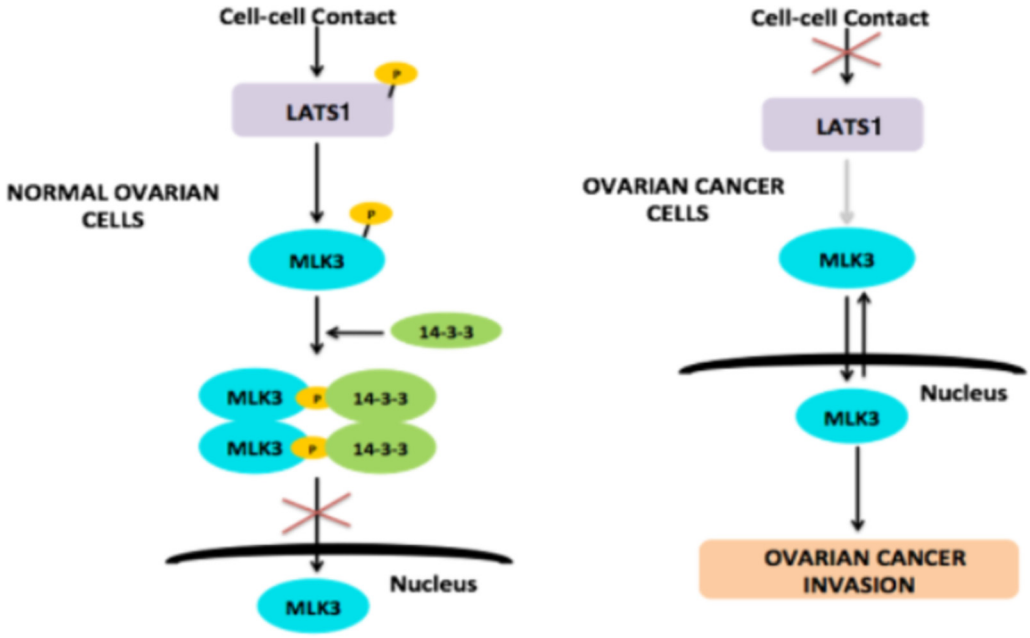

FIG 8 Proposed model of LATS1 regulation of MLK3 subcellular localization. LATS1 phosphorylates MLK3 on T477, enhancing MLK3/14-3-3ζ interaction and cytoplasmic retention of MLK3 in normal ovarian cells. In ovarian cancer cells with low levels of active LATS1, MLK3 localizes predominantly in the nucleus and promotes ovarian cancer cell invasion.

MLK3 localization; however, one possibility is that binding of 14-3-3ζ to phosphorylated T477 masks the NLS and thereby hinders the binding of the NLS to proteins required for MLK3 nuclear import.

A recent study on the dual leucine zipper kinase (DLK), a member of the MLK family of MAP3Ks, revealed that depending on its subcellular localization, DLK induces apoptosis of beta cells by inhibiting CREB-dependent gene transcription. Mutation of a putative nuclear localization sequence on DLK resulted in exclusion of DLK from the nucleus and a significant reduction in beta-cell apoptosis, identifying novel functions of DLK depending on its localization (45).

Humphrey et al. demonstrated that AKT-dependent phosphorylation of MLK3 on T477 is instrumental in destabilizing MLK3 in response to insulin in pancreatic beta cells (46). We did not observe a difference in T477A MLK3 protein steady-state levels when expressed in HEK293, T80, or SKOV3 cells. However, it is possible that AKT-dependent regulation of T477 and MLK3 stability is specific to insulin-stimulated pancreatic beta cells.

MLK3 is recognized as an important contributor to cell proliferation, migration, invasion, and metastasis of several cancers (5, 12, 38). We previously demonstrated a requirement for MLK3 in ovarian cancer cell invasion and matrix metalloproteinase 2 and 9 expression (9). This study focuses on a novel signaling pathway that contributes to ovarian cancer cell invasion. The finding that cells expressing the MLK3(T477A) mutant, which accumulates in the nucleus, had an approximately 7-fold increase in SKOV3 cell invasiveness compared to that of cells expressing wild-type MLK3 indicates that nucleus-localized MLK3 is a strong driver of ovarian cancer cell invasion. The mechanism(s) by which nuclear MLK3 promotes invasion has not been defined; however, one possibility is that interactions between MLK3 and nuclear proteins such as transcription factors could cause aberrant expression of genes involved in cell invasion.

This study sheds light on the importance of MLK3 subcellular localization to its role in regulating ovarian cancer cell invasion. Further research to elucidate nuclear MLK3 functions and the mechanisms that control MLK3 spatiotemporal signaling will be vital to defining its role in cellular transformation and tumorigenesis.

MATERIALS AND METHODS

Cell culture. Human ovarian cancer (SKOV3 and TOV112D) and human embryonic kidney (HEK293) cells were purchased from American Type Culture Collection (ATCC; Manassas, VA). T29 and T80 immortalized ovarian epithelial cells were a kind gift from Jinsong Liu (University of Texas, MD Anderson Cancer Center) (47). Ovarian cancer cells and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA) with 10% calf serum. T80 and T29 immortalized ovarian epithelial cells were cultured in 1:1 medium 199 (Cellgro) and MCDB 105 (Millipore Sigma) supplemented with 15% calf serum, penicillin, and streptomycin. Tissue culture media were supplemented with 2 mM L-glutamine, 25 μg/ml of streptomycin, and 25 IU of penicillin (Mediatech), and cells were grown in a humidified atmosphere with 5% CO_2 at 37°C.

Plasmid and siRNA transfections. Mammalian expression vectors pCMV-FLAG, pCMV-FLAG-MLK3, pCMV-FLAG-LATS1, and pEBG–GST–14-3-3ζ were used for the expression of human MLK3, 14-3-3ζ, and LATS1 proteins. Transient transfections in HEK293 cells were performed with Polyjet (SignaGen Laboratories, Rockville, MD) for 24 h. Nonspecific (NS) siRNA and MLK3 siRNA oligonucleotides were acquired from Dharmacon (Lafayette, CO). MLK3 siRNA sequences were as follows: siRNA 1 (sense), 5′-GCGCGAGAUCCAGGGUCUCdTdT-3′, and siRNA 2 (sense), 5′-GGGCAGUGACGUCUGGAGUdTdT-3′. LATS1 siRNA was acquired from Santa Cruz Biotechnology, Dallas, TX. siRNA transfections were performed using Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific, Waltham, MA) at a final concentration of 150 nM for 48 h.

Immunoblotting. Immunoblotting was performed with primary antibodies from Santa Cruz Biotechnology (Dallas, TX): MLK3 (C-20), MLK3 (H-3), LATS1 (G-12), β-actin (C-4), α-tubulin (10D8), GST (Z-5), 14-3-3ζ (C-16), and Yap (H-8). Activation state antibodies p-LATS1 (Thr1079), p-Yap (Ser127), and p-MST1/2 (Thr183/Thr180) were obtained from Cell Signaling Technology (Beverly, MA). FLAG antibody (200474-21) was from Agilent Technologies. Secondary antibodies were goat anti-mouse, goat anti-rat, and goat anti-rabbit Immun-Star IgG–horseradish peroxidase (HRP) conjugate (Bio-Rad, Hercules, CA).

Immunofluorescence. Cells were plated on sterile coverslips and then fixed and permeabilized using 100% acetone at −20°C or fixed with 3.7% paraformaldehyde for 20 min at room temperature (RT) and then permeabilized using 0.25% Triton X-100 in phosphate-buffered saline (PBS; pH 7.4). Blocking was performed with 5% goat serum (GS; Thermo Scientific) in PBS for 1 h. Fixed coverslips were incubated with primary antibodies at 37°C for 1 h. The following antibody dilutions in 5% GS in PBS were used: 1:500 for FLAG, 1:100 for MLK3 (C-20), and 1:75 for MLK3 (H-3). Slides were washed 3 times for 10 min in PBS. Anti-rabbit IgG–Alexa Fluor 488 and anti-mouse IgG–Alexa Fluor 647 (Life Technologies, Carlsbad, CA) were used at a 1:250 dilution in 3% GS in PBS and incubated in the dark at RT for 45 min, followed by 3 washes in PBS for 10 min each. Coverslips were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO). Cell images were taken with a confocal microscope (Leica SP8 system). Scale bars were added to each image using ImageJ (NIH), and the ratio of nuclear to cytoplasmic signal was also measured using ImageJ.

Cell fractionation. Cells were trypsinized and washed with 1× PBS. The pellets were resuspended in 5 pellet volumes of cytoplasmic extract (CE) buffer (10 mM HEPES [pH 7.6], 60 mM KCl, 1 mM EDTA, 0.075% NP-40, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The suspension was centrifuged and the supernatant (cytoplasmic fraction) was transferred to fresh microcentrifuge tubes. The nuclear pellets were washed with CE buffer without NP-40 detergent. The nuclei were incubated with 3 pellet volumes nuclear extract (NE) buffer (20 mM Tris Cl [pH 8.0], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF, and 25% [vol/vol] glycerol) for 10 min on ice. The NE and CE extracts were centrifuged at 12,000 rpm for 10 min, and the supernatants (NE and CE) were stored at −20°C.

Immunoprecipitation and *in vitro* binding assays. One microgram of His-tagged MLK3 protein purified from *Escherichia coli* BL21 and 1 μg of active FLAG-LATS1 recombinant protein obtained from Active Motif (Carlsbad, CA) were incubated together in cell lysis buffer for binding assays performed as described previously (48). For immunoprecipitations, T80 cells or HEK293 cells were transfected with appropriate amounts of plasmid DNA, and GST pulldowns and immunoprecipitations with MLK3 (D-11) antibody were performed as described previously (49).

***In vitro* kinase assay.** FLAG-MLK3 immunoprecipitates from HEK-293 cells were suspended in kinase assay buffer (50 mM Tris-HCl [pH 7.5], 2 mM EGTA, 10 mM $MgCl_2$, 0.1 mM DTT, and 0.1% Triton X-100) with 100 μM unlabeled ATP, 10 mM $MgCl_2$, and 3 μCi of [γ^{32}P]ATP (Perkin Elmer Health Sciences, Boston, MA). Purified full-length human active LATS1 (0.3 μg; Active Motif) was added to the FLAG-MLK3 immunoprecipitates, and kinase assays were performed for 30 min at 30°C and stopped with 1× SDS sample buffer and 95°C heat for 5 min (11).

Site-directed mutagenesis. Site-directed mutagenesis of human MLK3 was performed using the QuikChange Lightning site-directed mutagenesis kit according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). The T477A mutation was introduced into FLAG-MLK3 wild-type (WT) and K144R kinase-dead (KD) plasmids using the sense oligonucleotide 5′-CGCCCGCCGCCGCGGGGCATTCAA GCGCAGCAAG-3′ and the antisense oligonucleotide 5′-CTTGCTGCGCTTGAATGCCCCGCGGCGGCGGCG-3′. The MLK3 (R474A/R475A) NLS mutant was generated using sense oligonucleotide 5′-CGACCGCACGT GCGCGGCGGCCGCGGGACATTCAAG-3′ and the antisense oligonucleotide 5′-CTTGAAGTCCCGCGGCCGCC GCGCACGTGCGGGCT-3′ (mismatched base pairs from the wild-type template are underlined). The mutations were verified by sequencing (Genewiz, South Plainfield, NJ).

Invasion assay. Invasion assays were performed by plating SKOV3 cells on Transwell inserts with 8.0-μm pores (Corning, NY) that were coated with 100 μl of 1-mg/ml Matrigel (BD Biosciences, San Jose, CA) as described previously (9). A total of 250,000 cells were seeded in each upper chamber of the Transwell, which was then incubated for 16 h in a cell culture incubator. Invaded cells were fixed and

stained with Hema 3 Stat Pack (Fisher Healthcare) and counted using a bright-field Olympus microscope. Photomicrographs of invaded cells were taken using an EVOS cell imaging system (Thermo Fisher).

Quantification and statistical analysis. Immunoblots are representative of those from three independent experiments, and quantification and statistical analysis were performed on three independent biological replicates. Densitometric analysis of immunoblots was performed using ImageJ software (National Institutes of Health). Immunofluorescence staining of cells was quantified by analyzing 100 cells per replicate for three independent replicates, and the signal was quantified using the *Measure* tool of ImageJ software. Statistical analysis of two-sample assays was performed with unpaired Student's *t* test (two tailed). Comparison of more than two samples was done using Kruskal-Wallis one-way analysis of variance (ANOVA) followed by the Conover-Inman *post hoc* test. For samples included in this test, *P* values were not adjusted. In figures, bars depict means, error bars represent standard errors of the means (SEM), and statistical significance (*P* value) is indicated. A *P* value of <0.05 was considered statistically significant.

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