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## MLK3 phosphorylation by ERK1/2 is required for oxidative stress-induced invasion of colorectal cancer cells

April L. Schroyer, Nicholas W. Stimes, Widian F. Abi Saab, and Deborah N. Chadee\*

Department of Biological Sciences, University of Toledo, 2801 West Bancroft Street, Toledo, Ohio 43606, USA

### Abstract

Mixed lineage kinase 3 (MLK3) functions in migration and/or invasion of several human cancers; however, the role of MLK3 in colorectal cancer (CRC) invasion is unknown. MLK3 is a mitogen-activated protein kinase (MAPK) kinase kinase (MAP3K) which activates MAPK pathways through either kinase-dependent or -independent mechanisms. Human colorectal tumors display increased levels of reactive oxygen species (ROS) or oxidative stress. ROS, such as H<sub>2</sub>O<sub>2</sub>, are important for carcinogenesis and activate MAPK signaling pathways. In human colorectal carcinoma (HCT116) cells treated with H<sub>2</sub>O<sub>2</sub>, extracellular signal-regulated kinases 1 and 2 (ERK1/2) were activated and MLK3 exhibited reduced electrophoretic mobility (shift) in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was eliminated by phosphatase treatment. Pretreatment with the ROS scavenger N-acetyl-L-cysteine (NAC), the ERK1/2 inhibitor UO126, or ERK1/2 siRNA knockdown blocked the H<sub>2</sub>O<sub>2</sub>-induced shift of MLK3, while MLK3 inhibition with Cep1347 did not. In co-immunoprecipitation experiments performed on H<sub>2</sub>O<sub>2</sub>-treated HCT116 cells, endogenous MLK3 associated with endogenous ERK1/2 and B-Raf. Active ERK1 phosphorylated kinase dead FLAG-MLK3 in vitro, whereas ERK1 phosphorylation of kinase dead FLAG-MLK3-S705A-S758A was reduced. Both MLK3 siRNA knockdown and FLAG-MLK3-S705A-S758A expression decreased ERK1/2 activation in H<sub>2</sub>O<sub>2</sub>-treated cells. Prolonged H<sub>2</sub>O<sub>2</sub> treatment activated ERK1/2 and promoted invasion of colon cancer cells, which was attenuated by MLK3 siRNA knockdown. Furthermore, S705A-S758A-FLAG-MLK3 demonstrated decreased oxidative-stress induced colon cancer cell invasion, but increased interaction with GST-B-Raf as compared to wild-type-FLAG-MLK3 in H<sub>2</sub>O<sub>2</sub>-treated cells. These results suggest oxidative stress stimulates an ERK1/2-dependent phosphorylation of MLK3 on Ser<sup>705</sup> and Ser<sup>758</sup>, which promotes MLK3-dependent B-Raf and ERK1/2 activation; this positive feedback loop (PFL) enhances the invasion of colon cancer cells.

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**Correspondence:** Dr. DN Chadee, Department of Biological Sciences, University of Toledo, 2801 West Bancroft Street, Toledo, Ohio 43606, USA. Phone: 419-530-5077 Fax: 419-530-7737, [deborah.chadee@utoledo.edu](mailto:deborah.chadee@utoledo.edu).

### Conflict of interest

The authors declare no conflict of interest.

### Supplementary information

Supplementary information accompanies the paper on the *Oncogene* website (<http://www.nature.com/onc>).

## Keywords

ERK1/2; MLK3; B-Raf; invasion; oxidative stress; colon cancer

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## Introduction

Mixed lineage kinase 3 (MLK3) (1) is a ubiquitously expressed mammalian serine/threonine mitogen-activated protein kinase (MAPK) kinase kinase (MAP3K) (2). MLK3 belongs to the mixed lineage kinase family, which consists of three subfamilies: the MLKs, the dual-leucine-zipper bearing kinases (DLKs), and the zipper-sterile- $\alpha$ -motif kinase (ZAK) (3). The MLKs each contain an amino-terminus src-homology-3 (SH3) domain, a kinase catalytic domain with sequence similarity to both serine/threonine and tyrosine kinases, leucine zipper regions, a Cdc42/Rac-interactive binding (CRIB) motif, and a proline-rich carboxyl-terminus (2, 3). The Rho-family GTPases Rac and Cdc42 bind to the CRIB motif of MLK3 and promote MLK3 activation (4, 5), which involves zipper-mediated homodimerization (6) and trans/autophosphorylation of Thr<sup>277</sup> and Ser<sup>281</sup> within the activation loop (7, 8).

MLK3 activates the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways by phosphorylation and activation of the MAP2Ks MKK4/7 and MKK3/6, respectively (9–11). MLK3 also activates the extracellular signal-regulated kinases 1 and 2 (ERK1/2) MAPK pathway through both kinase-dependent (12) and -independent mechanisms (13). In the latter, MLK3 serves as a scaffold for B-Raf and Raf-1 transactivation in response to epidermal growth factor (EGF) treatment leading to ERK1/2 activation in colon cancer cells, lung fibroblasts, and human schwannoma cells bearing a loss-of-function mutation in the neurofibromatosis 2 (NF2) gene (13, 14). MLK3 also functions as a scaffold in limiting Rho activation, which promotes directed cell migration of human lung carcinoma cells (15). Upon direct protein-protein interaction of MLK3 and the Rho activator p63RhoGEF, MLK3 inhibits p63RhoGEF activation by G $\alpha_q$ . In addition to lung cancer cell migration, MLK3 is implicated in or is required for the migration and/or invasion of several human cancers including non-small cell lung (16), gastric (17), breast (18–21), ovarian (22), melanoma (23), glioblastoma (24), and hepatocellular carcinoma (25). The function of MLK3 in colorectal cancer (CRC) cell invasion is unknown.

CRC is the third most commonly diagnosed cancer and the third leading cause of cancer-related death in both men and women in the United States (26). Human colorectal tumors have increased endogenous reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub>, a marker of oxidative stress (27). ROS-induced oxidative modification of signaling proteins is important for carcinogenesis (27) and can initiate MAPK signaling by direct activation of receptor tyrosine kinases (RTKs), indirect activation of MAP3Ks, or direct inactivation of MAPK phosphatases (MKPs) which negatively regulate MAPKs (28). MLK3 missense mutations were identified in microsatellite instability (MSI) sporadic colorectal cancer (CRC) cases and MSI CRC cell lines (29). P252H A352R and A356V are located in the MLK3 kinase domain, but these residues are not highly conserved functional amino acids of the kinase catalytic core. Therefore, P252H, A352R, and A356V are predicted to affect the scaffold properties of the protein rather than its kinase activity. P252H exhibited transforming and

tumorigenic potential in vitro and in vivo, respectively, suggesting a scaffold function of MLK3 may be important for the malignant phenotype (29). In this study, we examined the regulation and function of MLK3 in human colorectal carcinoma (HCT116) cells exposed to oxidative stress. We found a ROS- and ERK1/2-dependent phosphorylation of MLK3 and demonstrated ERK1 phosphorylates MLK3 on Ser<sup>705</sup> and Ser<sup>758</sup> in vitro. Endogenous MLK3 interacted with endogenous ERK1/2 and B-Raf in H<sub>2</sub>O<sub>2</sub>-treated cells. Both oxidative stress-induced ERK1/2 activation and HCT116 cell invasion was partially MLK3-dependent in a mechanism reliant on phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup>. These results identify MLK3 as a direct ERK1/2 substrate and a positive regulator of both ERK1/2 activation and colon cancer cell invasion in response to oxidative stress.

## Results

### MEK1/2 and ERK1/2 are activated and MLK3 is phosphorylated in response to oxidative stress

Oxidative stress can influence MAPK signaling by affecting MAP3K function. ROS oxidation of thioredoxin (TRX) causes dissociation from the MAP3K apoptosis signaling kinase 1 (ASK1), which allows for TNF receptor-associated factor 2 (TRAF2) activation of ASK1 and leads to JNK and p38 signaling (30). To understand whether other MAP3Ks such as MLKs 1, 3, and 4 are regulated by ROS, HCT116 cells were treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min (Fig. 1A). MLK3, but not MLKs 1 or 4, exhibited reduced electrophoretic mobility (shift) by approximately 7 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and MEK1/2 and ERK1/2 were significantly activated. Pretreatment with 10 mM of the ROS scavenger N-acetyl-L-cysteine (NAC) abrogated the MLK3 mobility shift and significantly decreased the activation of ERK1/2 in H<sub>2</sub>O<sub>2</sub>-treated cells (Fig. 1B), suggesting the MLK3 mobility shift and ERK1/2 activation are ROS-dependent. To examine if a post-translational modification (PTM) such as a phosphorylation produced the shift, HCT116 cells were either untreated or treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. Endogenous MLK3 was immunoprecipitated and incubated with calf intestinal alkaline phosphatase (CIAP) buffer alone, buffer plus CIAP, or buffer/CIAP/and a phosphatase inhibitor cocktail containing the alkaline phosphatase inhibitor (-)-*p*-bromolevamisole oxalate (Fig. 1C). The H<sub>2</sub>O<sub>2</sub>-induced shift of MLK3 was not observed in the CIAP-treated MLK3 immunoprecipitates, but was evident in the MLK3 immunoprecipitates treated with CIAP and phosphatase inhibitor. These results indicate the ROS-induced shift is due to phosphorylation. MLK3 activation involves autophosphorylation on Thr<sup>277</sup> and Ser<sup>281</sup> and leads to phosphorylation and activation of the downstream MAPK JNK (8). JNK phosphorylates MLK3 in vitro and in vivo giving rise to positive feedback (31). To test if the ROS-induced phosphorylation of MLK3 corresponds to JNK feedback or autophosphorylation of Thr<sup>277</sup> and Ser<sup>281</sup> and thus MLK3 activation, HCT116 cells were pretreated with 100 nM (Fig. 1D) or 500 nM (Fig. S1) Cep1347, a small molecule competitive inhibitor of MLK3 kinase activity (32), for 1 h and then treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. Both 100 nM and 500 nM Cep1347 significantly reduced the activation of JNK in response to H<sub>2</sub>O<sub>2</sub>, indicating MLK3 activation was blocked. Inhibition of MLK3 kinase activity did not prevent the mobility shift of MLK3 or the activation of ERK1/2, which suggests the ROS-induced phosphorylation of MLK3 is separate from both JNK

feedback onto MLK3 and MLK3 autophosphorylation and activation. Collectively, we observed ROS-induced activation of MEK1/2 and ERK1/2 as well as phosphorylation of MLK3, which is independent of MLK3 activation and JNK feedback.

### **The ROS-induced phosphorylation of MLK3 is dependent on active ERK1/2, and endogenous ERK1/2 and MLK3 are associated in HCT116 cells**

Our Cep1347 results (Fig. 1D and S1) suggested a kinase other than MLK3 or JNK phosphorylates MLK3 in response to ROS. Significant ERK1/2 activation was observed in H<sub>2</sub>O<sub>2</sub>-treated cells (Fig. 1A–B, 1D, and S1); therefore, we investigated whether ERK1/2 could promote the ROS-induced phosphorylation of MLK3. HCT116 cells were either untreated, treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min, or pretreated with 10 μM of the MEK1/2 inhibitor UO126 and then treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min (Fig. 2A). The H<sub>2</sub>O<sub>2</sub>-induced mobility shift of MLK3 was not observed in cells with significant ERK1/2 inhibition, suggesting the ROS-induced phosphorylation of MLK3 is ERK1/2-dependent. To confirm the UO126 observations, HCT116 cells were transfected with either non-specific (NS) or ERK1/2 siRNA and then treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min (Fig. 2B). ERK1/2 siRNA knockdown significantly reduced ERK1/2 activation and blocked the MLK3 mobility shift in H<sub>2</sub>O<sub>2</sub>-treated cells, indicating the ROS-induced phosphorylation of MLK3 is an ERK1/2-specific event. To determine whether ERK1/2 interacts with MLK3, endogenous ERK1/2 was immunoprecipitated from HCT116 cells which were either left untreated or treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min (Fig. 2C). Endogenous MLK3 co-immunoprecipitated with endogenous ERK1/2, suggesting endogenous ERK1/2 and MLK3 interact in HCT116 cells. This interaction was also observed in H<sub>2</sub>O<sub>2</sub>-treated cells.

### **ERK1 phosphorylates Ser<sup>705</sup> and Ser<sup>758</sup> of MLK3 in vitro**

We observed a ROS- and ERK1/2-dependent phosphorylation of MLK3; thus, we hypothesized active ERK1/2 directly phosphorylates MLK3. Six MLK3 residues conform to the ERK1/2 phosphorylation consensus sequence of PXS/TP (33): Ser<sup>570</sup>, Thr<sup>605</sup>, Thr<sup>677</sup>, Ser<sup>705</sup>, Thr<sup>752</sup>, and Ser<sup>758</sup> (Fig. 3A). Both Ser<sup>705</sup> and Ser<sup>758</sup> are confirmed MLK3 *in vivo* phosphorylation sites (34). The MLKs share 75% sequence identity within the kinase catalytic domains and approximately 65% sequence identity from the SH3 domains to the CRIB motifs; however, the MLKs carboxyl termini sequences are divergent and might have different regulatory functions (3). Ser<sup>705</sup> and Ser<sup>758</sup> are located within the C-terminal sequence unique to MLK3. MLKs 1 and 4 did not exhibit slower mobility on SDS-PAGE in response to oxidative stress (Fig. 1A); thus, the potential ERK1/2 phosphorylation sites should not be present in these kinases. We performed a multiple sequence alignment of the human MLKs and found the residues of MLKs 1–2 and 4 which correspond to Ser<sup>705</sup> and Ser<sup>758</sup> of MLK3 did not conform to the preferred ERK1/2 phosphorylation consensus sequence of PXS/TP (Fig. 3B and S2). To investigate whether ERK1 could phosphorylate MLK3 *in vitro* on Ser<sup>705</sup> and Ser<sup>758</sup>, these amino acids of kinase dead (K144R) FLAG-MLK3 were mutated to non-phosphorylatable alanine residues. Empty vector (EV), wild-type-, K144R-, K144R-S705A-, K144R-S758A-, and K144R-S705A-S758A-FLAG-MLK3 were expressed in human embryonic kidney (HEK293) cells, immunoprecipitated, and used as substrate in an ERK1 kinase assay (Fig. 3C). ERK1 phosphorylated wild-type- and K144R-FLAG-MLK3 to a significantly greater extent than K144R-S705A- and K144R-

S758A-FLAG MLK3. ERK1 phosphorylation of K144R-S705A-S758A-FLAG-MLK3 was the least of all three mutants. K144R-FLAG-MLK3 phosphorylation was significantly less than wild-type-FLAG-MLK3, which is due to the inability of kinase dead K144R-FLAG-MLK3 to autophosphorylate on Thr<sup>277</sup> and Ser<sup>281</sup>. When quantified relative to phospho-K144R-FLAG-MLK3 (set at 1.0), the single mutants, phospho-K144R-S705A-FLAG-MLK3 and phospho-K144R-S758A-FLAG-MLK3, and the double mutant, phospho-K144R-S705A-S758A-FLAG-MLK3, were 0.63, 0.83, and 0.31, respectively, suggesting that ERK1 phosphorylates MLK3 in vitro on Ser<sup>705</sup> and Ser<sup>758</sup>. To verify the mutation of Ser<sup>705</sup> and Ser<sup>758</sup> to alanine did not affect MLK3 kinase function, wild-type FLAG-MLK3 and S705A-S758A-FLAG-MLK3 were expressed in HEK293 cells, and the amount of phosphorylated, activated JNK (p-JNK) was assessed (Fig. 3D). There was no significant difference between wild-type- and S705A-S758A-FLAG-MLK3 in the ability to promote JNK activation.

### **Oxidative stress promotes association of MLK3 and B-Raf: ROS-induced MEK1/2 and ERK1/2 activation requires MLK3 Ser<sup>705</sup> and Ser<sup>758</sup> phosphorylation**

MLK3, independent of its kinase activity, functions as a scaffold for B-Raf and Raf-1 transactivation leading to ERK1/2 activation in EGF-treated colon cancer cells (13, 14); therefore, we investigated whether this scaffold function of MLK3 is important for ERK1/2 activation in H<sub>2</sub>O<sub>2</sub>-treated colon cancer cells. To determine if endogenous MLK3 and B-Raf interact in response to oxidative stress, endogenous MLK3 was immunoprecipitated from HCT116 cells either untreated or treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 5 min (Fig. 4A). Co-immunoprecipitation of endogenous B-Raf with endogenous MLK3 was significantly increased in response to ROS. If oxidative stress enhances the MLK3 scaffold function associated with B-Raf activation, ERK1/2 activation should be dependent, at least in part, on MLK3. To test this hypothesis, HCT116 cells were transfected with either NS or MLK3 siRNA and then treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min (Fig. 4B). MLK3 siRNA knockdown significantly reduced the ROS-dependent activation of ERK1/2. To examine if phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup> promotes MLK3-dependent ERK1/2 activation, wild-type FLAG-MLK3 and S705A-S758A-FLAG-MLK3 were expressed in HEK293 cells, which were then treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min (Fig. 4C). No significant difference in JNK activation was detected between wild-type- and S705A-S758A-FLAG-MLK3 transfected cells; however, significantly less MEK1/2 and ERK1/2 activation was observed in cells expressing S705A-S758A-FLAG-MLK3 as compared to wild-type FLAG-MLK3. These results indicate phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup> are critical for MLK3-dependent MEK1/2 and ERK1/2 activation in H<sub>2</sub>O<sub>2</sub>-treated cells. To determine if MLK3 and B-Raf interaction is dependent on phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup>, HEK293 cells were transiently co-transfected with appropriate combinations of EV, wild-type-FLAG-MLK3, S705A-S758A-FLAG-MLK3, and GST-B-Raf. Cells were treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 5 min and a GST pull-down was performed (Fig. 4D). An interaction between wild-type-FLAG-MLK3 and GST-B-Raf was observed in H<sub>2</sub>O<sub>2</sub>-treated cells confirming our results from Fig. 4A. The association of S705A-S758A-FLAG-MLK3 and GST-B-Raf was significantly higher than wild-type-FLAG-MLK3, which suggests the phosphorylation of these residues regulates the binding of MLK3 to B-Raf. Collectively, the data indicates oxidative stress promotes an interaction between MLK3 and B-Raf, and the ERK1/2-

mediated phosphorylation of MLK3 function in the activation of the B-Raf—MEK1/2—ERK1/2 signaling module.

### **MLK3 promotes oxidative stress-induced invasion of colon cancer cells through a mechanism which requires phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup>**

MLK3 promotes the migration and/or invasion of several human cancers (15–25), and system biology approaches and computational studies suggest both MLK3 and ERK2 are master regulators of colon cancer cell invasion (35). To investigate whether MLK3 is important for colon cancer cell invasion in a ROS-, ERK1/2-, and phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup>-dependent mechanism, we first examined if prolonged H<sub>2</sub>O<sub>2</sub> treatment affected colon cancer cell invasion and ERK1/2 activation. HCT116 cells were either untreated or treated with 250 μM H<sub>2</sub>O<sub>2</sub> for 12 h and then invasion was analyzed by an in vitro FluoroBlok tumor invasion assay with Matrigel. In comparison to untreated cells, H<sub>2</sub>O<sub>2</sub> treatment significantly increased cell invasion (Fig. 5A). Significant ERK1/2 activation was observed in HCT116 cells treated with 250 μM H<sub>2</sub>O<sub>2</sub> for 12 h as compared to untreated cells (Fig. 5B). Next, we investigated whether MLK3 is required for oxidative stress-induced invasion of colon cancer cells. HCT116 cells were transfected with either 100 nM NS or MLK3 siRNA and treated with 250 μM H<sub>2</sub>O<sub>2</sub> for 12 h. In H<sub>2</sub>O<sub>2</sub>-treated cells, MLK3 siRNA knockdown significantly reduced cell invasion in comparison to cells transfected with NS siRNA (Fig. 5C). To determine if the phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup> is required for MLK3-dependent oxidative stress-induced colon cancer cell invasion, wild-type- and S705A-S758A-FLAG-MLK3 were expressed in HCT116 cells, and cells were treated with 250 μM H<sub>2</sub>O<sub>2</sub> for 12 h followed by cell invasion analysis. Wild-type-FLAG-MLK3 significantly increased colon cancer cell invasion in comparison to the EV control, while S705A-S758A-FLAG-MLK3 did not (Fig. 5D). Taken together, these results indicate MLK3 is required for the oxidative stress-induced invasion of colon cancer cells, and phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup> are critical for this process.

## **Discussion**

We propose a model (Fig. 6) in which ERK1/2 activated by oxidative stress phosphorylates MLK3 on Ser<sup>705</sup> and Ser<sup>758</sup>. Phosphorylated MLK3 binds B-Raf and promotes B-Raf, MEK1/2, and ERK1/2 activation, leading to maintenance of the ERK1/2-phosphorylated MLK3 and heightened activation of ERK1/2 signaling. This positive feedback loop (PFL) increases invasion of colon cancer cells in response to oxidative stress. Collectively, our data support the hypothesis of an ERK1/2—MLK3—B-Raf—MEK1/2—ERK1/2 PFL. We observed significant MEK1/2 (Fig. 1A) and ERK1/2 (Fig. 1A–B, 1D, S1, 2A–B, 4B, and 5B) activation in H<sub>2</sub>O<sub>2</sub>-treated HCT116 cells, indicating activation of the B-Raf—MEK1/2—ERK1/2 MAPK pathway. NAC pretreatment and MLK3 siRNA knockdown revealed ERK1/2 activation to be both ROS- and MLK3-dependent (Fig. 1B and 4B). In V600E-positive melanoma cell lines A375 and A2058, MLKs 1–4 act as MEK1 kinases that reactivate the MEK1/2—ERK1/2 pathway mediating resistance to RAF inhibitors (12). Inhibition of MLK3 kinase activity did not decrease ERK1/2 activation in response to oxidative stress (Fig. 1D and S1), suggesting the MLK3-dependent ERK1/2 activation in HCT116 cells under oxidative stress occurs through a kinase-independent mechanism. As

previously mentioned, MLK3 functions as a scaffold for B-Raf and Raf-1 transactivation leading to ERK1/2 activation (13, 14). We detected an interaction between wild-type-FLAG-MLK3 and GST-B-Raf as well as an interaction between endogenous MLK3 and both endogenous B-Raf and ERK1/2 in cells exposed to oxidative stress (Fig. 4D, 4A, and 2C). We identified a ROS- and ERK1/2-dependent phosphorylation of MLK3 on Ser<sup>705</sup> and Ser<sup>758</sup>, which was independent of MLK3 activation (Fig. 1B–D, 2A–B, and 3C). Furthermore, mutation of Ser<sup>705</sup> and Ser<sup>758</sup> to alanine significantly decreased the capacity of FLAG-MLK3 to activate both MEK1/2 and ERK1/2, but did not significantly affect FLAG-MLK3-dependent activation of JNK (Fig. 4C), which indicates phosphorylation of these residues are crucial for the MLK3 kinase-independent activation of B-Raf rather than MLK3 kinase activity. We observed a significant increase in the interaction between S705A-S758A-FLAG-MLK3 and GST-B-Raf compared to the interaction between WT-FLAG-MLK3 and GST-B-Raf (Fig. 4D), which suggests the ERK1/2-mediated phosphorylation of MLK3 limits the interaction of MLK3 with B-Raf; however, we still detect a significant interaction between WT-MLK3 and B-Raf in H<sub>2</sub>O<sub>2</sub>-treated cells (Fig. 4A). The ERK1/2-mediated phosphorylation of MLK3 is required for MLK3-dependent activation of MEK1/2 and ERK1/2 providing positive feedback onto the ERK1/2 signaling pathway, which is the mechanism reported in our model (Fig. 6). We propose the decrease in MEK1/2 and ERK1/2 activation observed with S705A-S758A-FLAG-MLK3 is due to its inability to activate B-Raf perhaps through influencing B-Raf homodimerization and/or heterodimerization with Raf-1.

Mathematical modeling of MAPK networks suggested the presence of an additional PFL involving MLK3 in response to ROS (36). An MKK4/7—JNK— (plenty of SH3s) POSH—active MLK3—MKK4/7 PFL is proposed to mediate the activating phosphorylations of ERK1/2 and JNKs by different ROS concentrations. JNK activation by this PFL promotes c-Jun-dependent expression of MKPs, which negatively regulate ERK1/2. Active JNK can cause direct positive feedback onto MLK3 through phosphorylation (31) and along with the MLKs through stabilization of POSH protein (37, 38). POSH can in turn stabilize the MLKs and act as a scaffold promoting their activation, thus inducing JNK (39). It is essential to note that ERK1/2 is not a component of the PFL reported in Lee et al., 2014, rather ERK1/2 activation is attenuated as a consequence of the PFL (36). In addition, the MKK4/7—JNK—POSH—active MLK3—MKK4/7 PFL is dependent on MLK3 kinase activity. Therefore, our proposed PFL is mechanistically distinct from that described in Lee et al., 2014 as it involves ERK1/2 activation and does not require MLK3 kinase activity. We propose MLK3, depending on its activation state, could act as a molecular switch to increase or decrease ERK1/2 activation in response to ROS. Inactive MLK3 could increase ERK1/2 activation through its scaffold function in activating B-Raf, while active MLK3 may decrease ERK1/2 activation through an active JNK—c-Jun—MKP pathway. In the latter, inhibition of MLK3 kinase activity would result in a decrease in MKP expression and therefore an increase in ERK1/2 activation. In our experiments, Cep1347 did not increase ERK1/2 activation in response to ROS (Fig. 1D and S1); hence, we propose that MLK3 is not activated by oxidative stress in HCT116 cells.

Prolonged H<sub>2</sub>O<sub>2</sub> treatment significantly increased both the activation of ERK1/2 (Fig. 5B) and invasion of colon cancer cells (Fig. 5A). MLK3 siRNA knockdown significantly

decreased the oxidative stress-induced invasion of HCT116 cells (Fig. 5C). Significantly reduced invasion was also observed with S705A-S758A-FLAG-MLK3 as compared to wild-type-FLAG-MLK3 in H<sub>2</sub>O<sub>2</sub>-treated HCT116 cells. Therefore, we propose MLK3 is required for oxidative stress-induced colon cancer cell invasion through a mechanism dependent on phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup>. In ovarian cancer cells, MLK3 is required for ERK1/2-dependent activation of matrix metalloproteinases (MMPs) and induction of AP-1-dependent MMP expression leading to an invasive phenotype (22). Possibly, the ERK1/2—MLK3—B-Raf—MEK1/2—ERK1/2 PFL in colon cancer cells exacerbates invasion through enhanced ERK1/2—AP-1—MMP signaling. The mobility shift of MLK3 was not detected in Fig. 5B; however, a slight mobility shift of MLK3 in response to 12 h H<sub>2</sub>O<sub>2</sub> treatment was observed in Fig. 5C. The electrophoretic shift of MLK3 is not as evident with the lower concentration of H<sub>2</sub>O<sub>2</sub> as compared to the higher concentration, in which the entire pool of MLK3 protein is phosphorylated and ERK1/2 activation is more robust.

Our findings indicate ERK1/2 is significantly activated by oxidative stress and promotes the phosphorylation of MLK3. In H<sub>2</sub>O<sub>2</sub>-treated HCT116 cells, MLK3 interacts with both B-Raf and ERK1/2 to promote further ERK1/2 activation in a manner that does not require MLK3 kinase activity, but is dependent on phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup>. We elucidated a requirement for MLK3 in the oxidative stress-induced invasion of colon cancer cells, which is also reliant on phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup>. We propose a PFL involving MLK3 and ERK1/2 which promotes the MLK3 kinase-independent activation of B-Raf, MEK1/2, ERK1/2, and invasion of colon cancer cells. This study identifies MLK3 as a direct ERK1/2 substrate and a positive regulator of both ERK1/2 activation and oxidative stress-induced colon cancer cell invasion. In addition, our findings offer insight into the ill-defined kinase-independent functions of MLK3 and define the ERK1/2—MLK3—B-Raf—MEK1/2—ERK1/2 PFL as a molecular mechanism by which MLK3 supports a malignant phenotype in CRC under oxidative stress.

## Materials and methods

### Cell culture

Human colorectal carcinoma (HCT116) cells and human embryonic kidney (HEK293) cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). HCT116 and HEK293 cells were cultured in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA), 2 mM l-glutamine, 25 µg/ml streptomycin, and 25 IU penicillin (Mediatech).

### Cell treatments

Cells were either left untreated or treated with dimethyl sulfoxide (DMSO) vehicle (Fisher Scientific, Hampton, NH, USA) or 2 mM or 250 µM H<sub>2</sub>O<sub>2</sub> (Fisher Scientific), and pretreated with 10 mM N-acetyl-L-cysteine (NAC) (Acros Organics, New Jersey, USA), 100 or 500 nM Cep1347 (Tocris Bioscience, Bristol, United Kingdom), or 10 µM UO126 (Promega, Madison, WI, USA). All pretreatments were for 1 h. Cells were harvested immediately after treatments, and whole-cell extracts were prepared with 6X SDS sample buffer. Samples



were boiled and subjected to western blot analysis with 10% SDS-PAGE to examine electrophoretic mobility of MLKs and 12 or 15% SDS-PAGE for other proteins.

### Immunoblotting

Immunoblotting was performed with the following Santa Cruz Biotechnology (Dallas, TX, USA) antibodies: MLK1 (N-20) sc-19120, MLK3 (C-20) sc-536, B-Raf (F-3) sc-55522, MEK1/2 (12-B) sc-436, ERK2 (C-14) sc-154, JNK1 (C-17) sc-474, GST (B-14) sc-138,  $\beta$ -actin (C-4) sc-47778, and goat anti-rat IgG-HRP sc-2032. Activation state antibodies from Cell Signaling Technology (Beverly, MA, USA) include: phosphorylated MEK1/2 (p-MEK) (Ser221) 166F8, phosphorylated ERK1/2 (p-ERK) (Thr202/Tyr204) #4370S, and phosphorylated JNK (p-JNK) (Thr183/Tyr185) 9251L. Other antibodies used in this study were MLK4 NBP1-41081 (Novus Biologicals, Littleton, CO, USA), rat anti-DYKDDDDK (FLAG) 200474-21 (Agilent Technologies, Santa Clara, CA, USA), anti-rabbit IgG HRP conjugate W401B (Promega), and Immun-Star goat anti-mouse IgG-HRP conjugate 1705047 (Bio-Rad, Hercules, California, USA).

### Phosphatase treatment

After H<sub>2</sub>O<sub>2</sub>-treatment and immunoprecipitation of endogenous MLK3, samples were suspended in either 1X calf intestinal alkaline phosphatase (CIAP) buffer alone, CIAP buffer plus CIAP enzyme (Promega), or CIAP buffer and CIAP enzyme plus phosphatase inhibitor cocktail 3 (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 1 h at 37°C. The reaction was stopped with 1X SDS sample buffer and 95°C heat for 5 min.

### Plasmid and siRNA transfections

In addition to the mutant plasmids described below, pRK5-FLAG-MLK3, pRK5-K144R-FLAG-MLK3, and pEBG-GST-B-Raf mammalian expression constructs were used for expression of human MLK3 and B-Raf. Transient transfections of HEK293 and HCT116 cells were performed with PolyJet (SignaGen Laboratories, Rockville, MD, USA) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively, according to the manufacturers' instructions. Small interfering RNA (siRNA) knockdown was also executed with Lipofectamine 2000. Non-specific (NS) siRNA, SignalSilence® p44/42 MAPK (ERK1/2) siRNA, and MLK3 sense 5'-GGGCAGUGACGUCUGGAGUdTdT-3' (nt 903–923) and antisense 5'-ACUCCAGACGUCACUGCCcdTdT-3' siRNA were obtained from Santa Cruz Biotechnology, Cell Signaling Technology, and GE Dharmacon (Lafayette, CO, USA), respectively. ERK1/2 and MLK3 siRNA transfection was performed with a final concentration of 100 nM for 72 and 36 h, respectively.

### Immunoprecipitations and GST pull-down

Immunoprecipitation of endogenous proteins was executed in HCT116 cells. HEK293 cells were transfected with appropriate plasmids to express human MLK3 and human B-Raf. Immunoprecipitations and GST pull-down was performed as described previously (40) using the following reagents and antibodies: protein A/G PLUS Agarose (Santa Cruz Biotechnology), protein G resin (GenScript, Piscataway, NJ, USA), rat anti-DYKDDDDK (FLAG) CAT# 200474-21 (Agilent Technologies, Santa Clara, CA, USA), anti-MLK3

(D-11) sc-166639 (Santa Cruz Biotechnology), anti-ERK2 (C-14) sc-154 (Santa Cruz Biotechnology), normal mouse IgG sc-2025 (Santa Cruz Biotechnology), normal rabbit IgG sc-2027 (Santa Cruz Biotechnology), and immobilized glutathione (Thermo Scientific, Waltham, MA, USA).

### Multiple sequence alignment

Multiple sequence alignment of human MLKs was generated with T-Coffee and the following NCBI reference sequences: NP\_149132.2 (MLK1), NP\_002437.2 (MLK2), NP\_002410.1 (MLK3), and NP\_115811.2 (MLK4). The alignment was formatted with Boxshade.

### Site-directed mutagenesis

Site-directed mutagenesis of human MLK3 was performed with a QuikChange Lightning Site-directed Mutagenesis kit from Agilent Technologies. The wild-type and kinase dead (K144R) MLK3 S705A mutant cDNA were generated with the sense oligonucleotide 5'-CTCAAGACGCCCGACCCCCGCCCACTCCTGCA-' and the antisense oligonucleotide 5'-TGCAGGAGTGGGCGGGGGTCGGGCGTCTTGAG-3' (mismatch with the wild-type MLK3 template is underlined) using either pRK5-wild-type or pRK5-K144R MLK3 as templates, respectively. The MLK3 kinase dead S758A, wild-type S705A-S758A, and kinase dead S705A-S758A mutant cDNA were generated with the sense oligonucleotide 5'-CCAGGCACCCACGTGACACCCTGGGCCTC-3' and the antisense oligonucleotide 5'-GAGGCCAGGGTGGTGACACGTGGGGTGCCTGG-3' using K144R MLK3, S705A MLK3, and K144R S705A MLK3 as templates. Mutants were verified by sequencing (Genewiz, South Plainfield, NJ, USA).

### In vitro kinase assay

FLAG-MLK3 immunoprecipitates were suspended in kinase assay buffer (50 mM Tris-HCl [pH 7.5], 2 mM EGTA, 10 mM MgCl<sub>2</sub>, 0.1 mM DTT, and 0.1 % Triton X-100) with 100 μM unlabeled ATP, 10 mM MgCl<sub>2</sub>, and 5 μCi γ-<sup>32</sup>P-ATP (PerkinElmer Health Sciences, Boston, MA, USA or MP Biomedicals, Solon, OH, USA). 0.01 μg purified full-length recombinant human active ERK1 (41) (SignalChem, Richmond, BC, CA) was added to appropriate samples. The assay was executed for 30 min at 30°C and stopped with 1X SDS sample buffer and 95°C heat for 5 min.

### In vitro FluoroBlok Invasion assay

The in vitro FluoroBlok invasion assay was performed as described in Partridge and Flaherty, 2009 (42). FluoroBlok 24 well transwell inserts with 8.0 μm colored PET membrane and Calcein AM fluorescent dye were obtained from Corning (Corning, NY, USA). 300,000 cells were seeded into the upper chamber of the transwell containing 100 μl of 1 mg/ml BD Matrigel matrix (BD Biosciences, San Jose, CA, USA). Fluorescence of invaded cells (relative fluorescence units [RFUs]) was read at 494/517 nm (Ex/Em) wavelengths on a SpectraMax M5 bottom-reading fluorescent plate reader with SoftMax Pro 5 microplate data acquisition and analysis software. Photomicrographs of invaded cells were

taken with an Olympus 1X81 inverted fluorescence microscope using Olympus cellSens microscope imaging software.

### Quantification and statistical analysis

For every data panel, immunoblots are representative of three independent experiments (n=3), and quantification and statistical analysis were performed on three independent experiments. Each experiment shown was replicated a minimum of three times in the laboratory. Densitometric analysis of immunoblot or autoradiography bands was executed using Image J software (National Institutes of Health). The signal for a phosphorylated protein was normalized to the total abundance of the protein of interest. Statistical analysis for comparison of two samples was performed with Student's *t* test (two-tailed). For comparison of three plus samples, Kruskal-Wallis one-way analysis of variance followed by the Conover-Inman post hoc test was performed. All samples were included in the analysis, and P-values were not adjusted. Bars depict means, error bars represent SEMs, asterisks indicate statistical significance (p-value < 0.05), and a.u.=arbitrary units.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

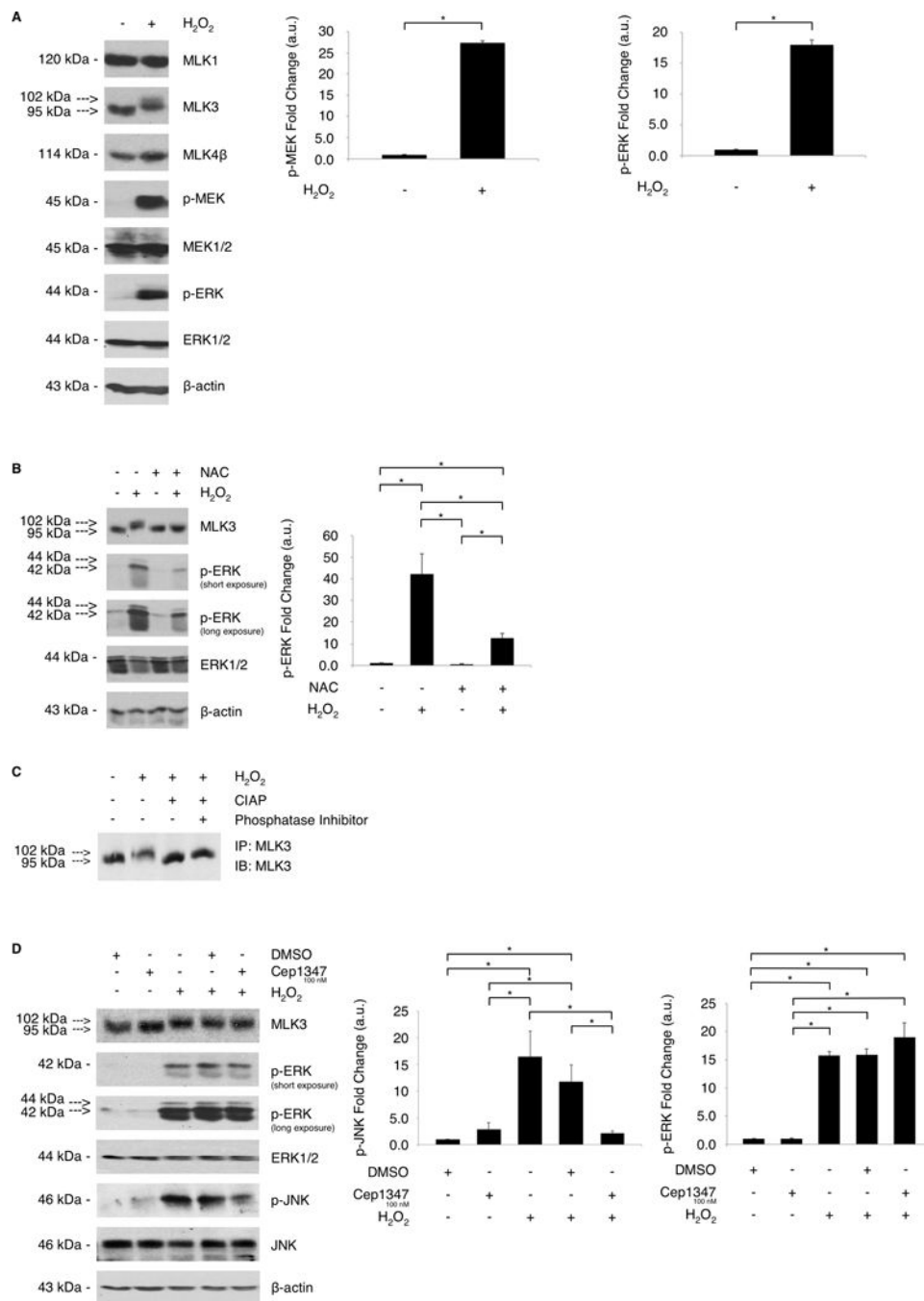
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**Fig. 1.** MEK1/2 and ERK1/2 are activated and MLK3 is phosphorylated in response to oxidative stress. (A) Western blot analysis of indicated proteins from HCT116 cell lysates treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. n=3 and a.u. stands for arbitrary units. (B) Western blot analysis of indicated proteins from HCT116 cell lysates pretreated with 10 mM NAC for 1 h followed by 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. n=3. (C) Western blot analysis of endogenous MLK3 immunoprecipitated from HCT116 cells treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min and then subjected to phosphatase treatment or treatment with phosphatase plus phosphatase inhibitor.

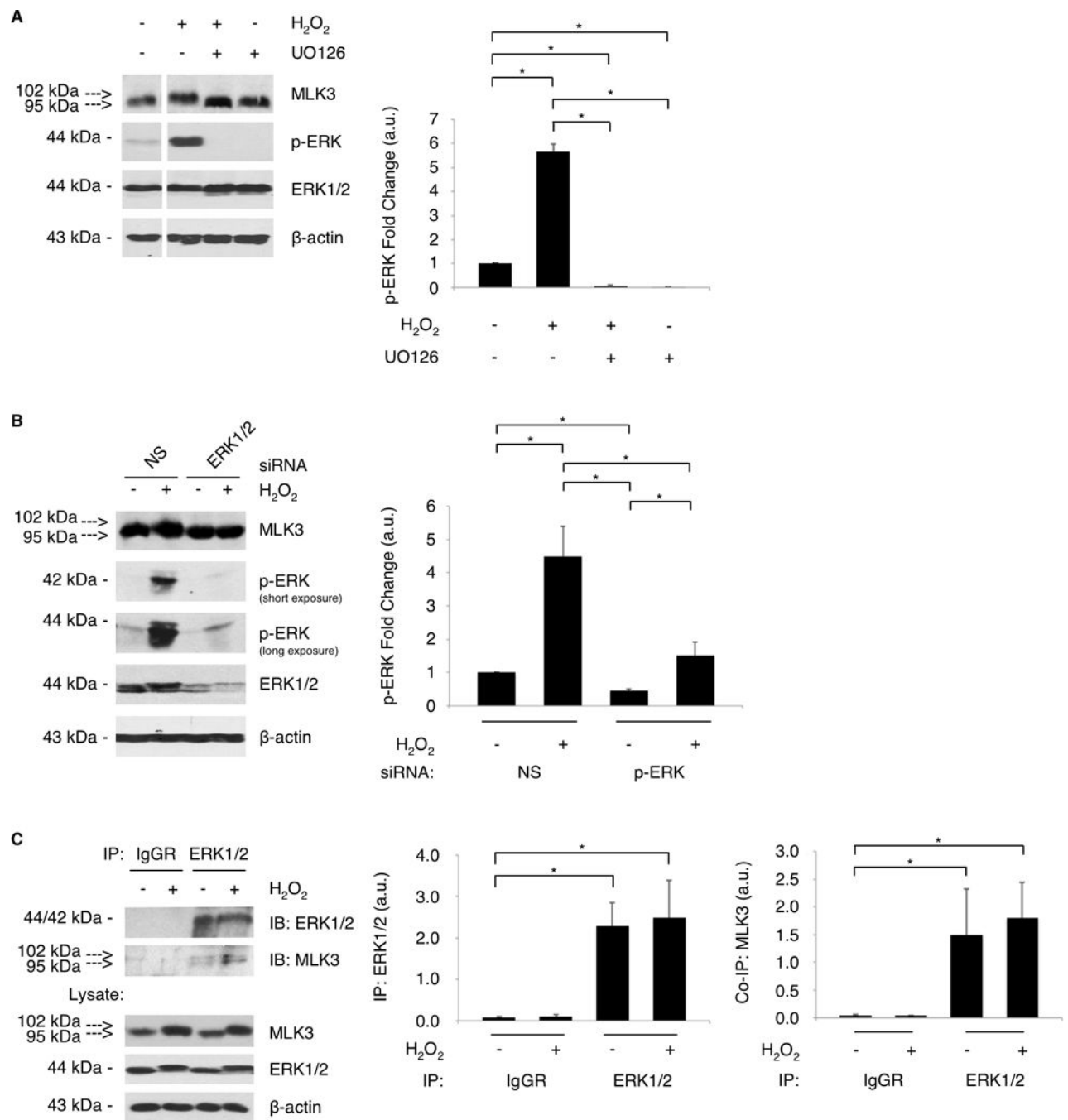
n=3. (D) Western blot analysis of indicated proteins from HCT116 cell lysates pretreated with 100 nM Cep1347 for 1 h followed by 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. n=3.

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**Fig. 2.**

The ROS-induced phosphorylation of MLK3 is dependent on active ERK1/2, and endogenous ERK1/2 and MLK3 are associated in HCT116 cells. (A) Western blot analysis of indicated proteins from HCT116 cell lysates pretreated with 10  $\mu$ M UO126 for 1 h followed by 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. Lanes 1 and 2 are non-contiguous. n=3. (B) Western blot analysis of indicated proteins from HCT116 cells transfected with non-specific (NS) or ERK1/2 siRNA and then treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. n=3. (C) Western blot



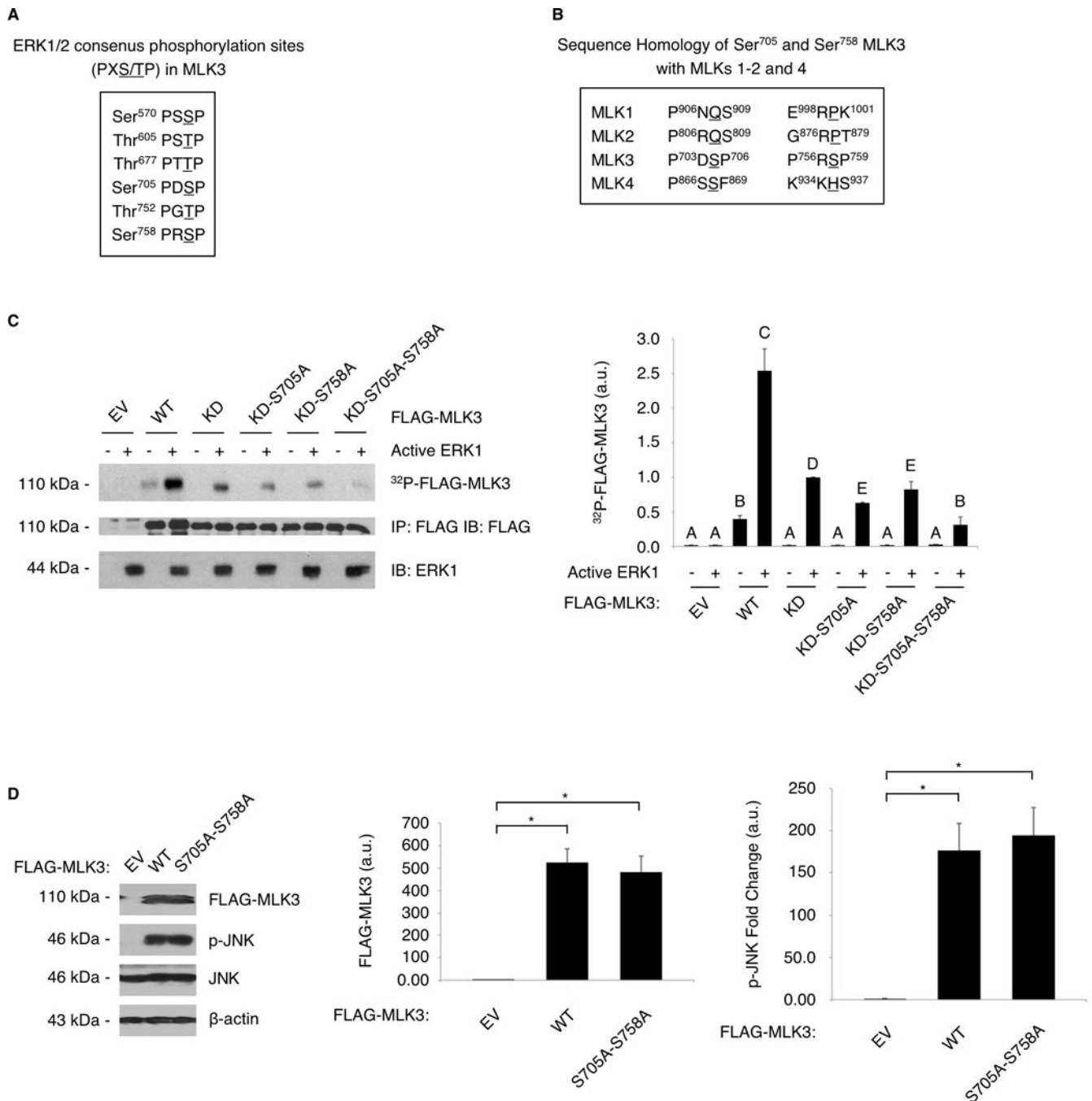
analysis of indicated proteins from HCT116 immunoprecipitates or cell lysates treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. n=3.

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**Fig. 3.** ERK1 phosphorylates Ser<sup>705</sup> and Ser<sup>758</sup> of MLK3 in vitro. (A) Residues in the MLK3 amino acid sequence that conform to the ERK1/2 phosphorylation consensus sequence of PXS/TP. (B) Results of a T-Coffee multiple sequence alignment of the human MLKs showing the sequences of MLKs 1–2 and 4 homologous to Ser<sup>705</sup> and Ser<sup>758</sup> of MLK3. (C) Autoradiography and western blot analysis of ERK1 kinase assays with EV and FLAG-MLK3 (wild-type, K144R, K144R-S705A, K144R-S758A, or K144R-S705A-S758A) substrates. KD=kinase dead=K144R. Different capital letters over the bars indicate statistical

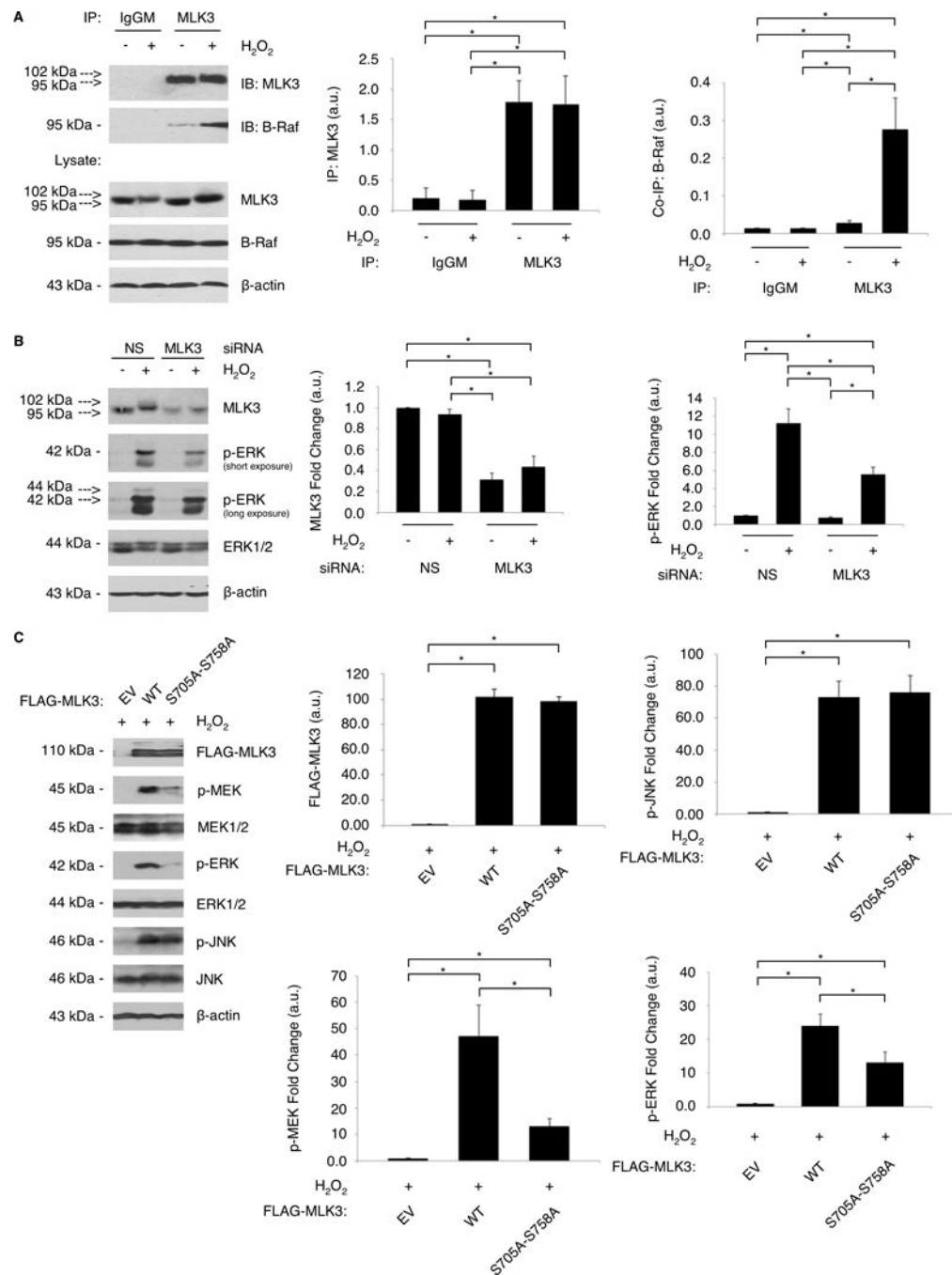
significance, while same letters do not. n=3. (D) Western blot analysis of indicated proteins from HEK293 cell lysates expressing EV, wild-type-FLAG-MLK3, and S705A-S758A-FLAG-MLK3. n=3.

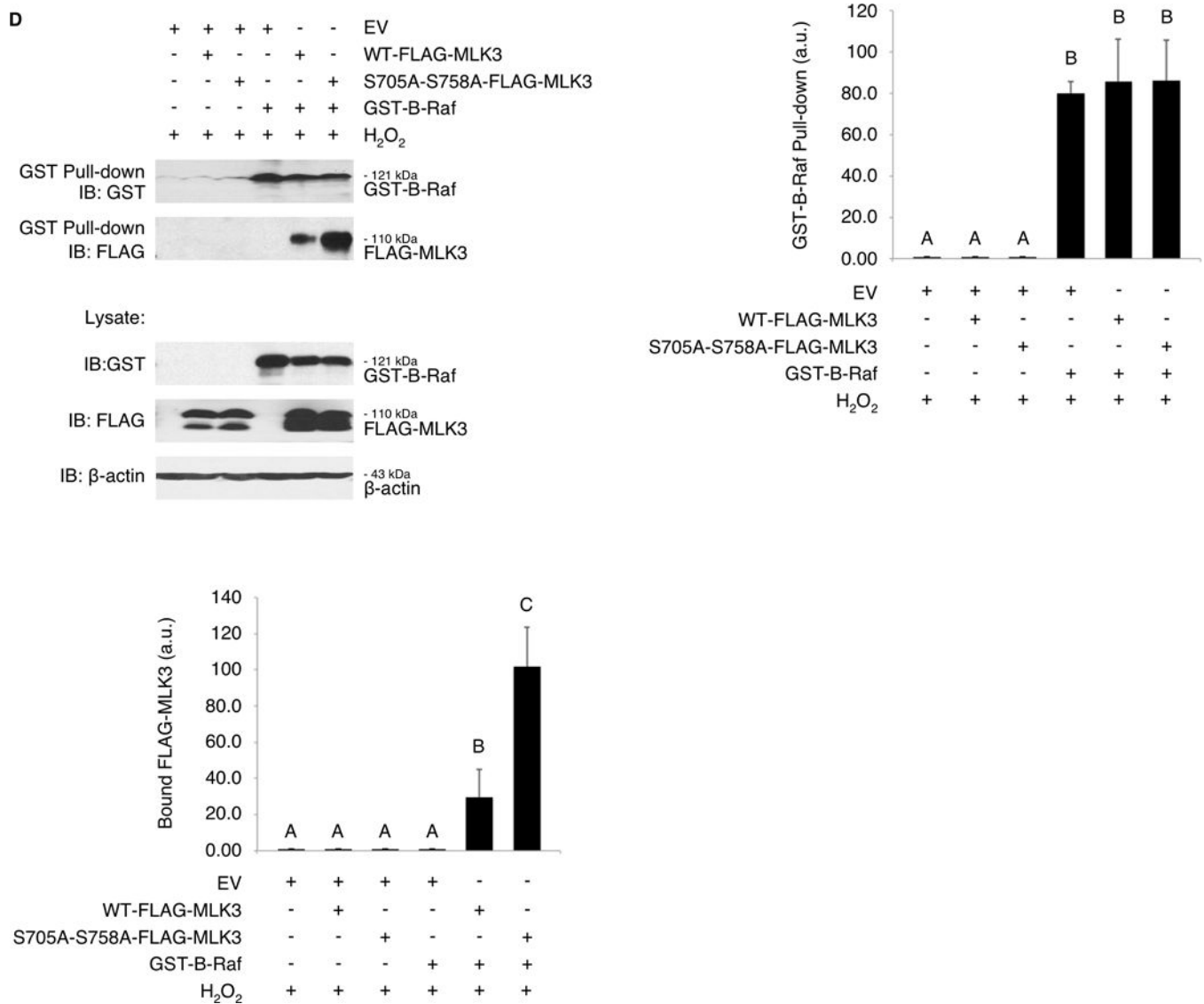
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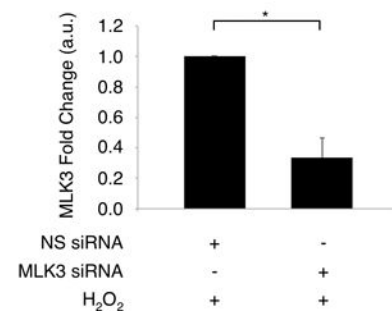
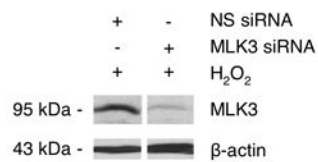
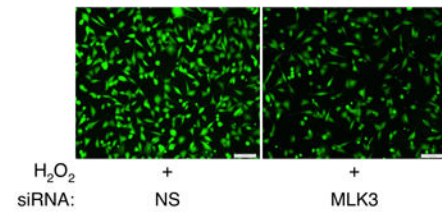
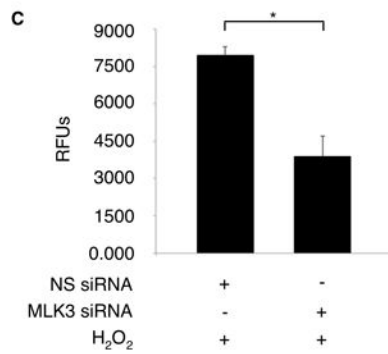
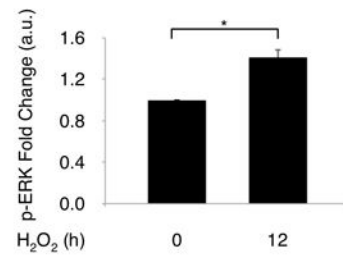
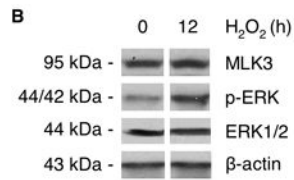
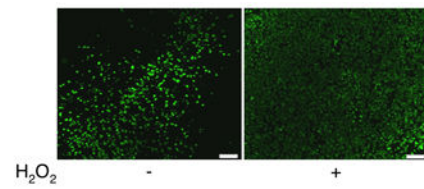
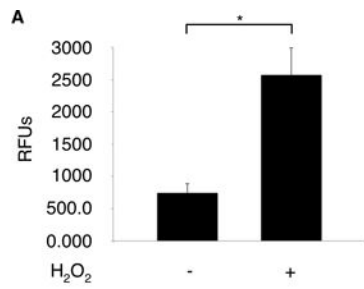
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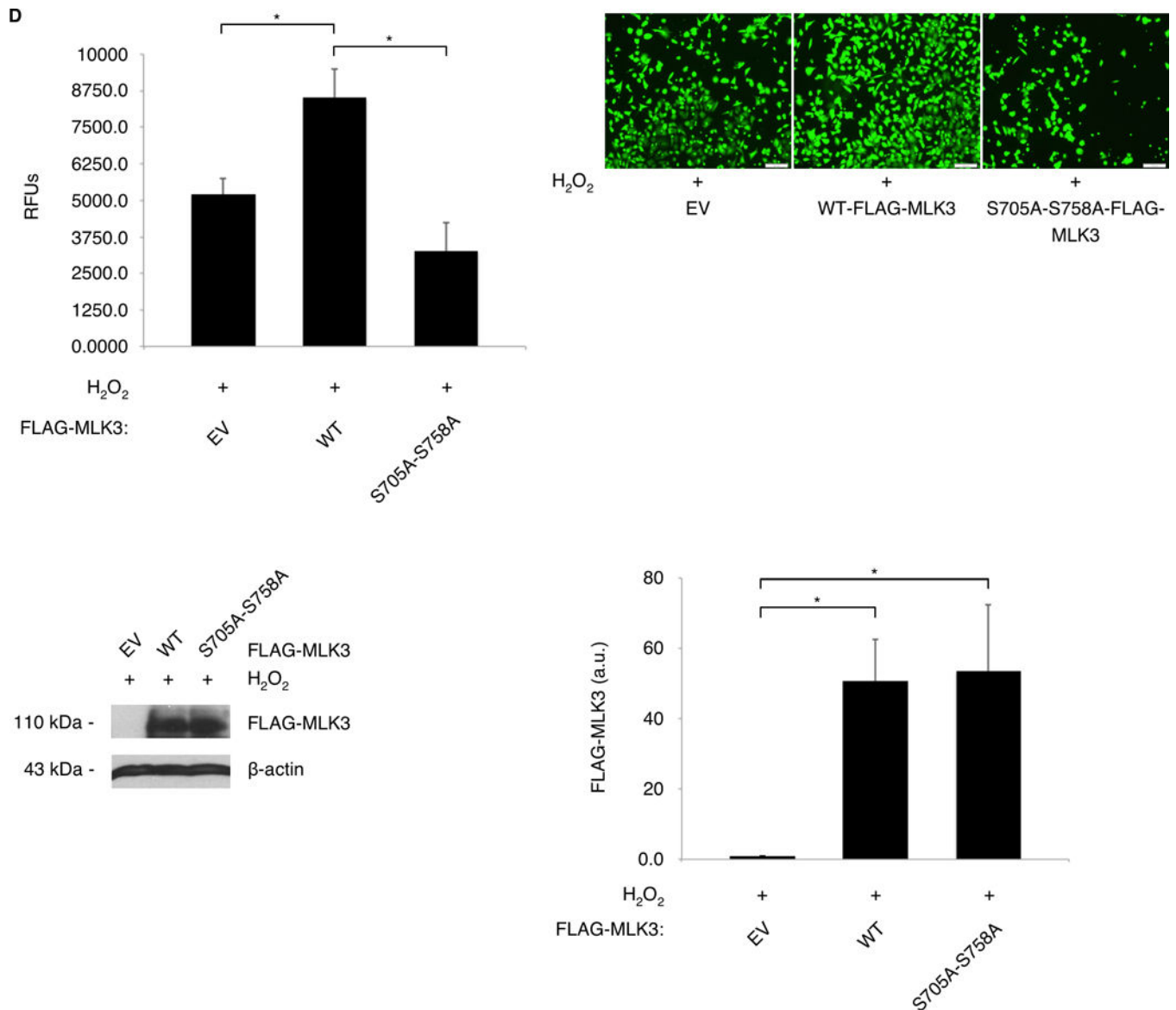
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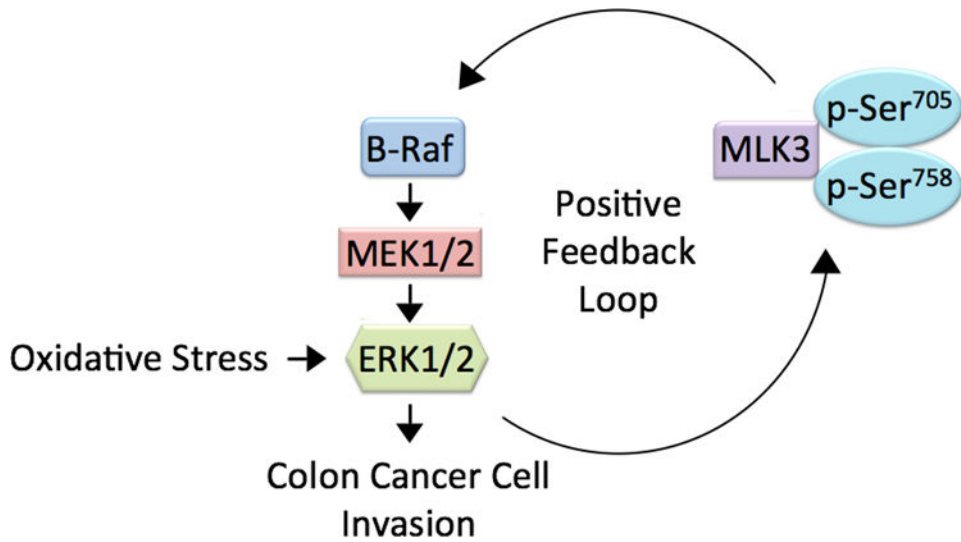
**Fig. 4.**

Oxidative stress promotes association of MLK3 and B-Raf: ROS-induced MEK1/2 and ERK1/2 activation requires MLK3 Ser<sup>705</sup> and Ser<sup>758</sup> phosphorylation. (A) Western blot analysis of indicated proteins from HCT116 immunoprecipitates or cell lysates treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 5 min. n=3. (B) Western blot analysis of indicated proteins from HCT116 cell lysates expressing either NS or MLK3 siRNA and treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. n=3. (C) Western blot analysis of indicated proteins from HEK293 cell lysates expressing EV, wild-type-FLAG-MLK3, and S705A-S758A-FLAG-MLK3 and treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. n=3. (D) Western blot analysis of indicated proteins from HEK293 cells co-expressing different combinations of EV, wild-type-FLAG-MLK3, S705A-S758A-FLAG-MLK3, or GST-B-Raf, treated with 2 mM H<sub>2</sub>O<sub>2</sub> for 30 min, and subjected to a GST pull-down. The amount of pull-down (GST-B-Raf) and bound (FLAG-MLK3) proteins was quantified. Different capital letters over the bars indicate statistical significance, while same letters do not. n=3.





**Fig. 5.** MLK3 promotes oxidative stress-induced invasion of colon cancer cells through a mechanism which requires phosphorylation of Ser<sup>705</sup> and Ser<sup>758</sup>. (A) Cell invasion, assessed by the FluoroBlok invasion assay and fluorescence microscopy, in HCT116 cells treated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 12 h. RFUs=relative fluorescence units. Scale bars represent 200  $\mu$ m. n=3. (B) Western blot analysis of indicated proteins from HCT116 cell lysates treated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 0 or 12 h. Lanes 1 and 2 are non-contiguous. n=3. (C) Cell invasion assessed as in 5A and western blot analysis in HCT116 cells expressing NS or MLK3 siRNA and treated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 12 h. Lanes 1 and 2 of are non-contiguous. Scale bars represent 100  $\mu$ m. n=3. (D) Cell invasion assessed as in 5A and western blot analysis in HCT116 cells expressing EV, wild-type-FLAG-MLK3, or S705A-S758A-FLAG-MLK3 and treated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 12 h. Scale bars represent 100  $\mu$ m. n=3.



**Fig. 6.** Proposed model for MLK3-mediated invasion of colon cancer cells under oxidative stress. Oxidative stress activates ERK1/2, which then phosphorylates MLK3 on Ser<sup>705</sup> and Ser<sup>758</sup>. Phosphorylated MLK3 interacts with and promotes B-Raf activation in a kinase-independent manner, and MLK3 induces further ERK1/2 activation and invasion of colon cancer cells in a positive feedback loop.