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Mixed lineage kinase 3 negatively regulates IKK activity and enhances etoposide-induced cell death

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Mixed lineage kinase 3 (MLK3) is a mitogen activated protein kinase kinase kinase (MAP3K) that activates multiple MAPK signaling pathways. Nuclear factor kappa B (NF-κB) is a transcription factor that has important functions in inflammation, immunity and cell survival. We found that silencing *mlk3* expression with RNA interference (RNAi) in SKOV3 human ovarian cancer epithelial cells and NIH-3T3 murine fibroblasts led to a reduction in the level of the inhibitor of kappa B alpha (IκBα) protein. In addition, we observed enhanced basal IκB kinase (IKK) activity in HEK293 cells transiently transfected with MLK3 siRNA and in NIH3T3 cells stably expressing MLK3 shRNA (shMLK3). Furthermore, the basal level of NF-κB-dependent gene transcription was elevated in shMLK3 cells. Silencing *mlk3* expression conferred resistance of cells to etoposide-induced apoptotic cell death and overexpression of wild type MLK3 (MLK3-WT) or kinase-dead MLK3 (MLK3-KD) promoted apoptotic cell death and cleavage of poly (ADP-ribose) polymerase (PARP). Overexpression of MLK3-WT or MLK3-KD enhanced etoposide-induced apoptotic cell death and cleavage of PARP. These data suggest that MLK3 functions to limit IKK activity, and depleting MLK3 helps protect cells from etoposide-induced cell death through activation of IKK-dependent signaling.

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1. Introduction

Mitogen activated protein kinases (MAPKs) respond to diverse stimuli to regulate expression of specific genes and modulate cellular processes such as proliferation, motility, differentiation, survival and apoptosis [1]. MAPKs are organized in a three-tiered signaling module whereby a MAP3K phosphorylates and activates a MAPK kinase (MAP2K) that, in turn, phosphorylates and activates a MAPK [1]. In response to various stimuli, activated MAPKs phosphorylate both cytoplasmic and nuclear substrates including transcription factors that ultimately result in the transcription of specific genes [1].

The MLKs are a family of MAP3Ks that activate the c-Jun N-terminal kinase (JNK) and p38 MAPK signaling pathways [2]. Upon activation, MLKs directly phosphorylate and activate MAP2Ks MKK4/SEK1, and MKK3/6 [3,4]. Activated MKK4/SEK1 and MKK3/6 directly phosphorylate and activate JNK and p38, respectively [1]. MLK3 is activated by tumor necrosis factor alpha (TNF-α), epidermal growth factor (EGF), T cell receptor co-stimulation, transforming growth factor beta (TGF-β), sorbitol, ceramide and nerve growth factor (NGF) deprivation in neuronal cells [2,5–9].

RNAi studies showed that MLK3 can promote microtubule instability, is required for cell proliferation in epithelial and fibroblast cells, and is required for TNF and EGF activation of extracellular signal-regulated kinase (ERK) and JNK [10,11]. Results from studies with mice that have a targeted disruption of *mlk3* confirmed a requirement for MLK3 in TNF activation of JNK [12]. MLK3 stimulates neuronal apoptosis through activation of JNK and neuronal apoptosis induced by nerve growth factor (NGF) withdrawal can be blocked by dominant negative MLK3 or pharmacological inhibitors of the MLKs [13–15]. Interestingly, AKT inhibits MLK3 kinase activity and blocks MLK3-dependent activation of JNK and apoptosis in HEK293 cells [16].

NF-κB is a transcription factor that has important functions in inflammation, immunity, apoptosis, cell cycle regulation and cell adhesion [17,18]. In the canonical pathway of NF-κB activation, NF-κB is retained in the cytoplasm by association with its inhibitor, IκBα [19]. Upon exposure of cells to activating stimuli such as TNF, interleukin 1 (IL-1) and ultraviolet radiation, an IKK kinase (IKKK) is activated that phosphorylates and activates IKK. Activated IKK then phosphorylates IκBα on Ser32 and Ser36 [19]. Phosphorylation of IκBα on these sites promotes ubiquitination and proteosomal degradation of IκBα, resulting in release of NF-κB from IκBα and exposure of the NF-κB nuclear translocation sequence. NF-κB then translocates to the nucleus and activates gene transcription [19].

Similar to other MAP3Ks like MEKK1 and NF-κB inducing kinase (NIK), MLK3 can phosphorylate IKKα and/or IKKβ *in vitro* [20–22]. Hehner et al. [6] observed that overexpression of MLK3 in T cells led to the activation of IKK. In that study, MLK3 was found to interact with IKKα and IKKβ and MLK3 phosphorylated IKK-α/β *in vitro* on Ser32

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and Ser36 residues [6]. In contrast, Zhao and Lee [21] reported that overexpression of MLK3 did not lead to activation of IKK in HeLa cells. Thus, the role of MLK3 in IKK activation remains elusive.

To investigate the function of MLK3 in regulating IKK activity, we depleted MLK3 protein with RNAi in HEK293, SKOV3 and NIH3T3 cells and analyzed the effect on IKK signaling. We found that *mlk3* silencing elevated basal IKK activity, reduced basal IκBα protein levels, and enhanced NF-κB-dependent gene expression. In addition, cells lacking MLK3 were partially protected from etoposide-induced apoptotic cell death. Consistent with these findings, overexpression of MLK3 promoted apoptotic cell death. Furthermore, overexpression of MLK3 enhanced apoptotic cell death induced by etoposide treatment. Taken together, our results suggest that MLK3 prevents NF-κB activation by inhibiting IKK activity.

2. Materials and methods

2.1. Short hairpin RNA (shRNA) MLK3 expression plasmid

Murine MLK3 shRNAi oligo sequences were: Forward 5′-gat ccc c GGG CAG CGA CGT GTC GAG CTT t tca aga gaa ctc cag acg tca ctg ccc ttt ttg gaa a-3′ and Reverse 5′-agc ttt t CCC GTC GCT GCA CAG CTC GAA cc aaa aag ggc agt gac gtc tgg agt tct ctt gaa act cca gac gtc act gcc cgg g-3′. Annnealed DNA oligos were digested with *Hin*dIII and *Bgl*II and ligated into pSUPER.retro.puro RNAi expression plasmid (Oligoengine, WA, USA) that contains a puromycin resistance gene. Sequencing of the ligation product was completed at the Ohio State University Plant Microbe Genomics Facility.

2.2. Plasmid DNA and siRNA oligo transfections

To generate MLK3 shRNA stable cell lines, NIH-3T3 fibroblasts were transfected with 5 μg of the pSUPER-Retro-shMLK3 plasmid using Lipofectamine 2000 from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. Cells were incubated in selection media containing 1 μg/ml puromycin and individual clones were screened for MLK3 expression. Transfection of MLK3 siRNA oligos were performed using Lipofectamine 2000 as previously described [10]. Transient transfection of all plasmid DNA was performed using Lipofectamine as previously described [23].

2.3. Real-time PCR (RT-PCR)

Total RNA from NIH3T3 shRNA clones was prepared using the Absolutely RNA Minipreparation kit from Stratagene (La Jolla, CA) according to the manufacturer's suggestions. RT-PCR was performed using IQ SYBR Green Supermix from Bio-Rad (Hercules, CA) as described previously [24]. The primer sequences were as follows: mouse β-Actin Forward 5′-AGGTGTGCACCTTTTATTGGTCTCAA-3′, Reverse 5′-TGTATGAAGTTTGGTCTCCCT-3′; mouse cIAP2 Forward 5′-GAGGTGCTGGGAATCTGGAG-3′, Reverse 5′-CACCAGGCTCCTACT-GAAGC-3′; mouse KC Forward 5′-ACCCGCTCGCTTCTCTGT-3′, Reverse 5′- CCAAGGGAGCTTCAGGGTCAAG-3′; and mouse IκBα Forward 5′-CTGCAGGCCACCAACTACAA-3′, Reverse 5′-CAGCACCCAAAGTCAC-CAAGT-3′.

2.4. Tissue culture

NIH3T3 mouse embryonic fibroblasts, SKOV3 human ovarian carcinoma cells and HEK293 human embryonic kidney cells were obtained from American Type Culture Collection (Manassas, VA). All cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum (NIH3T3) or 10% fetal bovine serum (SKOV3 and HEK293), 25 mg/mL streptomycin and 25 I.U. penicillin from Cellgro (Mediatech Inc. Herndon, VA).

2.5. Electrophoresis and immunoblotting

Cells were washed twice in phosphate buffered saline (PBS) and lysed in 500 μL 2× SDS sample buffer. Cell lysates were collected and subjected to 15% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to polyvinylidene difluoride (PVDF) membrane and probed with the appropriate primary and secondary antibodies. Antibodies against the following proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): MLK3, pp40/IκBβ, IKKγ, IκBα, and β-actin. Antibodies from Cell Signaling Technology (Beverly, MA) used in this study were: phospho-MLK3 (Thr277/Ser281), phospho-IκBα (Ser32/Ser36), cleaved PARP and cleaved Caspase 3. Anti-FLAG antibody was obtained from Stratagene. The secondary antibodies were Immun-Star Goat Anti-Mouse-horse radish peroxidase (HRP) conjugate and Immun-Star Goat Anti-Rabbit-HRP conjugate (Bio-Rad). Immobilon western chemiluminescent HRP substrate from Millipore (Bedford, MA) was used for chemiluminescent detection.

2.6. TNFα and K252a treatment of cells

Cells were treated with vehicle or 20 ng/mL TNFα from Biosource International (Camarillo, CA) for 10 min. For experiments where phosphorylated IκBα was analyzed, TNFα treatments were for 0, 5, 20, and 40 min. For treatment of HEK293 cells with K252a, cells were first transfected with plasmid DNA. 24 h after DNA transfection, cells were treated with 150 nM K252a for 5 h prior to treatment with DMSO or etoposide.

2.7. Analysis of cell death

4×10^5 of the HEK293 or shMLK3 cells were seeded on 6 cm tissue culture dishes and incubated overnight at 37 °C. The next day, cells were treated with etoposide (100 μM) from Sigma (St. Louis, MO) or DMSO for 26 h. After treatment, the cells were stained with trypan blue solution (0.4% w/v trypan blue in 1× PBS). Trypan blue-stained cells (non-viable) and unstained cells (viable) were counted with a hemocytometer and the percent of non-viable cells was calculated. Photomicrographs of cells were taken with an Olympus (model IX51) microscope using light microscopy under 400× magnification. Analysis of apoptotic cell death was performed using the APOPercentage apoptosis assay from Accurate Chemical (Westbury, NY) according to the manufacturer's instructions. Caspase 3 and Caspase 7 activities were measured using the Caspase-Glo 3/7 apoptosis assay system from Promega (Madison, WI) according to the manufacturer's instructions. Statistical analyses were performed using Microsoft Excel software. Data are presented as mean, with n representing 3 independent experiments. Comparison of two groups was performed using unpaired Student's t test. A P value of <0.05 was regarded as statistically significant.

2.8. IKK assay

GST-IκB protein preparation and IKK assay protocols were as previously described [25,26]. Briefly, 4×10^5 control or shMLK3 clones 4 and 2 cells were treated for 0, 20 and 40 min with 20 ng/ml TNFα. IKKγ was immunoprecipitated from cell lysates and incubated with 1 μg purified GST-IκBα, 10 mM $MgCl_2$ and 0.1 mM ATP at 30 °C for 30 min. Kinase assays were stopped by the addition of 2× SDS sample buffer and samples were boiled for 5 min before being loaded onto the gel. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and probed with anti-phospho-IκBα antibody.

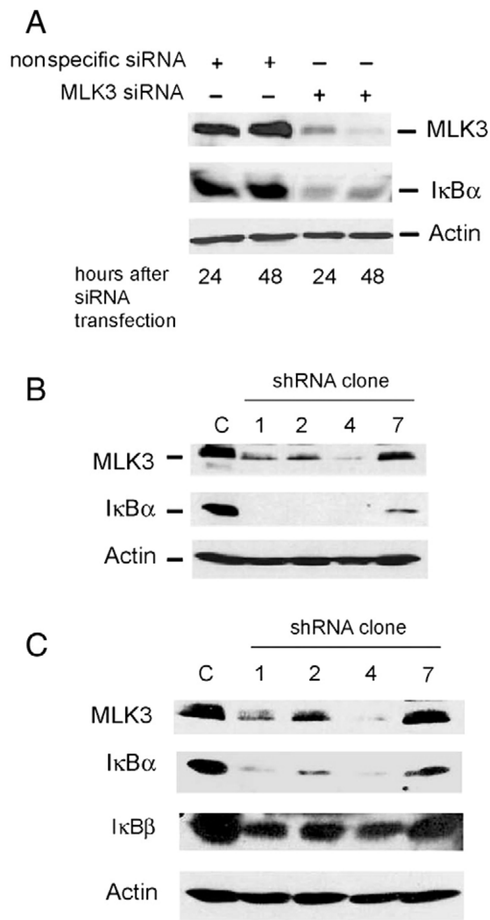

Fig. 1. Silencing *mlk3* leads to a reduction in the level of IκB protein. (A) Whole cell extracts from SKOV3 ovarian cancer cells transfected with nonspecific siRNA or human MLK3 siRNA were subjected to SDS-PAGE and Western blotting with MLK3, IκBα, and Actin (loading control) antibodies. (B) Whole cell extracts from control (transfected with vector alone) and shMLK3 clones 1, 2, 4 and 7 were subjected to SDS-PAGE and immunoblotted with MLK3, IκBα, and Actin antibodies. (C) Whole cell extracts from control and shMLK3 clones 1, 2, 4 and 7 were subjected to SDS-PAGE and Western blotting with MLK3, IκBα, IκBβ and Actin antibodies.

3. Results

3.1. Silencing mlk3 leads to a reduction in the level of IκBα protein

To investigate the role of MLK3 in NF-κB activation, we silenced *mlk3* expression in SKOV3 cells and analyzed the protein level of the NF-κB inhibitor, IκBα. Phosphorylation of IκBα by IKK occurs in response to TNF treatment and leads to ubiquitination and proteosomal degradation of IκBα [19]. Degradation of IκBα results in release of associated NF-κB and exposure of the NF-κB nuclear localization sequence which allows relocalization of NF-κB to the nucleus and activation of gene transcription [19]. Surprisingly, we observed a reduced level of IκBα protein 24 h and 48 h after transfection of SKOV3 cells with MLK3 siRNA in comparison to cells transfected with nonspecific siRNA oligos (Fig. 1A). To determine if a similar reduction in IκBα levels occurred in cells stably expressing MLK3 shRNA, NIH3T3 cells were prepared that stably expressed a MLK3 shRNA plasmid (shMLK3). Individual clones expressing the MLK3 shRNA were isolated in puromycin selection. Whole cell extracts were prepared from the shMLK3 clones and MLK3 protein levels were analyzed. shMLK3 clones 1, 2, 4, and 7 had reduced MLK3 protein levels in comparison to control cells expressing vector alone (Fig. 1B). Interestingly, the level of total endogenous IκBα protein was much lower in shMLK3 cells in comparison to control cells (Fig. 1B). However, in these cells, MLK3 knockdown had little effect on the level of endogenous IκBβ protein (Fig. 1C). The reduction of IκBα protein levels was not due to decreased synthesis of IκBα mRNA since RT-PCR of IκBα mRNA revealed an increase in the level of IκBα mRNA in shMLK3 cells (Fig. 3A). These results demonstrate that cells lacking MLK3 have reduced levels of IκBα protein. A reduction in the level of IκBα protein in cells could elevate the amount of free NF-κB that can translocate to the nucleus and activate gene transcription. Possibly, the reduction in IκBα protein levels in shMLK3 cells is a result of increased phosphorylation and degradation of IκBα.

3.2. Cells lacking MLK3 have elevated basal IKK activity

Next, we wished to determine if the decrease in IκBα protein levels was due to a change in IκBα protein stability or turnover. If MLK3 inhibits IKK activity, then loss of MLK3 would relieve this inhibition and augment endogenous IKK activity. To address this possibility, we analyzed IKK activity in HEK293 cells transfected with MLK3 siRNA or nonspecific siRNA oligos. IKK was immunoprecipitated with anti-IKKγ

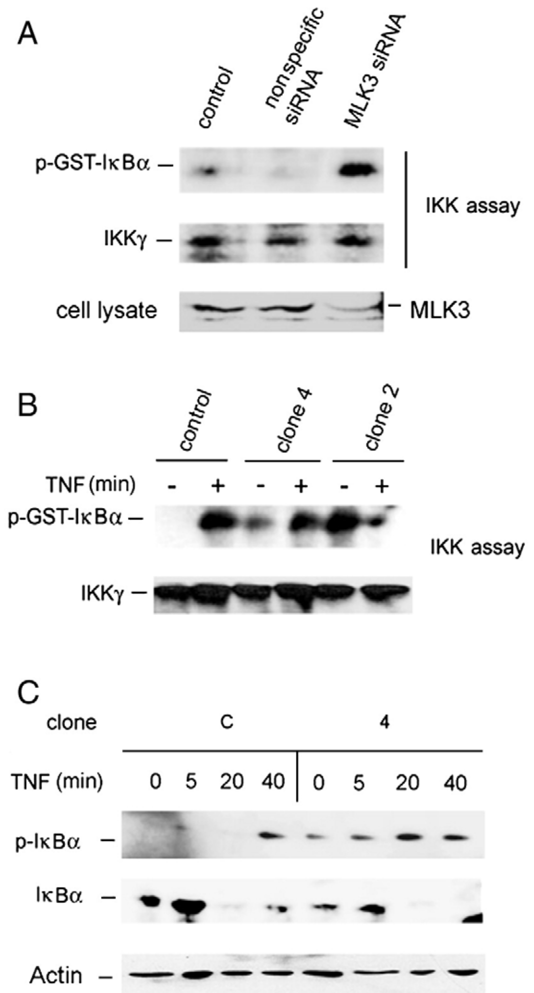

Fig. 2. Cells lacking MLK3 have elevated basal IKK activity. (A) HEK293 cells were transfected with nonspecific or MLK3 siRNA oligos. IKKγ was immunoprecipitated from cell lysates with IKKγ antibody and IKK assays were performed using GST-IκBα as substrate. (B) Control and shMLK3 clones 4 and 2 cells were treated with 20 ng/mL TNFα for 0 and 30 min. After TNFα treatments, IKKγ was immunoprecipitated from cell lysates with IKKγ antibody and IKK assays were performed. Samples were subjected to SDS PAGE and immunoblotted with IKKγ and phospho-IκBα antibodies. Phosphorylated GST-IκBα is indicated as p-GST-IκBα in the figure. (C) Control and clone 4 cells were treated with 20 ng/mL TNFα for 0, 5 and 20 and 40 min. Whole cell extracts were prepared and subjected to SDS PAGE and Western blotting with phospho-IκBα, IκBα and Actin antibodies.

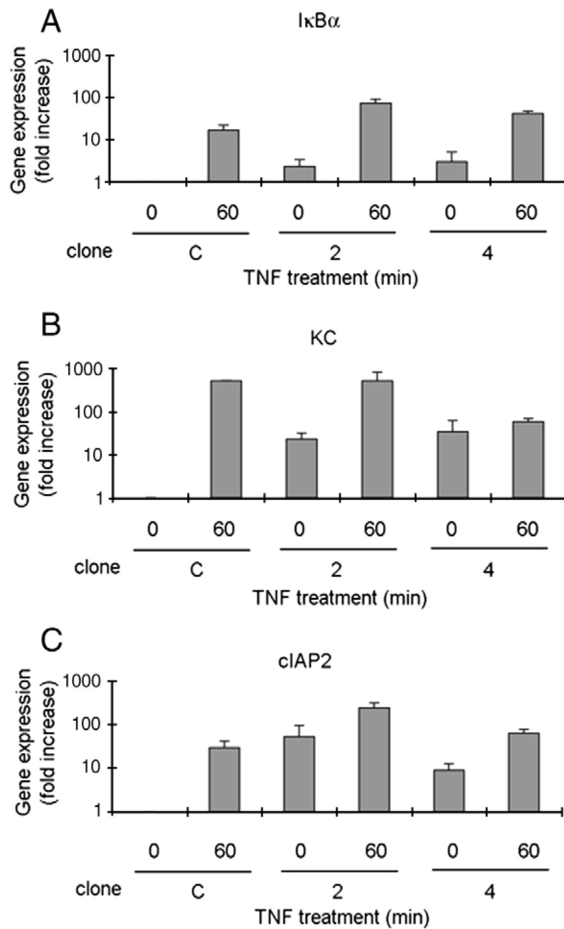

Fig. 3. Silencing *mlk3* leads to elevated expression of NF-κB regulated genes. (A-C) Control and shMLK3 (clones 4 and 2) cells were treated in triplicate with TNF for 0 and 60 min and then total RNA was isolated and real time PCR (RT-PCR) was performed with primers specific for mouse IκBα, cIAP, KC and Actin. RT-PCR products were analyzed and fold increase in gene expression was calculated.

antibody and IKK assays were performed using GST-IκBα as substrate. We observed an elevated basal level of endogenous IKK activity in cells transfected with MLK3 siRNA in comparison to cells transfected with nonspecific siRNA oligos (Fig. 2A). We then compared the TNF stimulation of IKK activity in control and shMLK3 cells. We observed that untreated shMLK3 cells had an elevated basal level of IKK activity (Fig. 2B). This was similar to the result we observed when MLK3 was silenced with siRNA oligos in HEK293 cells (Fig. 2A). TNF stimulation induced a further increase in IKK activity in shMLK3 clone 4 cells and in control cells (Fig. 2B). Interestingly, in comparison to shMLK3 cells, control cells had elevated levels of phosphorylated IκBα (p-IκBα) when treated with TNF for 0, 5 and 20 min (Fig. 2C). Total IκBα protein levels remained constant in control cells that were untreated or treated with TNF for 5 min. After TNF stimulation for 20 or 40 min, total IκBα protein levels declined (Fig. 2C). In the untreated, shMLK3 cells, total IκBα protein levels were lower than that of the untreated, control cells, and the IκBα levels decreased upon TNF treatment for 20 min and 40 min. At 40 min, there was no detectable IκBα protein in the shMLK3 clone 4 cells (Fig. 2C). These data indicate that silencing *mlk3* elevates basal IKK activity and enhances phosphorylation and degradation of IκBα.

3.3. Silencing mlk3 leads to elevated expression of NF-κB regulated genes

Since shMLK3 cells had elevated basal IKK activity, we predicted that these cells would also have elevated basal NF-κB-dependent gene expression. To test this possibility, we measured the transcription of specific NF-κB regulated genes in shMLK3 cells. Indeed, the basal levels of NF-κB regulated genes IκBα, cellular inhibitor of apoptosis 2 (cIAP2) and KC were higher in the shMLK3 cells in comparison to the control cells (Fig. 3A–C). Gene expression was further increased upon treatment of shMLK3 cells with TNF (Fig. 3A–C). These results are consistent with the MLK3 shRNA clones having elevated IKK activity. Furthermore, these results suggest that MLK3 may function to limit IKK activity and, therefore, loss of MLK3 could lead to elevated IKK activity and NF-κB dependent gene expression.

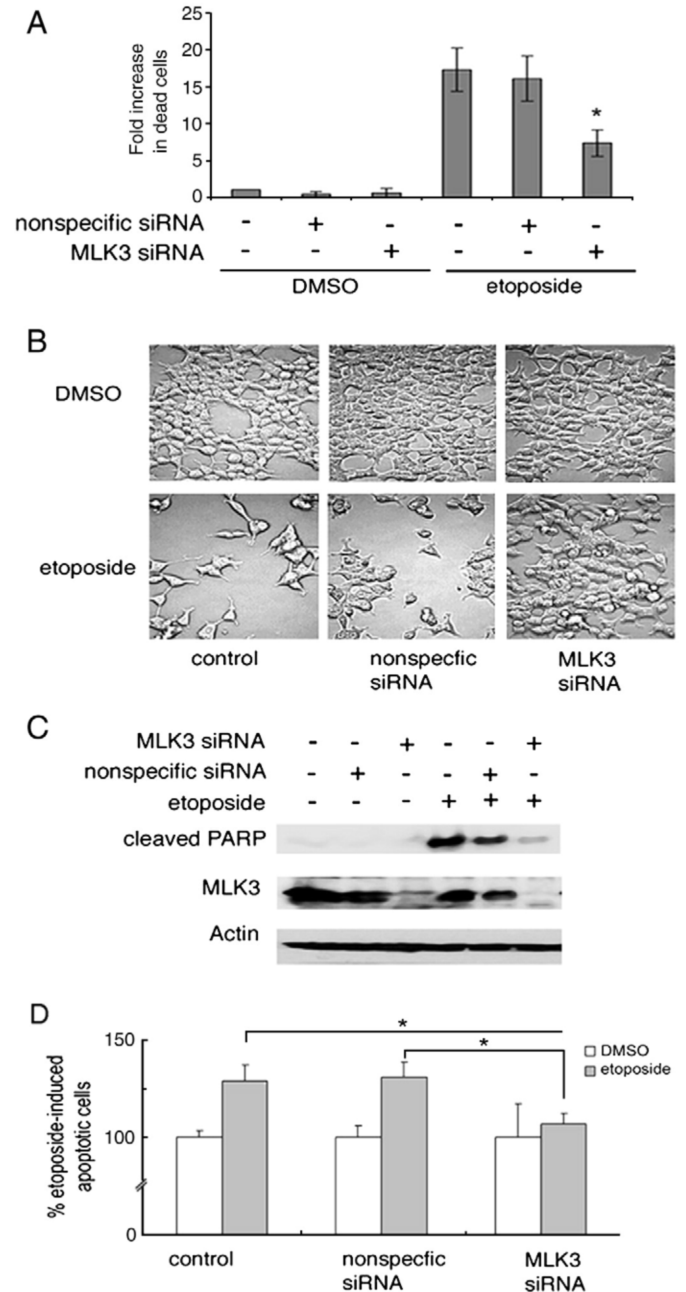

Fig. 4. Silencing *mlk3* renders cells resistant to etoposide-induced cell death. (A) HEK293 cells were transfected with MLK3 siRNA or nonspecific siRNA oligos. Cells were then treated with 200 μM etoposide or DMSO for 26 h. Dead cells were counted by trypan blue exclusion and the fold-increase in dead cells was calculated. (B) Photomicrographs of cells treated as described in A. (C) Extracts from cells in A, were subjected to SDS PAGE and Western blotting with MLK3, cleaved PARP and Actin antibodies. (D) Cells treated as described in A were analyzed with the APOPercentage apoptosis assay system and percent-increase in apoptotic cells was determined. Each bar represents the mean of data collected from 3 independent experiments and error bars represent the standard error of the mean. An asterisk (*) indicates a statistically significant difference between samples indicated in the figure ($P<0.05$).

3.4. Silencing mlk3 renders cells resistant to etoposide-induced cell death

We postulated that elevated NF-κB dependent, pro-survival gene expression in cells lacking MLK3 would confer resistance of these cells to etoposide-induced apoptotic cell death. To test this, *mlk3* was silenced with siRNA oligos in HEK293 cells. Cells were then treated with DMSO or etoposide for 26 h and cell death was analyzed. In cells transfected with nonspecific siRNA oligos, etoposide induced a 17.3-fold increase in the number of dead cells (Fig. 4A). In cells transfected with the MLK3 siRNA oligos, etoposide induced only 7.3-fold increase in the number of dead cells indicating that HEK293 cells lacking MLK3 are partially resistant to etoposide-induced cell death (Fig. 4A). Protection of MLK3 knockdown cells from etoposide-induced cytotoxicity was also evident in photomicrographs of DMSO- and etoposide-treated cells (Fig. 4B). Etoposide treatment caused cells to round up and dead cells lifted off the dish. The photomicrograph shows the remaining adherent cells after 26 h treatment with etoposide (Fig. 4B). To determine if cell death induced by etoposide treatment was apoptotic cell death, we analyzed cell lysates for the presence of cleaved PARP. Cleaved PARP was observed only in cells treated with etoposide. Furthermore, the level of cleaved PARP was much lower in cells transfected with MLK3 siRNA in comparison to cells transfected with nonspecific siRNA, indicating that the induction of apoptotic cell death by etoposide was impaired in cells lacking MLK3 (Fig. 4C).

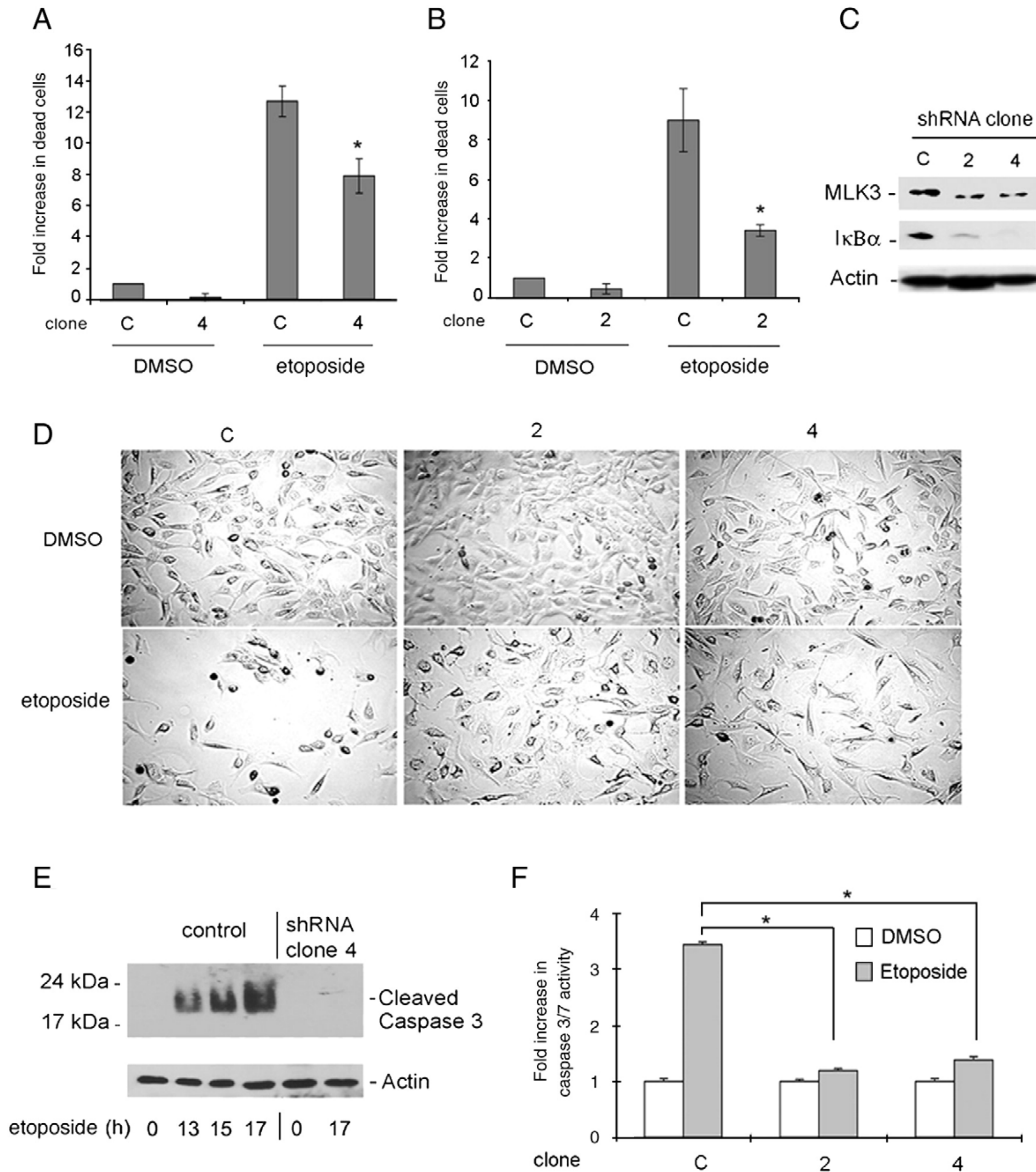

Fig. 5. shMLK3 cells are resistant to etoposide-induced apoptotic cell death and caspase activation. (A) Control and shMLK3 clone 4 cells were treated with 200 μM etoposide or DMSO for 26 h. Dead cells were counted by trypan blue exclusion and the fold-increase in dead cells was calculated. (B) Control and shMLK3 clone 2 cells were treated as described in A, and the fold-increase in dead cells was calculated. (C) Control and shMLK3 clone 4 and clone 2 cell extracts prepared from cells in A, were subjected to SDS PAGE and Western blotting with IκBα, MLK3 and Actin antibodies. (D) Photomicrographs of cells treated with DMSO and etoposide as described in A. (E) Control and shMLK3 clone 4 cells were treated with etoposide for various time intervals (as indicated) and cell extracts were prepared and subjected to SDS PAGE and Western blotting with cleaved Caspase 3 and Actin antibodies. (F) Cells treated with DMSO or etoposide as described in A, were analyzed with the Caspase-Glo 3/7 apoptosis assay system and the fold-increase in apoptotic cells was determined. Each bar represents the mean of data collected from 3 independent experiments and error bars represent the standard error of the mean. An asterisk (*) indicates a statistically significant difference between samples indicated in the figure ($P<0.05$).

Apoptotic cell death was also analyzed using the APOPercentage apoptosis assay system. Cells transfected with nonspecific siRNA oligos had a 30% increase in etoposide-induced apoptosis in comparison to cells transfected with MLK3 siRNA, which had a 7% increase in etoposide-induced apoptosis (Fig. 4D).

We also analyzed etoposide-induced cell death in control cells and shMLK3 cells that exhibited reduced MLK3 and IκBα protein levels (Fig. 5C). In the control cells, etoposide induced a 12.7-fold increase in the number of dead cells, while the shMLK3 clone 4 cells displayed a 7.9-fold increase in the number of dead cells upon etoposide treatment (Fig. 5A). Similarly, in shMLK3 clone 2 cells, etoposide induced only a 3.4-fold increase in cell death in comparison to 9.0-fold increase observed in the control cells (Fig. 5B). The protection of shMLK3 cells from etoposide-induced cell death is also evident in the photomicrographs in Fig. 5D that show the remaining adherent cells after 26 h DMSO or etoposide treatment. Cleaved Caspase 3 was also observed in control cells treated with etoposide at 13, 15 and 17 h, however, no cleaved Caspase 3 was observed in the shMLK3 cells treated with etoposide for 17 h (Fig. 5E). The Caspase Glo 3/7 apoptosis assay system was used to analyze Caspase 3 and 7 activities associated with apoptotic cell death. A 3.4-fold increase in Caspase 3/7 activity was induced by etoposide treatment in control cells in comparison to a 1.2- to 1.4-fold increase induced by etoposide in shMLK3 cells (Fig. 5F). Taken together, these results suggest that silencing *mlk3* expression inhibits etoposide-induced Caspase 3 and 7 activities and renders cells partially resistant to etoposide-induced apoptotic cell death.

3.5. Expression of MLK3 sensitizes cells to etoposide-induced cell death

Cells lacking MLK3 are resistant to etoposide-induced cell death, suggesting that MLK3 may have an important role in inducing cell death in response to etoposide treatment. Thus, we tested if overexpressing wild type FLAG-MLK3 (MLK3-WT) in HEK293 cells could enhance etoposide-induced cell death. Interestingly, expression of MLK3-WT alone in HEK293 cells induced 1.8-fold more cell death than control cells expressing vector alone (Fig 6A, B). Expression of a kinase-dead mutant (K144A) of MLK3 (MLK3-KD) resulted in 1.7-fold more cell death in comparison to vector alone (Fig. 6A, B). In addition, overexpression of either FLAG-MLK3-WT or FLAG-MLK3-KD enhanced etoposide-induced cell death (Fig. 6A, B). The kinase activity of MLK3 is important for enhancing etoposide-induced cell death since MLK3-WT was much more effective in enhancing etoposide-induced cell death than MLK3-KD (Fig. 6A, B). Furthermore, enhancement of etoposide-induced cell death by MLK3-WT was reduced by treatment of cells with K252a, a neuroprotective compound that is a potent inhibitor of MLK3 kinase activity (Fig. 6A, B) [27]. Cleaved PARP was observed in cells treated with etoposide or transfected with MLK3-WT or MLK3-KD, but not in cells transfected with empty vector suggesting that overexpressing MLK3 alone triggers apoptotic cell death (Fig. 6C). For cells treated with etoposide, MLK3-WT was more effective at inducing PARP cleavage than MLK3-KD (Fig. 6C). Consistent with these findings, enhancement of etoposide-induced cleavage of PARP by MLK3-WT was substantially reduced in cells treated with K252a (Fig. 6C). Our results suggest an anti-survival function for MLK3, which is in agreement with the observation that MLK3-WT, MLK3 WT + K252a treatment or MLK3-KD overexpression had no significant effect on IκBα protein levels. These results also indicate that inhibition of IKK signaling by MLK3 does not require MLK3 kinase activity. (Fig. 6C).

Using the APOPercentage apoptosis assay system, we observed that etoposide induced a 19% increase in apoptosis in HEK293 cells expressing vector alone. In cells expressing MLK3-WT, etoposide induced a 24% increase in apoptosis in comparison to DMSO treatment (Fig. 6D). For cells expressing MLK3-KD, etoposide induced a 24% increase in apoptosis, however, cells expressing MLK3-WT and treated with K252a had no significant increase in apoptotic cell death in comparison to DMSO treatment (Fig. 6D). The reduction in etoposide-induced PARP cleavage and apoptosis was more

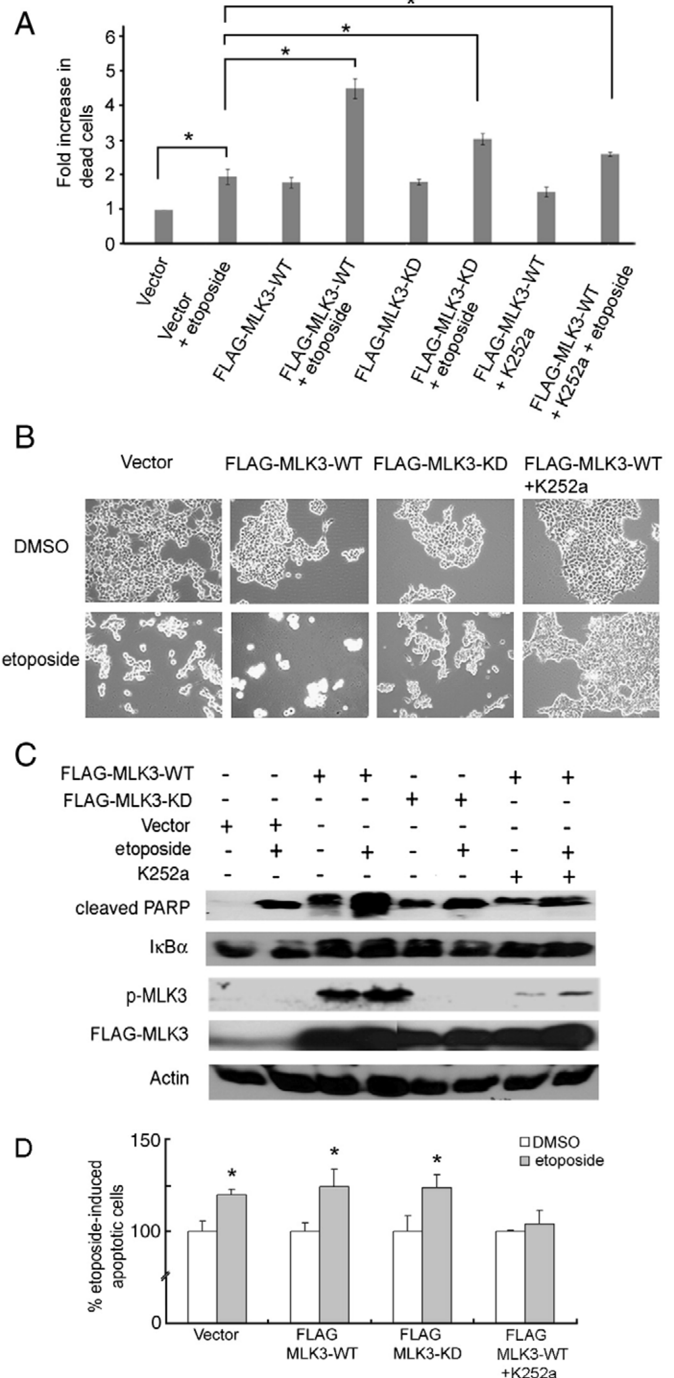

Fig. 6. Expression of MLK3 sensitizes cells to etoposide-induced cell death. (A) HEK293 cells were transfected with MLK3-WT or MLK3-KD expression plasmids or control pCMV5 vector. Cells were treated with 100 μM etoposide or DMSO for 24 h. Cells treated with the K252a inhibitor were incubated with the inhibitor for 5 h prior to treatment of cells with DMSO or etoposide. Dead cells were counted by trypan blue exclusion and the fold-increase in dead cells was calculated. (B) Photomicrographs of cells treated as described in A. (C) Cell lysates were prepared as in A, but collected 6 h prior to counting and then subjected to SDS PAGE and Western blotting with FLAG, cleaved PARP, pMLK3 and Actin antibodies. (D) Cells treated as described in A were analyzed with the APOPercentage apoptosis assay system and the percent-increase in apoptotic cells was determined. Each bar represents the mean of data collected from 3 independent experiments and error bars represent the standard error of the mean. An asterisk (*) indicates a statistically significant difference between samples indicated in the figure ($P<0.05$).

pronounced in cells expressing MLK3-WT and treated with K252a, in comparison to cells expressing MLK3-KD. This difference could be due to the fact that K252a treatment of cells can also cause activation of the AKT pro-survival signaling pathway [27]. Collectively, these results suggest that MLK3 overexpression induces cleavage of PARP and apoptosis. Furthermore, MLK3 overexpression can sensitize cells to etoposide-induced apoptotic cell death.

4. Discussion

To gain a better understanding of the function of endogenous MLK3 in regulation of IKK activation, we silenced *mlk3* expression by RNAi and analyzed the effect on IKK activity and NF-κB-dependent gene expression. We observed that both NIH3T3 cells stably expressing MLK3 shRNA and SKOV3 cells transiently transfected with MLK3 siRNA oligos had reduced levels of IκBα protein. Previously it was reported that IκBα protein levels were unaffected in mouse embryonic fibroblasts (MEFs) from MLK3 knockout mice in comparison to wild type MEFs [12]. Possibly, in the MLK3 knockout MEFs, one of the other members of the MLK family (MLK 1, 2 and 4) may have compensated for MLK3 function during development.

To determine the effect of reduced MLK3 protein levels on IKK activity, we compared the IKK activity in control cells and cells in which *mlk3* expression was silenced with RNAi. We observed that cells lacking MLK3 protein had an elevated level of basal IKK activity in comparison to control cells. These results suggest that MLK3 has an inhibitory effect on IKK activity that is released when *mlk3* expression is silenced. MLK3-dependent inhibition of IKK activity could occur indirectly through inhibition of an upstream IKKK such as NIK or TAK1. However, since MLK3 has been previously shown to directly associate with IKKα and IKKβ catalytic subunits, it is possible that MLK3 inhibits IKK activity directly through its interaction with the IKK catalytic subunits [6]. Hehner et al. [6] previously showed that overexpression of MLK3 stimulated IKK activity in Jurkat T cells. Our results using MLK3 RNAi in NIH3T3 and HEK293 cells suggest that MLK3 has an inhibitory effect on IKK activity. Possibly, the differences between the cell lines studied and the approaches employed could account for the variation in results obtained between these two studies [6,21].

In addition to having elevated basal IKK activity, cells depleted of MLK3 also had an elevated basal level of NF-κB-dependent gene expression (cIAP, IκBα, and KC). Consistent with these findings, elevated TNF-induced expression of IL-6, another NF-κB regulated gene, was previously observed in MLK3 deficient MEFs [12].

Upregulation of NF-κB pro-survival signaling could protect cells against cell death induced by various pro-apoptotic stimuli such as the DNA topoisomerase inhibitor, etoposide. Etoposide induces double strand breaks in DNA and triggers a p53-dependent activation of Bax transcription and apoptosis [28]. We observed that MLK3-deficient cells were partially resistant to etoposide-induced cell death in comparison to control cells. These data suggest that MLK3 has an anti-survival function and are in agreement with the enhanced IKK activity and NF-κB dependent gene expression observed in cells lacking MLK3.

MLK3 has been implicated in the activation of apoptotic cell death in neuronal and hepatoma cells. In serum-deprived neuronal cells, MLK3 induces apoptotic cell death through activation of JNK signaling [13,14]. Inhibition of MLK3 kinase activity with CEP1347 increases the survival of neurons that have been deprived of nerve growth factor [13]. In hepatoma cells, MLK3 activation of p38 was found to be critical for TGFβ-induced apoptosis [9]. Given the pro-apoptotic function of MLK3 in hepatoma and neuronal cells, we analyzed the effect of overexpressing MLK3 on the induction of apoptosis in HEK293 cells. We observed that MLK3 expression alone, triggered apoptotic cell death in HEK293 cells. Stimulation of apoptosis by MLK3 could occur through its direct activation of MAP2Ks that regulate JNK or p38 signaling. However, in HEK293 cells, overexpression of the kinase-dead form of MLK3 also activated apoptosis, suggesting that MLK3 can activate apoptosis through a mechanism that is independent of its kinase activity. Possibly, MLK3 can also function as a scaffold protein facilitating activation of other apoptotic signaling pathways independent of JNK or p38.

Interestingly, MLK3-WT expression was more effective in enhancing etoposide-induced cell death than MLK3-KD. These results suggest that MLK3 kinase activity is important for enhancement of etoposide-induced cell death. Since prolonged, rather than transient activation of JNK promotes apoptosis, it is possible that overexpression of MLK3 caused sustained JNK activation resulting in enhancement of etoposide-induced apoptotic cell death.

Collectively, our observations suggest an anti-survival role for endogenous MLK3 in NIH3T3, SKOV3 and HEK293 cells. TNF activates both pro-apoptotic signaling and pro-survival NF-κB signaling and ultimately, the decision between life and death is dependent on the balance between these opposing pathways [29]. In unstimulated cells, MLK3 may limit IKK activity and prevent inappropriate IKK activation. Upon cytokine stimulation, recruitment of MLK3 to JNK and p38 MAPK signaling pathways may relieve the inhibition on IKK activity. Similarly, depletion of MLK3 protein with RNAi removes the block on IKK activity and elevates basal IKK activity and NF-κB-dependent gene expression.

Our previous observations indicated that silencing *mlk3* in normal and tumor cells inhibited MAPK signaling and cell proliferation [10]. Herein, we observed that cells lacking MLK3 also have increased IKK activity and enhanced NF-κB-dependent gene expression. Thus, targeting MLK3 would have the combined effect of inhibiting cell proliferation and enhancing cell survival.

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