A Dissertation

entitled

Mixed Lineage Kinase 3 Signaling in Ovarian Cancer and Neurofibromatosis-2

by

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Submitted to the Graduate Faculty as partial fulfillment of the

requirements for the Doctor of Philosophy degree in Biology

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The University of Toledo

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An Abstract of

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Mixed lineage kinase 3 (MLK3), a 97 kDa Ser/Thr protein kinase, is a MAP kinase kinase kinase (MAP3K) that phosphorylates and activates MAP kinase kinases (MAP2Ks), resulting in the activation of JNK, p38 and ERK MAPK pathways. The Rho GTPases Rac and Cdc42, activate MLK3 via binding to the CRIB domain of MLK3, relieving the SH3 mediated auto-inhibition, and promoting MLK3 auto-phosphorylation and activation. Though the role of MLK3 in tumorigenesis is still under investigation, previous studies have indicated that MLK3 can transform NIH3T3 cells in a MEK dependent manner, and recently, MLK3 expression was found to induce invasion in mammary epithelial cells. In addition, our results indicate higher MLK3 expression and kinase activity in SKOV3, HEY and HEY1B ovarian tumor cells in comparison to immortalized ovarian epithelial T29 and T80 cells. Thus, we hypothesized that MLK3 could promote aberrant MAPK activity and cell invasion in ovarian cancer cells. In our
study, we found that silencing *mlk3* substantially reduced ERK and JNK signaling, cell invasion, and matrix metalloproteinases (MMP)-1, -2, -9 and -12 expression in SKOV3 cells. Thus we propose that MLK3 may promote invasion by up-regulating specific MMP expression in ovarian cancer cells.

The Neurofibromatosis Type 2 (*NF2*) gene encodes a 595 amino acid tumor suppressor protein, merlin, which has homology to the Ezrin, Radixin and Moesin (ERM) group of cellular proteins that link integral membrane proteins to the actin cytoskeleton. Merlin undergoes intramolecular interactions between the N-terminus and C-terminus, and phosphorylation on Ser518 inhibits this interaction and inactivates merlin. Activated merlin inhibits cell proliferation, cell cycle progression and motility, however, a detailed biochemical mechanism for the growth suppressive function of merlin remains elusive. We previously found that merlin is a MLK3 inhibitor, and we wished to investigate if MLK3 is required for merlin-mediated suppression of cell growth, invasion and MAPK signaling in ovarian and NF2 tumor cells.

Comparison of different human cell lines revealed high basal MLK3 activity in HEI193, SKOV3 and NCI-H460 cell lines. Interestingly, there was a correlation between high MLK3 activity and reduced merlin expression in the tumor cell lines. The carboxyl-terminus of merlin (340-590) is required for merlin-MLK3 interaction, and is sufficient to inhibit MLK3 kinase activity. Induction of merlin protein expression in RT4-NF2.17 cells (rat schwannoma cells with Tet-inducible merlin) inhibited MLK3, B-Raf, ERK and JNK activities. In contrast, depletion of merlin elevated MLK3, B-Raf,
ERK and JNK activities. Merlin expression blocked the interaction between MLK3 and the upstream activator, Rho GTPase, Cdc42. In addition, MLK3 was required for proliferation and invasion of cells that lacked functional merlin.

Our data suggests that merlin inhibits MLK3 by blocking the cdc42-MLK3 interaction, and that elevated basal MLK3 activity in NF2, ovarian and other tumor cells may be due to the loss of functional merlin in these cells.
DEDICATION

This dissertation is dedicated to my parents
Duan, Rongzhen and Zhan, Zhishan
for their sacrifices and love
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Firstly, I would like to thank my advisor, Dr. Deborah N. Chadee, whose guidance and advices make this dissertation possible. She has always been encouraging me to think independently and thoroughly, and discussing research with me. She helped me not only with my research, but also in my life. It is one of the luckiest things in my life that I have the opportunity to conduct exciting research under such a great advisor and under this wonderful environment. Also, I want to express my special thanks to Dr. Douglas Leaman, Dr. Fan Dong and Dr. Richard Komuniecki for giving me excellent supervision, advices and scientific training. I am appreciated all the helps from my other committee members, Dr. Ivana de la Serna, Dr. William A. Maltese, and to Dr. William Taylor, for their precious time and suggestions towards my dissertation research.

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<th>Description</th>
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<tbody>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42/Rac interactive binding</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DLKs</td>
<td>Dual leucine zipper-bearing kinases</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin, Radixin and Moesin</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGF receptor</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor binding protein 2</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HRS</td>
<td>Hepatocyte growth factor-regulated tyrosine kinase substrate</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factors</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>JIP</td>
<td>JNK-interacting protein</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KIU</td>
<td>Kallekrin inhibitory units</td>
</tr>
<tr>
<td>KSR</td>
<td>Kinase suppressor of Ras</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAP3K</td>
<td>MAPK kinase kinase</td>
</tr>
<tr>
<td>MAP2K</td>
<td>MAPK kinases</td>
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<tr>
<td>MEKK1</td>
<td>MEK kinase 1</td>
</tr>
<tr>
<td>MLK</td>
<td>Mixed lineage kinase</td>
</tr>
<tr>
<td>MLTKα</td>
<td>Mitogen-activated protein triple kinase alpha</td>
</tr>
<tr>
<td>MP1</td>
<td>MEK partner 1</td>
</tr>
<tr>
<td>MORG 1</td>
<td>MAPK organizer 1</td>
</tr>
<tr>
<td>MKPs</td>
<td>MAPK phosphatases</td>
</tr>
<tr>
<td>MSK</td>
<td>Mitogen and stress activated kinase</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MNK</td>
<td>MAPK interacting kinase</td>
</tr>
<tr>
<td>NF2</td>
<td>Neurofibromatosis Type 2</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethane sulphonyl fluoride</td>
</tr>
<tr>
<td>POSH</td>
<td>Plenty of SH3 scaffolds</td>
</tr>
</tbody>
</table>

POSH

Plenty of SH3 scaffolds
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>PVDF</td>
<td>Immobilon-P Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RSK</td>
<td>protein kinase p90 ribosomal S6 kinase</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress activated protein kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology 3</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>ZAKs</td>
<td>Zipper-sterile-alpha motif kinases</td>
</tr>
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1. INTRODUCTION

1.1. Mitogen and Stress-Activated Protein Kinase Pathways

The mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved cascades that transduce signals from extracellular stimuli to intracellular responses and regulate cell proliferation, differentiation, development, inflammation and apoptosis (Dhanasekaran and Johnson, 2007; Kyriakis et al., 2004). In response to multiple external stimuli, such as growth factors, proinflammatory cytokines and various stresses, MAPK kinase kinases (MAP3Ks), are phosphorylated and activated. Activated MAP3Ks then relay signals to specific MAPK kinases (MAP2Ks) by phosphorylating MAP2Ks on serine or threonine residues within the Ser-X-X-X-Ser/Thr motif (Cuevas et al., 2007; Doza et al., 1995; Payne et al., 1991; Zhang et al., 1995). MAP2Ks phosphorylate MAPKs on threonine and tyrosine residues within the Thr-X-Tyr motif, which results in MAPK activation (Cuevas et al., 2007; Doza et al., 1995; Payne et al., 1991; Zhang et al., 1995). Active MAPKs then either phosphorylate downstream cytoplasmic effectors or translocate to the nucleus and activate transcription factors to regulate gene expression (Avruch et al., 2001; Chang and Karin, 2001; Kyriakis and Avruch, 2001; Raman et al., 2007) (Figure 1).
Figure 1. ERK MAPK signaling pathway. Epidermal Growth factor binding to the EGF receptor, results in recruitment of Grb2 and SOS, and stimulation of Ras activation. Active Ras binds to B-Raf. B-Raf becomes activated and phosphorylates MEK. MEK, in turn, phosphorylates and activates ERK. Active ERK phosphorylates cytoplasmic effectors or transcription factors resulting in transcriptional activation of gene expression.
1.2. Activation of MAPK Signaling by Extracellular Stimuli

Three well studied MAPK signaling pathways in cells are: the extracellular signal-regulated kinase (ERK1/2 or p44/p42), the stress activated protein kinase (SAPK) or c-Jun N-terminal kinases (JNK1/2/3), and the p38 kinase (α/β/γ/δ) (Pearson et al., 2001). Upon exposure to specific external stimuli, cells elicit activation of specific MAPK cascades and channel MAPK signaling toward specific physiological responses. For instance, the ERK MAPK signaling pathway is predominantly activated by mitogens, such as epidermal growth factor (EGF) and fibroblast growth factor (FGF), whereas the JNK and p38 MAP kinase signaling pathways are mostly stimulated by cytokines and various stresses, such as osmotic shock, UV radiation, or oxidative stress (Kyriakis and Avruch, 2001; Robinson and Cobb, 1997). However, in response to EGF, other MAPK pathways in addition to ERK can also be activated, which is a result of recruitment of different downstream MAP3Ks. MAP2Ks are dual specificity kinases that phosphorylate MAPK substrates on serine/threonine and tyrosine residues (Ashworth et al., 1992). This dual specificity results in accumulation of inactive tyrosine-phosphorylated proteins, and subsequent phosphorylation of Thr residues of MAPK, leads to MAPK full activation (Ferrell, 1997; Prowse and Lew, 2001). Active MAPKs phosphorylate downstream effectors, such as cytoplasmic kinases, which in turn translocate to the nucleus and phosphorylate transcription factors (Lewis et al., 1998). In addition, MAPKs themselves can translocate to the nucleus and directly phosphorylate
transcription factors and regulate gene expression (Chen et al., 1992) (Figure 1).

An example of classical MAPK signaling is the ERK MAPK pathway activation by EGF. EGF undergoes dimerization, and binds to the extracellular surface of the transmembrane domain of the EGF receptor (EGFR), a receptor tyrosine kinase (RTK). This binding actually precedes RTK dimerization and causes RTK transphosphorylation on the cytoplasmic domains, which also further stimulates intrinsic kinase activity and transphosphorylation on tyrosine residues. Phosphorylated tyrosine residues on the receptor tyrosine kinases recruit adaptor proteins, such as growth factor receptor binding protein 2 (Grb2). Grb2 contains a Src homology 2 (SH2) domain and a SRC-homology-3 (SH3) domain, which interacts with the phosphotyrosine residues of the RTK and son of sevenless (SOS), a guanine nucleotide exchange factor (GEF), respectively. The SOS protein promotes the small GTPase, Ras, to switch from a GDP-bound, inactive form to a GTP-bound, active form (Downward, 2003). Activated Ras interacts with the MAP3K Raf, and this leads to Raf phosphorylation and activation. Raf phosphorylates and activates MEK1/2, and activated MEK phosphorylates and activates ERK (Buday and Downward, 1993a, b; Chadee and Kyriakis, 2004a).

ERK1/2, in response to mitogenic stimuli, phosphorylate cytoplasmic targets or translocate to the nucleus and activate transcription factors that regulate gene expression (Chen et al., 2001). Cytoplasmic targets of ERK1/2 include mitogen and stress activated kinase (MSK), MAPK interacting kinase (MNK) and p90 ribosomal S6 kinase (RSK). ERK1/2 can also translocate to the nucleus and activate specific
transcription factors, such as Elk-1, c-Fos and c-Myc (Raman et al., 2007).

In contrast to ERK1/2, JNK/SAPKs are predominantly stimulated by inflammatory cytokines and environmental stresses such as heat shock, oxidative stress and UV radiation (Kyriakis et al., 2004). As inferred by the name ‘c-Jun N-terminal kinases’, active JNK, in most cases, translocates to the nucleus and phosphorylates the N-terminus of the transcription factor, c-Jun, which leads to actin reorganization, cell transformation, cytokine production, and stress-induced and developmentally programmed apoptosis (Chen et al., 2001).

The activity of p38α/β/γ/δ MAP kinase is stimulated by various stressors including UV radiation, proinflammatory cytokines, osmotic stress, hypoxia, and hematopoietic growth factors (Chadee and Kyriakis, 2004b; Cowley et al., 1994; Kallunki et al., 1994; Minden et al., 1994; Wilkinson and Millar, 2000). Once active, p38 proteins can translocate to the nucleus where they phosphorylate serine/threonine residues on substrates, such as cyclin D3, p53 and ATF-2, thus, affecting cell cycle progression, apoptosis and gene transcription (Casanovas et al., 2004; Sanchez-Prieto et al., 2000; van Dam et al., 1995).

The duration of MAPK activation is usually transient and highly regulated. Cytoplasmic and nuclear MAPK phosphatases (MKPs) regulate the level of active MAPK in cells by dephosphorylating MAPKs on Thr/Tyr phosphoacceptor sites. There are ten MKPs in mammalian cells, each of which dephosphorylates a specific MAPK, and thereby negatively regulates MAPK signaling and gene expression (Owens and
MAPK signals are also regulated by their interaction with scaffold proteins that bind to members of the MAPK-MAP2K-MAP3K signaling cascades, and provide signal specificity and limit 'signal drifting' from one MAPK cascade to another (Leung and Lassam, 2001; Xu et al., 2003). ERK scaffold proteins include β-arrestin, kinase suppressor of Ras (KSR), MEK partner 1 (MP1), MAPK organizer 1 (MORG 1) and MEK kinase 1 (MEKK1), whereas JNK pathway scaffolds include the JNK-interacting protein (JIP) family and plenty of SH3 scaffolds (POSH) (Dhanasekaran et al., 2007). Some MAP2Ks and MAP3Ks also possess both scaffold function and kinase activity. For example, MLK3 functions as a scaffold protein for ERK MAPK and a protein kinase that phosphorylates and activates MKK4/7 and MKK3/6 which, in turn, activates SAPK/JNK and p38 MAPKs respectively (Chadee and Kyriakis, 2004b; Chadee et al., 2006; Gallo and Johnson, 2002; Rana et al., 1996; Tibbles et al., 1996). MEKK1 is another dual function MAP3K in ERK1/2 signaling pathway. As a scaffold protein, MEKK1 associates with Raf-1, MEK1 and ERK2, and activates ERK2; and as a MAP3K, MEKK1 can phosphorylate MEK1 and MEK2 (Dhanasekaran et al., 2007).

Additionally, MAPK signaling is also regulated by feedback loop mechanisms. An activated MAPK can phosphorylate and inhibit MAP3K activity and block MAPK signaling (review in Dhanasekaran and Johnson, 2007). For example, JNK, which is activated by MLK3, can phosphorylate and inactivate MLK3 (Schachter et al., 2006). Interestingly, ERK, the downstream effector of B-Raf, can phosphorylate B-Raf, and
negatively regulate B-Raf activity (Brummer et al., 2003).

In summary, the activity of MAPKs and the duration of their activation are regulated by phosphatases, scaffolds proteins and feedback loop mechanisms. Together, these mechanisms function to turn MAPKs pathways ‘on’ and ‘off’, and ensure tight regulation of MAPK signaling.

Ras and Rho small GTPases can activate MAPK signaling pathways by activating MAP3Ks. In mammals, the Rho GTPase family includes three subfamilies: Cdc42 (Cdc42Hs and G25k), Rac (1, 2 and 3 isoforms) and Rho (A, B and C isoforms) (Jaffe and Hall, 2005). Different effectors interact with the Rho GTPases to regulate cellular responses, such as reorganization of the actin cytoskeleton. Activation of Rho regulates stress fiber and focal adhesion formation; Rac induces actin polymerization and lamelllopodia formation, and Cdc42 causes filopodia formation (Hall, 1998). For example, the direct interaction between small-GTPases (K-Ras, N-Ras and H-Ras) and the Raf MAP3Ks (Raf-1, A-Raf and B-Raf), recruits Rafs to the plasma membrane, a critical event that results in Raf phosphorylation and activation (Mercer and Pritchard, 2003)

1.3. Mutations of the Ras/MAPK pathway in cancer

MAPK signaling is critical for biological response, such as proliferation, survival, inflammation, and metabolism, and deregulated MAPK activity is associated with a number of different pathological states, including cancer (Gollob et al., 2006).
To date, one of the best characterized signaling pathways that are activated by Ras is the Ras/Raf/MEK/ERK pathway. The conserved Ras-activated protein kinase cascade Ras/Raf/MEK/ERK regulates cell growth, proliferation, and differentiation in response to growth factors, cytokines, and hormones (Robinson and Cobb, 1997).

There are three members of the Ras family: H-Ras, K-Ras and N-Ras, which are closely related. H-Ras, K-Ras and N-Ras are all widely expressed, and K-Ras is expressed in almost all cell types (Downward, 2003). Activating mutations of these Ras family members are frequently found in human tumors (Downward, 2003). RAS mutations have been found in approximately 20% of all tumors, including 90% of pancreatic and 45% of colorectal cancers and 50% of colon carcinomas (Roberts and Der, 2007). Almost all RAS activation in tumors is caused by mutations in codons 12, 13 and 61 (Bos, 1989), and these activating point mutations in RAS are most frequently found in KRAS (about 85% of total), but less frequently found in NRAS (about 15%) and HRAS (less than 1%) (Downward, 2003). In most cases, these mutation results in hyperactivated forms of Ras, which constitutively activate downstream pathways, enhance transcription and accelerate cell cycle progression (Bos, 1989; Downward, 2003).

Most solid colon, lung and pancreatic tumors with activated Ras and also display elevated levels of phosphorylated ERK (Hoshino et al., 1999). Specifically, the Ras proteins K-Ras, N-Ras and H-Ras, directly interact with the three Raf MAP3Ks, Raf1, A-Raf and B-Raf (Kolch, 2000). This interaction is mediated by an N-terminal Ras binding domain in the Rafs that facilitates their recruitment to the plasma membrane.
where phosphorylation by multiple kinases contributes to their full enzymatic activation (Mercer and Pritchard, 2003). Raf was the first downstream effector of RAS to be identified, and MEK1/2 are the only commonly accepted downstream substrates for the Raf proteins (Avruch et al., 2001; Malumbres and Barbacid, 2003; Marais and Marshall, 1996; Marshall, 1994). Activated Rafs phosphorylate and activate the MAP2Ks, MEK1 and 2, which in turn, phosphorylate and activate the MAPKs, ERK1 and 2 (Shaul and Seger, 2007).

There are three common Raf proteins in human: A-Raf, B-Raf, and C-Raf (Raf-1) (Li et al., 2001; Zhang and Guan, 2000). B-Raf has a much higher basal kinase activity than either A-Raf or C-Raf (Mason et al., 1999). Raf proteins are not only effectors of oncogenic Ras but also have a high frequency of mutations in human cancer. Screening of tumor samples indentified the V600E (T–A change at nucleotide 1797) mutations of the B-Raf in approximately 70% of human malignant melanomas and 15% of colorectal cancers (Davies et al., 2002; Roberts and Der, 2007). However, this mutation was detected at a low frequency in other tumor cancers, such as breast cancers, gliomas, liver cancers, lung cancers, sarcomas and ovarian carcinomas (Davies et al., 2002). It has been shown that 98% of the B-Raf mutations are present in the activation domain, 92% of which were found to be the V600E mutation. In contrast, Raf-1 constitutive activation has also been observed, though not as high as B-Raf, in some cancer samples (Berger et al., 1997). This mutation causes enhanced proliferation in a Ras-independent fashion in multiple cancers (i.e. melanoma) (Davies et al., 2002). Interestingly, MLK3 was found to
be required for mitogen induced B-Raf activation and cell proliferation. However, loss of MLK3 does not affect the proliferation of cells that have a V600E mutation.

1.4. Mixed Lineage Kinase 3 (MLK3)

The mixed lineage kinase (MLK) family are a group of proteins that have sequence homology to both tyrosine kinases and serine/threonine kinases, thus the name "mixed lineage". However, it was later determined that they are indeed Ser/Thr kinases. The MLK family consists of seven members, which have all been shown to activate JNK (Gallo and Johnson, 2002). The three subfamilies of the MLK family are the dual leucine zipper-bearing kinases (DLKs), the MLKs and the zipper-sterile-alpha motif kinases (ZAKs) (Figure 2) (Gallo and Johnson, 2002).

MLK1, MLK2, MLK3 and MLK4 are all belong to the MLK subfamily (Gallo and Johnson, 2002). MLK subfamily members contain an SH3 domain at the N-terminus, which is followed by a kinase domain, dual leucine zipper, Cdc42/Rac interactive binding (CRIB) domain, and a proline rich C-terminus (Figure 2). The C-terminus of MLK subfamily members varies, but all are proline-rich, and specifically, MLK3 has a Gly-pro-rich amino terminus that is absent from some other MLK subfamily members (Gallo and Johnson, 2002).
Figure 2. The mixed lineage kinase (MLK) family (adapted from Gallo and Johnson, 2002). The MLK family has three subfamilies: the MLKs, the DLKs and the ZAKs. All the MLKs have an SH3 domain and a CRIB domain, while the DLKs and the ZAKs lack these domains. The DLKs have two leucine-zipper domains and ZAKα has a SAM domain.

MLK3 kinase activity is regulated by autophosphorylation. The sequence TTXXS on residues 277-281 in the activation loop contains the autophosphorylation sites, T277 and S281, that are required for MLK3 activity (Leung and Lassam, 2001). Without stimulation, MLK3 is autoinhibited through an interaction between the N-terminal SH3 domain and the proline residue 495, causing a ‘closed’ structure, which prevents MLK3 oligomerization and autophosphorylation (Zhang and Gallo, 2001). Leucine zippers facilitate MLK3 dimerization by forming stabilized coiled
coils, and deletion of the leucine zipper domain will prevent MLK3 dimerization and autophosphorylation (Leung and Lassam, 1998).

The MLKs are important effectors of the Rho GTPases Rac and Cdc42 and potential physiological targets (Teramoto et al., 1996; Zhang and Gallo, 2001). Rac and Cdc42 interact with the CRIB domain and promote MLK3 autophosphorylation and activation (Du et al., 2005; Gallo and Johnson, 2002; Teramoto et al., 1996; Vacratsis and Gallo, 2000; Zhang and Gallo, 2001). Specifically, binding of Cdc42 to the CRIB domain of MLK3 blocks the N-terminal interaction with the pro495 residue, which relieves the autoinhibition and promotes autophosphorylation and activation of the enzyme. Cdc42 also relocalizes MLK3 to the plasma membrane (Du et al., 2005). It has also been shown that MLK3 kinase activity is negatively regulated by Akt phosphorylation on Ser674 and this interaction is regulated by insulin (Barthwal et al., 2003).

In response to mitogenic and stressful stimuli, MLK3 becomes phosphorylated and activates MKK4/7 and MKK3/6 which, in turn, activate SAPK/JNK and p38 MAPKs respectively (Gallo and Johnson, 2002; Rana et al., 1996; Tibbles et al., 1996). MLK3 also phosphorylates and activates, to a lesser extent, the MAP2K, MEK1, which is a direct upstream activator of the ERK MAPK (Hartkamp et al., 1999).

1.5. MLK3 signaling in cancer

MLK3 is required for mitogen activation of B-Raf, ERK and cell proliferation in
normal lung and colon fibroblasts and in specific tumor cells (Chadee and Kyriakis, 2004a). Furthermore, stable expression of wild-type MLK3 can transform NIH3T3 cells in a MEK-dependent manner (Hartkamp et al., 1999), whereas stable expression of mixed lineage kinase-like mitogen-activated protein triple kinase alpha (MLTKα), another MLK family member, can transform B6 C141 skin spidermal cells leading to fibrosarcoma formation in nude mice (Cho et al., 2004). Recently, MLK3 missense mutations with transforming capacity in vitro have been identified in gastric tumors (Velho et al., 2010). In addition, other recent reports indicate a requirement for MLK3 in invasion of mammary epithelial cells and migration of gastric tumor cells and A459 lung cells (Chen et al., 2010a; Mishra et al., 2010; Swenson-Fields et al., 2008). Collectively, these findings suggest that MLK3 has oncogenic potential, however, the role of MLK3 in tumorigenesis remains to be determined.

1.6. Matrix metalloproteinases (MMPs)

Cells grow in a microenvironment that includes extracellular matrix (ECM), growth factors and cytokines. In the dynamic matrix, cells interact with each other, and with the ECM. Matrix metalloproteinases (MMPs) are groups of zinc-dependent endoproteinases, which are implicated in a variety of physiological and pathological processes, including wound healing, uterine involution, inflammatory and carcinogenesis (Nagase et al., 2006; Page-McCaw et al., 2007; Parks et al., 2004). The MMPs function
to degrade components of the extracellular matrix (ECM), thus resulting in remodeling extracellular matrix, which is a critical event in cancer development (Kessenbrock et al., 2010).

1.6.1. MMP family

More than 20 MMP family members expressed in human have been categorized by their structures. In general, almost all the MMPs have three conserved domains: the pro-peptide with a cysteine residue, the catalytic domain with metal-binding sites, and the C-terminal zinc-binding active site (Figure 3) (Kleiner and Stetler-Stevenson, 1999). Most of the human MMPs have a signal peptide sequence (except MMP-17), and a C-terminal domain with homology to the protein hemopexin (except MMP-7). The structural blueprint of MMPs is shown in Figure 3 (Chakraborti et al., 2003; Nagase et al., 2006). Structurally, there are two subclasses of MMPs. The gelatinases (MMP-2 and MMP-9) have a gelatin-binding domain, and their activity can be analyzed by gel zymography. The other group of MMPs, which includes MMP-14, -15, -16, and -17, are membrane-associated MMPs which have transmembrane domains at the C-terminus (Kleiner and Stetler-Stevenson, 1999).
Figure 3. The domain structure of MMPs (adapted from (Chakraborti et al., 2003). S-signal peptide; P-propeptide; C-catalytic domain; F-fibronectin type II domain; L-linkage domain; H-hemopexin like domain; FR-furin recognition site; T-transmembrane domain.

1.6.2. Roles of MMPs in cancer progression

Migration, invasion, metastasis and angiogenesis are hallmarks for cancer development (Hanahan and Weinberg, 2000). The ability to degrade the basement membrane was correlated to the metastatic potential of cancers (Hanahan and Weinberg, 2000). In order for cancer cells to expand locally or invasion to a distant location, they require proteolytic enzyme activity to degrade the physical barriers of ECM. MMPs have the ability to degrade the basement membrane, which is considered to be one of the critical processes for tumor cell invasion (Liootta et al., 1980). For example, MMP-2 and
MMP-9 promote invasion by degrading a variety of ECM components (Weaver, 2006).

The MMPs have different roles during cancer progression. In general, they promote or inhibit cancer development and metastatic process. For example, MMP-8 has been found frequently mutated in melanomas, suggesting a protective role of MMP-8 in melanomas (Lopez-Otin et al., 2009; Palavalli et al., 2009).

MMPs also affect cells in many other ways, including proliferation, apoptosis and angiogenesis (Gialeli et al., 2011). It has been reported that MMPs can affect cell proliferation by modulating the binding of growth factors and cell surface receptors. In particular, MMPs can release growth factors by cleaving them from cell membrane precursors, such as insulin-like growth factors (IGFs) and the epidermal growth factor receptor (EGFR) ligands, which will promote proliferation (Nakamura et al., 2005). MMPs also affect apoptosis by inactivating apoptosis signal receptors. For example, MMP-7 can cleave Fas ligand, a transmembrane stimulator of the death receptor Fas, and thus transmit anti-apoptotic signals to cancer cells, and cause cancer cells resistance to apoptosis and chemotherapies (Strand et al., 2004). In addition, MMPs are found to be regulators of angiogenesis. MMP-1, MMP-2, MMP-7, MMP-9 and MMP-14 specifically participate in tumor angiogenesis (Gialeli et al., 2011). Due to their role in cancer development, cell growth, inflammation, and angiogenesis, MMPs have been viewed as potential biomarkers in different types and stages of cancer (Kessenbrock et al., 2010; Roy et al., 2009).
1.7. Neurofibromatosis Type 2 (NF2)

NF2 is an autosomal dominant disorder of the nervous system that occurs in about 1 of 30,000 individuals (Gusella et al., 1999; McClatchey, 2003). Patients with NF2 develop meningiomas, as well as bilateral vestibular schwannomas that arise due to loss of function mutations or deletions of the NF2 gene (Reed and Gutmann, 2001). Merlin is the protein product of the NF2 gene (Rouleau et al., 1993; Trofatter et al., 1993). Loss of function mutations or deletions in NF2 produce nonfunctional or reduced levels of merlin, and are associated with the development of the disease (Gutmann et al., 2001b; Horiguchi et al., 2008; Poulakakos et al., 2006; Thurneysen et al., 2009). Interestingly, some kinds of mutations, such as deletions are associated with more severe disease and other types of mutations such as point mutations are associated with milder disease (Gutmann et al., 2001b). Understanding the biochemical and biological functions of merlin will be critical for the development of novel therapies for patients with NF2.

1.7.1. Merlin

Merlin belongs to Protein 4.1 superfamily and has homology with the Ezrin, Radixin and Moesin (ERM) group of cellular proteins (Ramesh, 2004; Sun et al., 2002). Merlin, a 595 amino acid protein, contains a FERM domain (1-302) at the N-terminus, a central α-helical domain (303-478), and a C-terminal domain (479-595) (Figure 4) (Sun et al., 2002). The N-terminal domain is required for merlin membrane localization, and the C-terminal domain fragment can associate with the N-terminal domain, resulting in
merlin activation and subcellular localization (Scoles, 2008).

ERM proteins bind actin through an extended structure at the C-terminus and link integral membrane proteins to the actin cytoskeleton (Ramesh, 2004; Sun et al., 2002). Merlin is associated with the cytoskeleton, and the FERM domain facilitates the interaction with membrane-binding partners, such as CD44 and other ERM proteins (McClatchey, 2003; Ramesh, 2004). However, merlin lacks the C-terminal extended structure that is necessary for actin cytoskeleton binding, and therefore may interact indirectly with cytoskeletal proteins via an interaction with other ERM proteins (McClatchey, 2003). Importantly, an intermolecular association between the N-terminus and the C-terminus is required for the tumor suppressor function of merlin.
1.7.2. Inactivating mutations in NF2

Eight naturally occurring non-conservative missense mutations in merlin have been identified thus far and are characterized for their growth suppressive function and ability to disrupt actin cytoskeleton-mediated events (Gutmann et al., 2001b). Gutmann et al. (2001a) demonstrated that three mutations at the highly conserved N-terminus (L64P, K79E and E106G) and the two mutations in the unique C-terminus (L535P and L538P) impaired merlin suppression of cell proliferation, anchorage-independent growth and actin cytoskeleton organization functions (Figure 5). The three mutations within the predicted α-helical domain (Q324L, T352M and K413E) retained structural and functional properties observed with wild-type merlin (Gutmann et al., 2001b).

Figure 4. Structure of merlin. Merlin has a FERM domain at the N-terminus, and a central α-helical domain. The phosphorylation at the residue S518 affects merlin activity and localization. FERM domain is involved in membrane interaction.
Patients with NF2 develop nervous system tumors, including schwannomas, meningiomas, and ependymomas (Mota et al., 2001). In addition, mutations and loss of merlin expression have also been found in other types of cancers (Horiguchi et al., 2008; Thurneysen et al., 2009). For example, NF2 mutations, including deletions and insertions that lead to truncated and inactivated merlin, have been found in 40% of mesotheliomas (Baser et al., 2002; Baser et al., 2005; Bianchi et al., 1995; Cheng et al., 1999; Poulakakos et al., 2006; Robinson et al., 2005; Schipper et al., 2003; Sekido et al., 1995; Thurneysen et al., 2009). Merlin has also been shown to be inactive in DU145 prostate cancer cells (Horiguchi et al., 2008). Moreover, it has been reported that merlin is a potent growth inhibitor of malignant gliomas. Merlin expression is reduced in human gliomas and reexpression of functional merlin inhibits glioma cell proliferation (Lau et al., 2008). The functional significance of merlin inactivation in other cancers remains to be determined.
Figure 5. Naturally occurring mutations in merlin in NF2 patients (adapted from (Gutmann et al., 2001b)). Eight mutations occurring in NF2 patients have been identified. Three of them are at the N-terminal FERM domain, while three of them are at the central α-helical domain, and two of them are locate at the C-terminus of merlin.

1.7.3. Phosphorylation of merlin

Phosphorylation of merlin on Ser518 plays a critical role in controlling merlin intramolecular interactions, binding to its effector proteins, and regulation of cell growth (Rong et al., 2004; Surace et al., 2004). The Ser518 phosphorylated form of merlin is inactive, with respect to its growth suppressive activity, and fails to form productive intramolecular and intermolecular interactions (Rong et al., 2004; Shaw et al., 2001). Merlin can be phosphorylated on Ser518 by p21 activated kinase (PAK), and elevated PAK activity could lead to phosphorylation and inhibition of merlin (Kissil et al., 2003; Xiao et al., 2002). In response to growth factors, merlin is also phosphorylated by Akt and this phosphorylation leads to merlin proteosomal degradation (Tang et al., 2007).
Thus, exposure of cells to mitogens or persistent activation of Akt/PI3K signaling, as observed in vestibular schwannomas, may lead to merlin degradation (Gusella et al., 1999).

Merlin is dephosphorylated by MYPT-1-PP1δ on Ser518 resulting in merlin activation (Jin et al., 2006). However, MYPT1 is inhibited by the oncoprotein CPI-17 which results in merlin phosphorylation and inactivation (Jin et al., 2006). Therefore, deregulation of MYPT-1-PP1δ activity by altered CPI-17 protein levels in some tumor cells might lead to increased Ser518 phosphorylation and inactivation of merlin (Jin et al., 2006). Thus, the kinases and phosphatases that phosphorylate and dephosphorylate merlin play important roles in the regulation of merlin tumor suppressor function and merlin-dependent signaling pathways.

1.7.4. Merlin and growth suppression

Similar to the ERM proteins, which have important functions in cellular remodeling, merlin localizes to membrane ruffles, and regulates the cytoskeleton by altering actin organization (Ikeda et al., 1999; McClatchey, 2003; Okada et al., 2005; Shaw et al., 2001; Sherman and Gutmann, 2001; Tsukita and Yonemura, 1997). For example, actin cytoskeleton organization is altered in NF2-deficient schwannoma cells, and transient induction of wild-type merlin expression causes F-Actin organization alteration during cell spreading, abnormalities in cell attachment and reduced cell motility in rat schwannoma cells (Shaw et al., 2001; Sherman and Gutmann, 2001; Sun et al., 2002). Adherens-junctions in fibroblasts and keratinocytes are also disrupted as a result
of the loss of merlin (McClatchey, 2003). Activated merlin inhibits cell proliferation, cell cycle progression and motility and merlin mutation or reduced expression is associated with NF2 development (Gutmann et al., 2001b). Notably, tissue-specific inactivation of NF2 in murine schwann cells is sufficient for schwannoma development (Giovannini et al., 2000).

Rho GTPases play important roles in merlin-dependent growth suppression. Rac-dependent signaling and anchorage-independent growth of Rac-transformed cells can be blocked by overexpression of merlin (Shaw et al., 2001; Tikoo et al., 1994). Thus, physiological targets of merlin are potential downstream effectors of Rac signaling (Kissil et al., 2003; Shaw et al., 2001).

1.7.5. Merlin binding partners

It has been suggested that the growth suppressive function of merlin may be mediated through its interaction with its binding partners, which include CD44 (hyaluronan receptor), hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), paxillin, syntenin, β1 integrin, and SCHIP (Fernandez-Valle et al., 2002; Goutebroze et al., 2000; Jannatipour et al., 2001; Obremski et al., 1998; Scoles et al., 2000). The heteromeric interactions between merlin and binding partners might regulate merlin function, perhaps by forming complexes that differentially modulate the ability of merlin to bind to critical effectors or regulatory molecules (Sun et al., 2002). Understanding the functional significance of the interactions between merlin and its binding partners will be critical to elucidating the growth suppressive function of merlin.
2. MATERIALS AND METHODS

2.1. Cell culture

Human embryonic kidney (HEK293), colon cancer (HT29), ovarian cancer (Hey1B, SKOV3, OVCAR3 and TOV21G), lung cancer (NCIH460), breast cancer (MCF7), and mouse normal schwann (SW10) cells were obtained from the American Type Culture Collection (ATCC). RT4 cells are rat schwannoma cells. The RT4-NF2.17 cell line was derived from RT4 cells that have been engineered with a tetracycline-inducible merlin (Gutmann et al., 2001a). HEI193 are patient-derived NF2 schwannoma cells (Hung et al., 2002). T29 and T80 are immortalized human ovarian epithelial cells (Liu et al., 2004). RT4, RT4-NF2.17, NCIH460, HEI193, Hey1B, SKOV3, OVCAR3, SW10, and HT29 and MCF7 cells were cultured in DMEM (Mediatech, Herndon, VA, USA) supplemented with 10% FBS (fetal bovine serum) (HyClone, Logan, UT, USA). T29 and TOV21G cells were cultured in medium 199 (Mediatech, Inc.), with 10% MCDB 105 (Sigma-Aldrich, St. Louis, MO, USA) and 10% FBS. All tissue culture media were supplemented with 25 μg/ml streptomycin and 25 I.U. penicillin (Mediatech). Cells were cultured in a humidified atmosphere with 5% CO₂ at 37°C.

2.2. Expression vectors

The following mammalian expression vectors were used in this study:

pEBG-GST-MLK3, pCMV-HA-MLK3, pCMV5-FLAG-MLK3(WT),
pCMV5-FLAG-MLK3(K144R), pCMV5-Myc-merlin, pCDNA3-HA-merlin(WT),
pCDNA3-HA-merlin(S518A), pCDNA3-HA-merlin(S518D),
pCMV5-FLAG-Cdc42(WT), pCMV5-FLAG-Cdc42(N17), pCMV5-FLAG-Cdc42(V12),
and pEBG-MLK3 (GST fusion protein).

2.3. Site-directed mutagenesis

pCMV5-FLAG-MLK3(K114R) is made from pCMV5-FLAG-MLK3(WT) by using
Quickchange II site-directed mutagenesis kit (Stratagene). The primer sequences (human)
were as follows:

forward 5’-GGTGAGCTGGTGGCTGTGGCGGCAGCTCGCCAGGACCCC-3’
reverse 5’-GGGGTCCTGGCGAGCTGCCGCCACAGCCACCAGCTCACC-3’

The reaction included 5 µl of 10x reaction buffer, 2 µl (100ng) of dsDNA template, 2
µl of primer mix (250 ng total), 1 µl of dNTP mix, and 40 µl of the ddH2O to make a
final volume of 50 µl. Next 1 µl of PfuUltra HF DNA polymerase (2.5 U/µl) was added.
The amplification reaction cycles were as following:

1 cycle
Step 1: 95 ºC, 30 seconds

18 cycles
Step 1: 95 ºC, 30 seconds
Step 2: 55 ºC, 1 minute
Step 3: 68 ºC, 9 minute

After the reaction, the tubes were placed on ice for 2 minutes to cool. 1 µl Dpn I
restriction enzyme was added to each amplification reaction and then the tubes were incubated at 37 °C for 1 hour. 1 µl of the Dpn I-treated DNA was transferred to 50 µl of the XL1-blue supercompetent cells, heat shocked at 42 °C for 45 seconds, and then incubated on ice for 30 minutes. After adding 0.5 ml of NZY+ broth, the reaction was incubated at 37 °C for 1 hour at 225 rpm. The reactions were plated onto 2 agar plates containing ampicillin.

2.4. DNA and siRNA transfections

DNA transient transfections were performed by using Lipofectamine (Invitrogen) reagent according to the manufacturer’s protocol. Cells were seeded in either 6 or 10 cm dishes, and transfected when cells reached 60-70% confluence. Briefly, 2-5 µg of DNA was diluted in serum free medium (2 µg for 6 cm dishes, 5 µg for 10 cm dishes). 8 µl of Lipofectamine was added to the DNA suspension, mixed well and incubated for 15 min. Cells were washed with serum free medium once, and lipofectamine and DNA complexes were overlayed onto cells in serum free medium (2.5 ml total for 6 cm dishes, 5 ml for 10 cm dishes), and then placed in a 5% CO2 incubator at 37°C. After 5 hours of incubation, an equivalent volume of medium containing 20% FBS was added to each dish. Cells were cultured overnight and allowed to recover for at least 16 hours before harvesting.

SiRNA transfections were performed by using Lipofectamine 2000 (Invitrogen) reagent. 8-15 µl siRNA oligos was added to the serum free medium to make a final siRNA
concentration of 30 nM. Lipofectamine 2000 was added to the siRNA suspension, mixed well and incubated for 15 min. Cells were washed with serum free medium once, and Lipofectamine 2000 and siRNA complexes were overlayed onto cells in serum free medium (2.5 ml total for 6 cm dishes, 5 ml for 10 cm dishes), and then placed in a 5% CO₂ incubator at 37°C. After 5 hours incubation, an equivalent volume of medium containing 20% FBS was added to each dish. Cell were cultured overnight and allowed to recover for at least 16 hours before harvesting. The siRNA oligonucleotide sequences used in this study were as following:

murine merlin (Morrison et al., 2007):

<table>
<thead>
<tr>
<th>sense</th>
<th>5’-UACCGAGCUUCGACAUUAUUG-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>antisense</td>
<td>5’-AUUAUGUCAAGCUCGGUAUG-3’</td>
</tr>
</tbody>
</table>

human merlin (Okada et al., 2005):

<table>
<thead>
<tr>
<th>sense</th>
<th>5’-UGGCCAACGAAGCACUGAU-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>antisense</td>
<td>5’-AUCAGUGCUCUUGGCCA-3’</td>
</tr>
</tbody>
</table>

murine MLK3 (Chadee and Kyriakis, 2004a):

<table>
<thead>
<tr>
<th>sense</th>
<th>5’-GGGCAGCGACGCGTGTGCGG-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>antisense</td>
<td>5’-AAGCUCGACACGUCCUGCCC-3’</td>
</tr>
</tbody>
</table>

human MLK3 (Chadee and Kyriakis, 2004a):

<table>
<thead>
<tr>
<th>sense</th>
<th>5’-GGGCAGUGACGCGUCGAGUUU-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>antisense</td>
<td>5’-AAACUCAGACGACGUGCUGCC-3’</td>
</tr>
</tbody>
</table>

siRNA with non-targeting sequence (Dharmacon) (Morrison et al., 2007):
sense 5’-AAUCGGGUUGCAUAGUUCAUG-3’
antisense 5’-UGAACUAUGCAACCGGAUUUG-3’

2.5. Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for immunoblotting. 15% polyacrylamide running gels were prepared by using 5 ml of 30% acrylamide (29.2 g acrylamide and 0.8 g bisacrylamide in 100 ml ddH$_2$O), 2.5 ml of 4X Tris-Cl/SDS, pH 8.8 (0.5 M Tris base, 13.87 mM SDS), 40 µl of 50% ammonium persulfate (APS), 4 µl of TEMED, and 2.5 ml of H$_2$O to make the final volume of 10 ml for 1 gel. Polyacrylamide stacking gel were prepared by using 0.9 ml of 30% acrylamide, 1.7 ml of 4X Tris-Cl/SDS, pH 6.8 (0.5 M Tris base, 13.87 mM SDS), 23.3 µl of 50% APS, 6.7 µl of TEMED, and 4.1 ml of H$_2$O to make the final volume of 6.7 ml for 1 gel.

Electrophoresis was conducted under constant amps (12.5 mA for one gel) in running buffer (25 mM Tris base, 192 mM glycine, and 6.94 mM SDS). Proteins were then transferred from the SDS gel to Immobilon-P Polyvinylidene Fluoride (PVDF) membrane in transfer buffer (25 mM Tris base, 192 mM glycine and 20% methanol) at 50 V for 2 hours. Membranes were stained with Brilliant Blue R250 solution (40% methanol, 10% acetic acid, and 0.04% Brilliant R250 Blue) for 2 minutes and destained in destain solution containing 70% methanol destain solution. The membrane was then incubated in blocking buffer containing 5% nonfat powder milk in 1X PBS (diluted from 10 X PBS: 28
137 mM NaCl, 27 mM KCl, 43 mM KH$_2$PO$_4$, 14 mM Na$_2$HPO$_4$ pH 7.4) with rotation. After blocking for 1 hour at room temperature, membranes were incubated with primary antibodies overnight with shaking at 4°C. Primary antibodies were diluted 1:1000 in antibody buffer containing 5% nonfat dry milk and 0.05% Tween 20 in 1X PBS. Phospho specific antibodies were diluted 1:500 in 1X PBS containing 5% bovine serum albumin (BSA) and 0.05% Tween 20. After 16 hours incubation with primary antibodies, membranes were washed with fresh antibody buffer (without milk) 3 times for 15 min, and then incubated in secondary antibody that was diluted 1:5000 in antibody buffer containing 5% nonfat dry milk and 0.05% Tween 20 in 1X PBS for 2 hours at room temperature. Next, membranes were washed three times for 15 min in fresh antibody buffer without milk. The membranes were developed with Enhanced Chemiluminescence (ECL) with 2.5 mM Luminol, 0.4 mM p-Coumaric acid and 16.5% Hydrogen Peroxide in 100 mM Tris-HCl, pH 8.8; or with Immobilon-P development solutions (millipore).

The following antibodies were used: GST (Z-5), Myc (9E10), B-Raf (F-3), ERK2 (C-14), MLK3(C-20), β-Actin (C-4), Cyclin E (M-20), phospho-B-Raf (p-B-Raf) (Thr598/Ser601), merlin (A-19), JNK (C-17), His probe (G-18), HA probe (Y-11) and normal rabbit IgG (Santa Cruz Biotechnology). Activation-state phospho-ERK (p-ERK) (Thr202/Tyr204), phospho-MLK3 (p-MLK3) (Thr277/Ser281) and phospho-JNK (p-JNK) (Thr183/Tyr185) antibodies were from Cell Signaling Technology. Phospho-NF2 (p-merlin) (Ser518) antibody was from Rockland Immunochemicals and FLAG antibody was from Stratagene. Anti-Myc (9E10) monoclonal antibody was prepared by growing
9E10 cells in 10 cm dishes and collecting medium containing the Myc antibody. The secondary antibodies were Immun-Star Goat Anti-Mouse (GAM) - Horseradish Peroxidase (HRP) Conjugate and Immun-Star Goat Anti-Rabbit (GAR) - HRP Conjugate (Bio-Rad).

2.6. Preparation of protein extracts from tissue culture cells

Cells were washed with PBS, and then, the whole cell extracts were made by adding 600 µl of 6X SDS sample buffer (70% Tris-Cl, pH 6.8, 30% glycerol, 346.8 mM SDS, 602.9 mM DTT) directly to dishes. Cells extracts were collected, and then transferred to 1.5 ml microcentrifuge tubes. Protein extracts were boiled for 5 minutes and subjected to SDS-PAGE or stored at -20 °C.

2.7. Ovarian tumor tissues specimens

Human ovarian serous adenocarcinomas (stages 3 and 4), serous cystadenomas, and normal matched tissues were obtained from the Gynecologic Oncology Group Tissue Bank (Children’s Research Institute, Columbus, OH).

2.8. Preparation of protein and RNA from ovarian tumor tissues

Preparation of protein and RNA was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). 50 mg of tissue was homogenized in 1 ml of TRIzol reagent, and
0.2 ml of chloroform was added followed by centrifugation at 12,000 x g for 15 min at 4°C to separate phases. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol. The RNA pellets were washed once with 75% ethanol, dried and dissolved in RNase-free water. Proteins were precipitated from phenol-ethanol supernatants with 1.5 ml isopropanol. Protein precipitates were washed 3 times in 0.3 M guanidine hydrochloride in 95% ethanol. Pellets were washed with 2 ml of ethanol, vacuum dried and dissolved in 2X SDS sample buffer.

For isolation of RNA from tissue culture cells, cells were cultured in 6 cm dishes. 1 ml of TRIzol reagent was added directly to each dish, and incubated at room temperature for 3 minutes. Cell extracts collected and transferred to tubes. 0.2 ml of chloroform was added followed by centrifugation at 9300 rpm for 15 min at 4°C to separate phases. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol. The RNA pellets were washed once with 75% ethanol, dried, and dissolved in 25 µl RNase-free water.

2.9. Immunoprecipitation

For immunoprecipitation of endogenous or overexpressed proteins, TOV21G or HEK293 cells were grown to 80-90% confluency and lysed with lysis buffer containing 20 mM TRIS, pH 7.4, 2 mM EGTA, 10 mM MgCl₂, 0.1% β-mercaptoethanol, 1% Triton X-100, 100 µM phenylmethane sulphonyl fluoride (PMSF), 2 µM leupeptin and 2 µM
pepstatin, 10 kallekrin inhibitory units (KIU)/ml aprotinin. After incubating on ice for 5 min, cells extracts were collected, and cell lysates were transferred to 1.5 ml centrifuge tubes. Cell lysates were centrifuged at 13,000 rpm for 20 min and supernatant was removed and transferred to a new tube. 2 µl of antibody was added to 500 µl cell lysates together with 25 µl Protein-G Sepharose beads (Pierce). The lysates were incubated with antibody and rocked for 3 hours at 4 °C. Beads were pelleted at 13,000 rpm for 2 min and supernatant was removed, and then washed with lysis buffer once, and twice in wash buffer (20 mM Tris pH 7.4, 2 mM EGTA, 10 mM MgCl₂, 0.1% β-mecaptoethanol, 0.1% Triton X-100, 100 µM PMSF, 10 KIU/ml aprotinin, 2 µM leupeptin and 2 µM pepstatin). For the MLK3 kinase assay, an additional high salt wash (wash buffer + 1 M LiCl) was performed. Protein G beads were resuspended in 25 µl 6X SDS sample buffer, and then boiled for 5 min before subjecting to SDS-PAGE.

2.10. GST fusion protein preparation and binding assays

GST fusion merlin truncation mutant constructs (aa 1-590, aa 1-322, aa 308-590, and aa 340-590) were obtained from Dr. V. Ramesh (Gonzalez-Agosti et al., 1999). These GST fusion merlin truncation mutants were expressed in bacteria and purified with glutathione-sepharose beads (PIERCE, Thermo Fisher Scientific Inc.). Cells were lysed in lysis buffer (50 mM TRIS pH 7.8, 60 mM NaCl, 1 mM EDTA, 2 mM DTT, 4 mM benzamidine, 0.05% Triton X-100, 1 mM PMSF, 2 µM leupeptin and 2 µM pepstatin,
10KIU/ml aprotinin), and lysates were incubated with GSH beads and rotated at 4°C for 1 hour; and then eluted with 50 mM glutathione in lysis buffer. His-MLK3 protein was also expressed by bacteria, and then purified on nickel agarose beads, and eluted with imidazole (80 mM imidazole, 10 mM Heps, pH 7.9, 5 mM MgCl₂, 0.1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol and 17% glycerol). GST-merlin fusion proteins were incubated with His-MLK3 for 3 hours at 4°C. GST beads were pelleted and protein complexes bound with GST-sepharose beads were subjected to 15% SDS-PAGE, and then transferred to PVDF membranes. His-MLK3 that coimmunoprecipitated with GST-merlin mutants was detected by immunoblotting with GST and MLK3 antibodies.

2.11. Invasion assays

Cell invasion assays were performed using an artificial extracellular matrigel matrix (BD Bioscience). Matrigel was diluted to 100 µl of 1 mg/ml with cold serum free medium in 24-well transwells with 8.0 µm pore size and 24 mm diameter polycarbonate membrane (Corning Inc.) and incubated for 2 hours at 37°C. Cells were washed in 0.5% FBS in DMEM. Ten thousand cells were counted and seeded in 0.5% FBS onto the upper chamber, and 0.5 ml of growth medium was added in the well as attractant. After 16 hours incubation, cells remaining in the upper chamber were removed and cells on the bottom side of the membrane were fixed in Diff Quick Stain Kit (IMEB Inc.) and counted. All experiments were run in triplicate and repeated at least three times.
2.12. Assay of MLK3 kinase activity

Preparation of cell lysates and immunoprecipitation of FLAG-MLK3 for kinase assays was performed. Briefly, FLAG-MLK3 (wild-type or K114D) was transfected to HEK293 cells, and cells were lysed in lysis buffer (20 mM TRIS pH 7.4, 2 mM EGTA, 10 mM MgCl₂, 0.1% β-mercaptoethanol, 1% Triton X-100, 100 μM phenylmethane sulphonyl fluoride (PMSF), 2 μM leupeptin and 2 μM pepstatin, 10 KIU/ml aprotinin), and then lysates were incubated with protein G-agarose beads and FLAG antibody to immunoprecipitate FLAG-MLK3. Beads were washed with high salt wash buffer (1 M LiCl, 20 mM Tris pH 7.4, 2 mM EGTA, 10 mM MgCl₂, 0.1% β-mercaptoethanol, 0.1% Triton X-100, 100 μM PMSF, 10 KIU/ml aprotinin, 2 μM leupeptin and 2 μM pepstatin) to eliminate non-specific binding. Immunoprecipitate FLAG-MLK3 was incubated with or without C-terminal (340-590) merlin (0.35, 0.88 or 1.25 μg) with 100 μM ATP and 10 mM MgCl₂ for 30 minutes at 30°C. 6X SDS sample buffer containing EDTA was added to stop the reaction, and samples were boiled 5 min and subjected to 15% SDS-PAGE and immunoblotting with p-MLK3 antibody to detect active MLK3 phosphorylated on Thr277/Ser281.

2.13. Assay of Cdc42 activity

T29 cells were transfected with or without merlin siRNA oligos. Cell lysates were prepared by using Cdc42 assay lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1%
Lgepa CA-630, 10% glycerol, 25 mM NaF, 10 mM MgCl₂, 1 mM EDTA, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin). 10 µg of Rac/Cdc42 Assay Reagent was added to 1 ml of the cell lysate, and incubated at 4 ºC for 1 hour. For controls, half of the cell lysates were incubated with EDTA and GDP (negative control) or GTPγS (positive control) for 15 min at 30ºC, and then incubated with Pak1 PBD-agarose beads for 1 hour at 4 ºC. Active FLAG-Cdc42 that bound to Pak1-agarose beads were pelleted and suspended in 2X sample buffer. Samples were subjected to 15% SDS-PAGE and immunoblotting with anti-FLAG antibody to detect active Cdc42 that was bound to Pak1 PBD-agarose beads.

2.14. Real-time PCR (RT-PCR)

RT-PCR was performed using IQ SYBR Green Supermix (Thermo Fisher Scientific, Waltham, MA, USA). Total volume for each reaction is 20 µl, including 10 µl of SYBR Green, 1 µl of the cDNA, and 9 µl of the primer mix (500 nM). The primer sequences (human) were as follows:

- β-Actin  
  Forward 5’-GGACTTCGAGCAAGAGATGG-3’
  Reverse 5’-AGCACTGTGTGCGCGTACAG-3’

- MLK3  
  Forward 5’-GTCATAAGATGCCAGTGG-3’
  Reverse 5’-CACGTCACCTTCCTCA-3’

- MMP-1  
  Forward 5’-ATGCTGAACCCTGAAGGTG-3’
Reverse 5’-CTGCTTGACCCTCAGAGACC-3’

MMP-2 Forward 5’-TTTCCATTCCGCTTCCAGGGCAC-3’

Reverse 5’-TCGCACACCACATCTTTCCGTCACT-3’ (Kohrmann et al., 2009)

MMP-9 Forward 5’-GCCATTCACGTCGTCCTTA T-3’

Reverse 5’-TTGACAGCGACAAGAAGTGG-3’

MMP-12 Forward 5’-CCTTCAGCCAGAAGACCTG-3’

Reverse 5’-ACACATTTCGCCTCTCTGCT-3’.

The Real-time PCR was performed under following cycles:

1 cycle Step 1: 95 °C, 3 minute

40 cycles Step 1: 95 °C, 10 seconds

Step 2: 55 °C, 30 seconds

All the experiments were performed in triplicate and repeat 3 times.

2.15. Gelatin zymography

SDS-PAGE gelatin zymography was used to determine MMP-2 and MMP-9 enzymatic activities in the cell culture medium. Briefly, 5x10⁵ cells were seeded in 6 cm dishes. Equal amounts culture media from cells (100 µg) was mixed with the sample buffer without reducing agents, and electrophoresed in 10% SDS-PAGE containing 0.1% (w/v) gelatin (Acros Organics, Morris Plains, New Jersey, USA). The SDS-PAGE gels
were incubated in 2.5% Triton X-100 at room temperature for 2 h, and then transferred to digestion buffer containing 1% Triton X-100, 10 mM CaCl$_2$, 0.15M NaCl, and 50 mM Tris (pH 7.5), and incubated at 37°C for 40 h. Gels were stained with Coomassie Blue R-250 and destained in destain buffer containing 10% methanol and 5% acetic acid. Proteolysis was detected as a white band against a blue background (Schmalfeldt et al., 2001).
3. RESULTS

Part I: MLK3 is required for MMP expression and invasion in ovarian cancer

3.1. MLK3 protein levels in ovarian tumor cell lines

MLK3 expression transforms NIH3T3 cells in a MEK dependent manner, and stable expression of MLK3 promotes neoplastic transformation in mammary epithelial cells (Chen et al., 2010a; Hartkamp et al., 1999). Thus, we postulated that elevated MLK3 expression could persistently promote ERK and JNK activation, and contribute malignancy. To analyze MLK3 protein levels in human ovarian tumor cell lines, immunoblotting of MLK3 was performed with cell extracts prepared from immortalized ovarian epithelial T80 and T29 cells, and ovarian tumor HEY1B, HEY and SKOV3 cell lines which are derived from human ovarian epithelial carcinomas. The results of this analysis indicated that SKOV3, HEY1B and HEY cells had substantially higher levels of MLK3 protein in comparison to T80 and T29 cells (Figure 6).

To compare MLK3 activity in human ovarian tumor cells and normal ovarian epithelial cells, whole cell extracts were immunoblotted with an activation state MLK3 antibody (p-MLK3) that detects the phosphorylation on Thr277 and Ser281 residues at the activation loop. The results of this analysis revealed that p-MLK3 levels in SKOV3, HEY1B and HEY cells are higher than that observed in T80 or T29 cells (Figure 6).
Figure 6. Elevated MLK3 levels in human ovarian tumor cell lines. Cell lysates were prepared from immortalized ovarian epithelial T80 and T29 cells, and ovarian cancer SKOV3, HEY1B, HEY cells. Immunoblotting of cell extracts was performed with MLK3, p-MLK3 and β-Actin antibodies.
3.2. MLK3 loss of contact inhibition in ovarian tumor cells

In normal fibroblasts, the levels of active MEK and ERK decline when cell become contact inhibited (Wayne et al., 2006). Whereas tumor cells, which lack contact inhibition, do not exhibit a density-dependent negative regulation of ERK signaling (Wayne et al., 2006). To determine if MLK3 activity is also regulated by cell density in ovarian epithelial cells, p-MLK3 levels were analyzed in T29 cells at different stages of cell confluence. Interestingly, in T29 cells, p-MLK3 levels decreased as cells became confluent. In contrast, high levels of p-MLK3 were observed in SKOV3 cells at both low and high confluence (Figure 7). These results indicate that cell density negatively regulates MLK3 activity in T29 cells, but not in SKOV3 tumor cells.
Figure 7. MLK3 loss of contact inhibition in ovarian tumor cells. T29 and SKOV3 cells were seeded at a density of 5x10^5 cells per 10 cm dish, and cultured for 24, 48, and 72 hours. Cell lysates were prepared at each time point and samples were immunoblotted with p-MLK3, MLK3 and β-Actin antibodies.
3.3. MLK3 expression levels in human ovarian tumor tissues

To determine if MLK3 is also elevated in human ovarian tumor tissues, MLK3 protein levels in benign and malignant ovarian tumor specimens were compared with MLK3 from normal matched tissues isolated from the same patient. MLK3 levels were elevated in 4 out of 5 benign tumor samples and p-MLK3 levels were elevated in all five benign tumor samples in comparison to normal matched tissues (Figure 8A). To ascertain if the elevated MLK3 levels was due to increased gene expression, MLK3 mRNA was analyzed in the ovarian (normal and tumor) tissues by RT-PCR. Strikingly, MLK3 mRNA levels were significantly higher in the benign cystadenoma ovarian tumor tissues in comparison to normal matched tissues (Figure 8B). Furthermore, MLK3 protein levels were higher in 3 out of 5 malignant ovarian tumor tissues in comparison to normal tissues; and MLK3 mRNA levels were also increased in 3 out of 5 malignant tumor tissue samples (Figure 8C and 8D). The levels of p-MLK3 were also elevated in 1 out of 5 malignant tumors analyzed (Figure 8C). These results indicate that MLK3 is activated in a subset of both benign and malignant ovarian tumors, and possibly that MLK3 may play a role in the early stages of ovarian tumor development.
Figure 8. Elevated MLK3 expression in human ovarian tumor tissues. (A) Protein extracts were prepared from human benign ovarian tumor and normal matched tissues. Tumor tissue samples are designated as T and normal matched tissue samples are designated as N. Samples were subjected to immunoblotting with p-MLK3, MLK3 and β-Actin antibodies (upper panel). Quantitation of MLK3 and p-MLK3 levels (lower panel) was determined using Image J software (NIH, Bethesda, Maryland, USA). MLK3 or p-MLK3 levels were first normalized to β-Actin levels in each sample and the values
presented represent the fold increase in MLK3 or p-MLK3 levels in the tumor samples in comparison to normal matched tissue from the same patient. (B) Total RNA was isolated from human benign ovarian tumor tissues and RT-PCR was performed with MLK3 and β-Actin primers. Experiments were performed in triplicate and repeated three times. Values obtained for MLK3 expression was normalized to β-Actin expression in each sample. Each bar represents the mean of data collected from three independent experiments and error bars represent standard deviations from the mean. (C) Protein extracts were prepared from human malignant ovarian tumor and normal matched tissues and subjected to immunoblotting as described in A. Tumor tissue samples are designated as T and normal matched tissue samples are designated as N. Quantitation of MLK3 and p-MLK3 levels (lower panel) was performed as described in A. (D) Total RNA was isolated from human malignant ovarian tumor tissues and RT-PCR was performed and data analyzed as described in B.
3.4. MLK3 is required for SKOV3 cellular invasion

Overexpression of wild-type MLK3 transforms NIH-3T3 cells suggesting that high MLK3 expression in tumor cells may promote invasion (Hartkamp et al., 1999). Therefore MLK3 expression may promote cellular transformation. Thus, we wished to investigate if depleting MLK3 expression would impair ovarian cancer cell invasion. SKOV3 cells were derived from an invasive ovarian epithelial carcinoma and have high levels of basal active MLK3 (Figure 6). Depleting MLK3 in SKOV3 cells with RNAi significantly inhibited invasion, while the control non-specific siRNA oligo treatments had little or no effect (Figure 9, performed by Nidhi Modi). These results suggest that MLK3 is required for SKOV3 ovarian cancer cell invasion.
Figure 9. MLK3 knockdown reduced SKOV3 cell invasion. SKOV3 cells were untreated or treated with murine (mm) or human (hs) MLK3 siRNA. Cell invasion was analyzed using a modified Boyden chamber with matrigel. Cells that traversed the membrane were stained and counted (This experiment was performed by Nidhi Modi). Experiments were performed in triplicate and repeated three times.
3.5. Upregulation of MMPs in SKOV3 cells

To begin to elucidate the mechanism by which MLK3 regulates cell invasion in ovarian tumor cells, we focused on genes that are regulated by ERK and JNK MAPK signaling pathways and are important for cell invasion. ERK and JNK stimulate activator protein 1 (AP-1)-mediated gene transcription, and MMPs are AP-1 regulated-genes that are critical for tumor cell invasion (Karin, 1995). MMPs are proteolytic enzymes that degrade components of the extracellular matrix, and increased expression of MMP-2 and MMP-9 is associated with progression from benign to advanced ovarian cancer (Ala-aho and Kahari, 2005; Nagase and Woessner, 1999; Schmalfeldt et al., 2001). RT-PCR analysis of MMP-1, -2, -9 and -12 in T29 and SKOV3 cells indicate that the expression of all four of these MMPs was upregulated in SKOV3 cells in comparison to T29 cells (Figure 10).
Figure 10. MMP-1, -2, -9, and -12 gene expression in T29 and SKOV3 cells. Total RNA was isolated from T29 and SKOV3 cells and RT-PCR was performed with human β-Actin, MMP-1, -2, -9 and -12 primers. MMP expression levels were normalized to β-Actin expression levels in each sample. Each bar represents the mean of data collected from three independent experiments and error bars represent standard deviations from the mean.
3.6. MLK3 is required for ERK, JNK and MMP activities in SKOV3 cells

We postulated that MLK3 could promote SKOV3 invasion by modulating MMP gene expression. To test this possibility, we first assessed the impact of silencing *mlk3* on ERK and JNK MAPK signaling in SKOV3 cells. Consistent with our previous findings in colorectal and lung fibroblasts, *mlk3* silencing significantly reduced basal levels of active ERK and JNK (Figure 11A) (Chadee and Kyriakis, 2004a). These results underscore the role of MLK3 as a major activator of ERK and JNK signaling pathways in ovarian tumor cells. Next, to determine the effect of silencing *mlk3* on MMP-2 and/or -9 enzyme activities, the levels of active MMP-2 and -9 were analyzed by gel zymography in SKOV3 cells transfected with nonspecific MLK3 siRNA, human MLK3 siRNA or no siRNA. The results demonstrate that silencing *mlk3* significantly reduced the levels of MMP-2 and MMP-9 gelatinase activities present in the media of SKOV3 cells (Figure 11B). Furthermore, RT-PCR analysis of SKOV3 MMP mRNA revealed a 75-90 percent decrease in MMP-1, MMP-2 and MMP-9 and MMP-12 mRNA in MLK3 knockdown cells in comparison to cells treated with nonspecific siRNA (Figure 11C). These results indicate that MLK3 is required for MMP-1, -2, -9, and -12 expression in SKOV3 cells.
Figure 11. Silencing mlk3 in SKOV3 cells inhibits ERK, JNK and MMP activation.

(A) SKOV-3 cells were transfected with nonspecific, MLK3, or no siRNA and cell extracts were immunoblotted with MLK3, p-ERK, p-JNK, ERK, JNK and β-Actin (loading control) antibodies. (B) SKOV3 cells were transfected with siRNA as described in A. Cell culture medium was collected and subjected to gelatin zymography analysis (upper panel). Cell extracts were prepared and subjected to immunoblotting with MLK3 and β-Actin antibodies (lower panel). (C) Total RNA was isolated from SKOV3 cells that were transfected with nonspecific or MLK3 siRNA, and RT-PCR was performed with human β-Actin, MMP-1, -2, -9 and -12 primers. MMP expression levels were normalized to β-Actin expression levels in each sample. Each bar represents the mean of data collected from three independent experiments and error bars represent standard deviations from the mean.
3.7. Reduced levels of merlin and increased MLK3 activity in tumor cell lines

Previously we found that exogenous merlin expression inhibited MLK3-dependent JNK activation in HEK293 cells (Chadee et al., 2006). Therefore, we postulated that if merlin were a physiological inhibitor of MLK3, cells with lower levels of merlin protein would have elevated basal MLK3 kinase activity. To test this, we analyzed merlin protein expression and MLK3 kinase activity in different tumor cell lines.

NF2 schwannoma HEI193 and ovarian cancer SKOV3 cells had low or undetectable merlin protein and high basal MLK3 kinase activity (Figure 12A and 12B). HEI193 expresses a splice variant of merlin (isoform 3) that has impaired growth suppressive activity (Hung et al., 2002; Lepont et al., 2008). Lung cancer NCIH460 cells had intermediate levels of merlin protein expression in comparison to the other cell lines and high levels of basal MLK3 kinase activity (Figure 12A and 12B). In contrast, colon cancer HT29, breast cancer MCF7, ovarian cancer TOV21G and OVCAR3, and human embryonic kidney HEK293 cells all had relatively high merlin protein expression, and low or undetectable levels of basal active MLK3, indicating an inverse correlation between merlin expression and MLK3 kinase activity, which is consistent with merlin being a physiological inhibitor of MLK3 (Figure 12A and 12B).

We observed a similar inverse relationship between active MLK3 levels and merlin protein levels in cells that were harvested at low (Figure 12A) or high confluence (Figure 12B). Interestingly, at higher confluence, SKOV3 cells had significantly less active MLK3 and higher merlin protein. This suggests that while merlin protein levels in these
cells could vary depending on cell confluence, the relationship between merlin protein levels and MLK3 kinase activity remained consistent. Additionally, these results indicate that MLK3 kinase activity is deregulated in some tumor cells which could promote persistent MAPK signaling and tumorigenesis.
Figure 12. Reduced levels of merlin and increased MLK3 activity in human tumor cell lines. (A and B) Tumor cells (HEI193, HT29, MCF7, NCIH460, HEK293, SKOV3, OVCAR3 and TOV21G) were cultured and whole cell extracts were prepared when cells were 60% confluent (A) or 90% confluent (B) and subjected to immunoblotting with antibodies that recognize p-MLK3, total MLK3, and total merlin as indicated.
Part II: Regulation of MLK3 by tumor suppressor protein merlin in human cancer

3.8. Merlin associates with MLK3 in cells

In previous studies, it was observed that exogenous merlin and MLK3 co-immunoprecipitated from HEK293 cells (Chadee et al., 2006). These results were confirmed in Figure 13A where we observed that overexpressed, full-length merlin (1-595) co-immunoprecipitated with overexpressed full-length MLK3 from HEK293 cells. In addition, endogenous MLK3 co-immunoprecipitated with endogenous merlin from human ovarian (TOV21G) tumor cells (Figure 13B), indicating that endogenous merlin and MLK3 are associated in cells.
Figure 13. Endogenous association of merlin and MLK3. (A) Full-length FLAG-merlin (1-595) interacts with HA-MLK3. FLAG-merlin and HA-MLK3 were overexpressed in HEK293 cells and HA-MLK3 was immunoprecipitated from cell lysates. Immunoprecipitates were immunoblotted with anti-HA and anti-FLAG antibody to detect FLAG-merlin and HA-MLK3. FLAG-merlin was also immunoprecipitated from cell lysates and immunoblotted with anti-FLAG antibody to verify FLAG-merlin expression. (B) Endogenous merlin was immunoprecipitated from TOV21G cells. Immunoprecipitates were immunoblotted with anti-MLK3 antibody to detect co-immunoprecipitated MLK3. Control immunoprecipitations were performed with rabbit IgG and Cyclin E antibodies.
3.9. Merlin interacts with MLK3 in vitro

Merlin has an N-terminal FERM domain (aa 1-302) involved in membrane interactions, a central coiled-coil alpha helical region (aa 303-478) and a unique C-terminal domain (aa 479-595) (Sun et al., 2002). Merlin isoform 2 is generated by alternative splicing and gives rise to a 590 residue protein that differs by 11 residues at the C-terminus (aa 580-590) (Sun et al., 2002). To investigate if merlin interacts directly with MLK3, \textit{in vitro} binding assays were performed with GST-merlin and His-MLK3 proteins purified from bacteria. GST-merlin full length (aa 1-590) or deletion mutants (aa 1-332, aa 308-590 and aa 340-590) (Figure 14A) were incubated with full length His-MLK3 \textit{in vitro}. GST pulldown assays revealed that full-length GST-merlin bound to His-MLK3. The deletion mutants that contain the alpha helical region and the C-terminus, aa 340-590 and aa 308-590, also bound to His-MLK3, however, the N-terminal deletion mutant aa 1-332 did not bind to His-MLK3 (Figure 14B). Together, the results suggest that the aa 340-590 C-terminal region of merlin is required for binding to MLK3.
Figure 14. The C-terminus of merlin interacts with MLK3. (A) Diagram of merlin GST-fusion proteins. (B) Bacterial purified His-MLK3 wild-type protein was incubated with bacterial purified GST-merlin full-length protein and deletion mutants in an in vitro binding assay. GST-pulldowns were performed and bound MLK3 protein was analyzed by immunoblotting with anti-MLK3 antibody. Upper panel shows His-MLK3 associated with GST-merlin fusion proteins. Lower panel shows GST-merlin protein expression.
3.10. The C-terminus of merlin inhibits MLK3 kinase activity

To determine whether the C-terminal region of merlin that bound to MLK3 was sufficient for the inhibition of MLK3 activity, increasing quantities of purified GST-merlin (residues 340-590) were incubated with FLAG-MLK3-wild-type (WT) or a FLAG-MLK3-kinase dead (KD) K144R mutant, and a MLK3 kinase assay was performed. FLAG-MLK3-KD was not detected by the p-MLK3 antibody, which verifies the specificity of the antibody for active MLK3 enzyme. Incubation of FLAG-MLK3-WT with GST-merlin (residues 340-590) dramatically reduced p-MLK3 levels (Figure 15), suggesting that residues 340-590 in the C-terminus are sufficient for the direct inhibition of MLK3 activity by merlin.
Figure 15. C-terminus of merlin inhibits MLK3 kinase activity. A MLK3 kinase assay was performed with immunopurified FLAG-MLK3 protein expressed in HEK293 cells and increasing amounts (0.35-1.25 μg) of bacterial purified C-terminal GST-merlin mutant aa 340-590. P-MLK3 antibody was used to detect active MLK3. Immunoblotting with anti-FLAG and anti-GST antibodies was also performed to show the level of FLAG-MLK3 and Myc-merlin in the assay.
3.11. Merlin S518D mutant exhibits impaired binding to MLK3

Phosphorylation of merlin on Ser518 inhibits merlin intramolecular interactions and inactivates merlin-dependent growth suppressive activity (Rong et al., 2004). Thus, we sought to determine whether phosphorylation of merlin impaired its interaction with MLK3. The interaction between MLK3 and merlin was evaluated using wild-type merlin and mutant merlin proteins with either a Ser to Ala substitution at residue 518 (S518A) that cannot be phosphorylated, or a Ser to Asp substitution at residue 518 (S518D) that is phosphomimetic. Wild-type and S518A merlin proteins, but not the S518D mutant, co-immunoprecipitated with MLK3 (Figure 16A). These results indicate that phosphorylation of merlin on Ser518 impairs the merlin-MLK3 interaction. Furthermore, exogenous wild-type and S518A merlin, but not the S518D mutant substantially reduced p-MLK3 and p-JNK levels in SKOV3 cells (Figure 16B). These results indicate that phosphorylation of merlin on Ser518 impairs merlin inhibition of MLK3 activity (Figure 16B).
Figure 16. The S518D mutant of merlin impairs the MLK3-merlin interaction.

(A) GST-MLK3 and HA-merlin (wild-type, S518D and S518A) were overexpressed in HEK293 cells. GST pulldowns were performed and HA-merlin and GST-MLK3 were detected by Western blotting with the indicated antibodies.

(B) HA-merlin (wild-type, S518D and S518A) were overexpressed in SKOV3 cells and cell extracts were immunoblotted with the indicated antibodies.

3.12. Merlin inhibits MLK3, B-Raf, ERK and JNK activation

MLK3 is present in a multi-protein complex in cells that includes Raf-1 and B-Raf (Chadee et al., 2006). Furthermore, MLK3 is required for the full activation of B-Raf by growth factors (Chadee et al., 2006). Previously, it was observed that merlin disrupted the MLK3-B-Raf complex. Thus, we wished to test if induction of merlin expression in cells would inhibit both MLK3 and B-Raf kinase activities. For these
experiments we induced merlin expression in RT4-NF2.17 rat schwannoma cells that have doxycycline-inducible expression of wild-type merlin, and a low level of endogenous merlin. Induction of merlin expression in these cells significantly reduced the basal levels of p-MLK3 and p-JNK with no effect on total MLK3 or total JNK protein levels. Furthermore, induction of merlin expression reduced the basal levels of B-Raf phosphorylation (activation) on residues Thr599/Ser601 (p-B-Raf), and p-ERK with no effect on total B-Raf or total ERK protein levels (Figure 17A, right panel). Treatment of control RT4 cells that do not express an inducible merlin with doxycycline had no effect on total and phosphorylated MLK3, B-Raf, ERK and JNK levels (Figure 17A, left panel). These data suggest that merlin is a potent inhibitor of endogenous MLK3, B-Raf, ERK and JNK kinase activities in schwann cells. Next, we wished to test the effect of expression of functional merlin in cells (SKOV3 and HEI193) that have reduced or inactive merlin and elevated levels of p-MLK3. Consistent with the results obtained for RT4-NF2.17 cells, overexpression of merlin in SKOV3 and HEI193 cells significantly reduced the levels of p-MLK3, p-B-RAF, p-ERK and p-JNK (Figure 17B).
Figure 17. Merlin inhibits MLK3, B-Raf, ERK and JNK activities. (A) RT4 (control, left panel) and RT4-NF2.17 (inducible merlin, right panel) cells were treated with 1.0 μg/ml doxycycline for 24 h and immunoblotting of cell extracts was performed with antibodies that detect p-MLK3, p-B-Raf, p-JNK, MLK3, JNK and B-Raf. (B) SKOV3 and HEI193 cells were transfected with Myc-merlin expression plasmid and cell extracts were prepared and immunoblotting was performed with antibodies that detect p-MLK3, p-ERK, p-B-Raf, p-JNK, total MLK3, total ERK, B-Raf, JNK and β-Actin.
3.13. Loss of merlin elevates MLK3 and B-Raf kinase activities.

Since merlin expression in SKOV3 ovarian tumor cells inhibited MLK3, B-Raf, ERK and JNK activities, we sought to test if loss of merlin in immortalized ovarian epithelial cells that have functional merlin, would elevate MLK3, B-Raf, ERK and JNK activities. Merlin was depleted from T29 immortalized ovarian epithelial cells by siRNA-mediated knockdown and p-MLK3, p-B-Raf, p-ERK and p-JNK levels were analyzed (Figure 18). In T29 cells treated with merlin siRNA, p-MLK3, p-B-Raf, p-ERK and p-JNK levels were increased compared to control cells treated with nonspecific siRNA, supporting our findings above that merlin negatively regulates MLK3, B-Raf, ERK and JNK activation.
Figure 18. Loss of merlin elevates MLK3, B-Raf, ERK and JNK kinase activities.

T29 cells were untreated, or transfected with nonspecific or merlin siRNA oligos and cell extracts were immunoblotted with the indicated antibodies.
3.14. Merlin expression does not affect Cdc42 activity

Cdc42 is a key upstream regulator of MLK3 activity, such that binding of Cdc42 to the MLK3 CRIB domain relieves MLK3 autoinhibition and allows MLK3 autophosphorylation and activation (Du et al., 2005). The effect of merlin on Cdc42 activity is unknown. To determine whether merlin inhibits MLK3 indirectly by inhibiting Cdc42 activity, we examined Cdc42 activity in cells overexpressing merlin or treated with merlin siRNA. Cdc42 activity was unaffected by merlin overexpression or by merlin knockdown, suggesting that merlin suppression of MLK3 activity does not occur through inhibition of Cdc42 activity (Figure 19A). Consistent with this finding, overexpression of merlin also did not alter the levels of activated wild-type or constitutively active V12 Cdc42 (Figure 19B).
**Figure 19. Merlin does not affect Cdc42 activity.** (A) T29 cells were transfected with FLAG-Cdc42 and nonspecific or merlin siRNA. GTP-bound FLAG-Cdc42 was isolated from cell lysates with Pak-1 binding domain (PBD) beads and detected with FLAG antibody. For control samples, cell lysates were incubated with GTP γS or GDP prior to the addition of PBD beads. Protein expression was verified by Western blotting with the indicated antibodies. (B) HEK293 cells were transfected with FLAG-Cdc42 (wild-type, V12 or N17) with or without Myc-Merlin. GTP-bound FLAG-Cdc42 was isolated from cells lysates with PBD beads and detected with FLAG antibody. Western blotting of cell extracts was performed with FLAG and Myc antibodies to verify protein expression.
3.15. Merlin inhibits the interaction between Cdc42 and MLK3.

If merlin does not affect Cdc42 activity, then an alternative possibility is that merlin blocks MLK3 activation by hindering the interaction between MLK3 and Cdc42. To test this possibility, Cdc42-MLK3 binding was analyzed in the absence or presence of merlin expression in RT4 cells. As shown in Figure 20A, without doxycycline treatment, the basal merlin expression in RT4 cells was undetectable and endogenous MLK3 co-immunoprecipitated with FLAG-Cdc42. However, when merlin expression was induced with doxycycline, the interaction between MLK3 and FLAG-Cdc42 was dramatically reduced (Figure 20A). Induction of merlin expression caused a dramatic reduction in the interaction between endogenous MLK3 and FLAG-Cdc42 in RT4 cells (Figure 20A). A similar reduction in Cdc42-MLK3 binding was observed when GST-MLK3 and FLAG-Cdc42 were co-expressed in HEK293 cells with or without co-expression of merlin (Figure 20B). Thus, inhibition of the Cdc42-MLK3 interaction is one potential mechanism by which merlin could inhibit MLK3 kinase activity.
Figure 20. Merlin inhibits the interaction between Cdc42 and MLK3. (A) FLAG-Cdc42 was overexpressed in RT4 cells. Cells were left untreated or treated with 1.0 µg/ml doxycycline for 24 hours to induce Merlin expression. FLAG-Cdc42 was immunoprecipitated and immunoprecipitates were analyzed by immunoblotting with MLK3 antibody to detect associated endogenous MLK3. (B) Myc-Merlin, FLAG-Cdc42, and GST-MLK3 were overexpressed in HEK293 cells and FLAG-Cdc42 was immunoprecipitated and immunoprecipitates were probed with GST antibody to detect associated GST-MLK3.
3.16. Merlin expression reduces MLK3 activity by inhibiting the Cdc42-MLK3 interaction

Cdc42 is the key upstream regulator of MLK3. GTP-bound Cdc42 interacts with the MLK3 CRIB domain and promotes MLK3 autophosphorylation and activation (Du et al., 2005; Gallo and Johnson, 2002; Teramoto et al., 1996; Vacratsis and Gallo, 2000; Zhang and Gallo, 2001). Cdc42 not only relieves MLK3 autoinhibition, but it also relocalizes MLK3 to the plasma membrane, thereby facilitating its activation (Du et al., 2005). To determine whether merlin reduces the amount of active MLK3 bound to Cdc42, Cdc42 and MLK3 was transfected with or without merlin in HEK293 cells, and then coimmunoprecipitations were performed. Interestingly, merlin expression led to a substantial reduction in the amount of MLK3 and p-MLK3 bound to Cdc42 (Figure 21). These findings suggest that merlin inhibits Cdc42-mediated activation of MLK3 by blocking the Cdc42-MLK3 interaction.
Figure 21. Merlin inhibits MLK3 activity by inhibiting the interaction between Cdc42 and MLK3. Myc-merlin, FLAG-Cdc42, and HA-MLK3 were overexpressed in HEK293 cells. FLAG-Cdc42 immunoprecipitates and cell lysates were analyzed by Western blotting with the indicated antibodies.
3.17. Increased cell proliferation, due to a loss of merlin, requires MLK3.

We previously observed that silencing MLK3 expression inhibited cell proliferation of normal and tumor cells (Chadee and Kyriakis, 2004a). Conversely, knockdown of merlin stimulated cell proliferation in different cell types (Morrison et al., 2007; Okada et al., 2005; Xiao et al., 2005). Thus, to determine if cell proliferation, induced by the loss of merlin, is MLK3-dependent, T29 cells were transfected with merlin siRNA, MLK3 siRNA or both MLK3 and merlin siRNA. When compared to control cells transfected with nonspecific siRNA, cells with merlin knockdown had an increased rate of cell proliferation, and cells with MLK3 knockdown had a reduced rate of cell proliferation (Figure 22). Interestingly, the proliferation of cells with knockdown of both merlin and MLK3 was much less than cells with merlin knockdown alone, suggesting that MLK3 is required for merlin dependent suppression of cell proliferation.
Figure 22. Increased cell proliferation, due to a loss of merlin, requires MLK3. 10 x10⁴ T29 cells were seeded in 6 cm tissue culture dishes and transfected with nonspecific, human merlin, human MLK3 or human merlin plus human MLK3 siRNA. Cell proliferation was determined by counting cells at various times (as indicated in the figure) after transfection. Experiments were performed in triplicate and repeated three times. Cell extracts were also prepared from cells at each time interval and proteins from cell extracts were separated by 15% SDS PAGE and immunoblotted with merlin, MLK3 and β-Actin antibodies to determine the efficiency of knockdown of MLK3 and merlin.

3.18. Merlin overexpression reduces cell invasion.

Since SKOV3 cells have reduced merlin protein in comparison to other tumor derived cell lines that we analyzed, we wished to determine if overexpression of merlin in SKOV3 cells would impair cell invasion. Indeed, we found that overexpression of merlin in SKOV3 cells significantly reduced SKOV3 cell invasion (Figure 23).
Figure 23. Merlin overexpression reduced cell invasion. SKOV3 cells were transfected with empty vector or Myc-merlin expression plasmid. Cell invasion was analyzed using a modified Boyden chamber with matrigel and cells that traversed the membrane were stained and counted. Experiments were performed in triplicate and repeated three times.

3.19. MLK3 is required for cell invasion in cells lacking merlin.

We hypothesize that merlin loss in specific tumor cells causes elevated MLK3 and B-Raf kinase activities and persistent MAPK signaling, which could facilitate cellular transformation and tumorigenesis. Studies by others revealed that loss of NF2 gene expression increased cellular proliferation in NIH3T3 cells, increased cell invasion in MEFs and was sufficient for metastatic tumor development in murine schwann cells (Giovannini et al., 2000; Morrison et al., 2007; Poulakakis et al., 2006). To test if MLK3 is required for cellular transformation induced by merlin loss, we depleted merlin and MLK3 in normal SW10 murine schwann cells and analyzed cell invasion.
Previous studies showed that merlin silencing reduced cell invasion in mesothelioma cells (Poulakakos et al., 2006). Consistent with this, we observed a significant increase in the cell invasion upon depletion of merlin in SW10 cells. Interestingly, when both merlin and MLK3 were depleted, we observed an almost complete inhibition of cell invasion. These results indicate that MLK3 is required for invasion of cells that lack merlin (Figure 24).
**Figure 24. MLK3 is required for invasion of cells lacking merlin.** SW10 cells were treated with nonspecific siRNA oligo (lane 1), *NF2* siRNA (lane 2), or both *mlk3* and *NF2* siRNA. Cell invasion was analyzed using a modified Boyden chamber and cells that traversed the membrane were stained and counted. Experiments were performed in triplicate and repeated three times.
4. DISCUSSION

4.1. Part I: MLK3 in ovarian cancer

Ovarian cancer is the leading cause of death from gynecological malignancies with a high mortality rate that is primarily due to the late stage of diagnosis and limited treatment options (Dutta et al., 2010). Therefore, understanding the early events of ovarian tumorigenesis is important for the development of novel therapies to treat this disease. Recently, high MLK3 expression was reported in breast cancer cell lines (Chen et al., 2010a). Here we report elevated MLK3 expression and activity in SKOV3, HEY and HEY1B ovarian cancer cell lines in comparison to T29 and T80 immortalized ovarian epithelial cells (Figure 6). Furthermore, the level of active MLK3 decreased in confluent T29 cells, but remained high in confluent SKOV3 cells (Figure 7), suggesting that SKOV3 cells may have lost a density-dependent mechanism to down-regulate MLK3 activity. In addition, we observed elevated MLK3 levels in both benign and malignant ovarian tumor specimens. Collectively these results suggest that deregulation of MLK3 expression and activity could be early events in ovarian cancer development (Figure 8).

Interestingly, mutation of another MAP3K, BRAF (V600E), has also been observed in 31% of low-grade ovarian serous carcinoma and borderline tumors, but not in high-grade tumors (Mayr et al., 2006). Possibly, aberrant MAP3K activities in the early stages promote persistent MAPK signaling and tumorigenesis of ovarian cancer development.

Using RNAi mediated MLK3 gene silencing, we found that MLK3 expression is
crucial for invasion of SKOV3 cells (Figure 9). Indeed, other recent reports indicate a requirement for MLK3 in invasion of mammary epithelial cells and migration of gastric tumor cells and A459 lung cells (Chen et al.; Mishra et al.; Swenson-Fields et al., 2008). These results point to an emerging role for MLK3 in invasion and migration of numerous cell types.

We observed high MMP-1, -2, -9, and -12 gene expression in SKOV3 cells in comparison to T29 cells (Figure 10) and silencing MLK3 substantially reduced MMP -1, -2, -9 and -12 expression and MMP-2/9 activity in SKOV3 cells (Figure 11). Possibly MLK3 dependent activation of MMP expression could promote invasion of ovarian cancer cells. While MMP expression is an indicator of ovarian tumor malignancy, prominent MMP gene expression was also found in benign tumor cells and preneoplastic lesions (Cai et al., 2007). Therefore, it is possible that elevated MLK3 levels may also be contributing to enhanced MMP expression in benign ovarian tumors.

MMPs are regulated by a number of different transcription factors, including Nuclear Factor-kappa B (NFκB) and AP-1. AP-1 is a c-Fos/c-Jun heterodimer, and c-Fos and c-Jun are activated by ERK and JNK respectively (Shen and Brown, 2003). Aberrant Ras/MAPK signaling is frequently observed in ovarian cancer and treatment of ovarian cancer cells harboring B-Raf or KRAS mutations with the MEK inhibitor CI-1040 induces profound growth inhibition and apoptosis, which emphasizes the critical role for ERK1/2 signaling in tumor growth and survival of ovarian cancers (Nakayama et al., 2008). Since MLK3 is required for ERK and JNK activation in SKOV3 cells, possibly,
high levels of active MLK3 in ovarian tumor cells could promote constitutive JNK and ERK signaling and aberrant cell proliferation (Rennefahrt et al., 2002; Schutte et al., 1989).

Based on these findings, we propose a model for the role of MLK3 in ovarian tumor development. Inappropriate MLK3 expression and deregulated levels of active MLK3 in ovarian tumor cell lines activates downstream ERK and JNK signaling, and MMPs expression, which promotes invasion of ovarian tumor cells (Figure 25).
MLK3 activates ERK and JNK MAPK signaling pathways, and promotes MMP expression and activation.

Figure 25. Role of MLK3 in MMP expression and ovarian cancer development.
4.2. Part II: MLK3 is regulated by merlin

The tumor suppressor protein merlin links extracellular cues to multiple intracellular signaling pathways to regulate cell size, motility, proliferation and survival (review by Stamenkovic and Yu, 2010). It has been proposed that the growth suppressive function of merlin may be mediated by its binding partners (Scoles, 2008). For example, merlin binding partners that interact with the C-terminus of merlin, such as Syntenin (binds to 566-595 of merlin), EG1/Magician (binds to 340-590 of merlin), HEI10 (binds to 306-339 of merlin) and hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) (binds to 453-557 of merlin), are all growth activators and their function is suppressed by merlin (Scoles, 2008). Our results demonstrate that merlin and MLK3 are associated in situ and the C-terminal residues 340-590 of merlin directly interact with MLK3 (Figure 14). Interestingly, this region of merlin (340-590) is sufficient for the direct inhibition of MLK3 kinase activity in vitro (Figure 15). Furthermore, merlin suppression of T29 cell proliferation requires MLK3 (Figure 22), which suggests an important functional significance of the merlin-MLK3 interaction.

Merlin phosphorylation at residue Ser518 regulates its growth suppressive function. Phosphorylation of this residue inactivates merlin and changes merlin intramolecular structure, and predominantly localized at the plasma membrane, while the wild-type of merlin is active and is mainly localized to the cytoplasm (Kissil et al., 2002). In this study, we demonstrate that S518D inactive mutant of merlin exhibits markedly
reduced binding to MLK3 and does not inhibit MLK3 activity in comparison to wild-type merlin, indicating that only active merlin can bind to MLK3 and inhibit MLK3 activity (Figure 16). Merlin is activated upon dephosphorylation of Ser518 by the myosin phosphatase MYPT-1-PP1δ (Jin et al., 2006). MYPT1 is inhibited by the oncoprotein CPI-17, and altered CPI-17 protein levels in tumor cells leads to deregulation of MYPT-1-PP1δ activity and increased levels of phosphorylated, inactive merlin (Jin et al., 2006). Possibly, inactivation of merlin through this mechanism could also elevate the level of active MLK3 in cells.

In our study, we found that merlin expression inhibits Cdc42-MLK3 association (Figure 20 and Figure 21). These results suggest that merlin may compete with Cdc42 for binding to MLK3, and thereby, inhibit MLK3 phosphorylation and activation. We considered the possibility that merlin inhibits Cdc42 activity. For instance, merlin interacts with and inhibits RhoGD1, Ras- and Rac- dependent signaling and transformation (Maeda et al., 1999; Morrison et al., 2007; Shaw et al., 2001; Tikoo et al., 1994). However, our results indicate that Cdc42 was not affected by merlin. Thus, merlin inhibition of MLK3 is not a result of inhibition of Cdc42 by merlin.

MLK3 protein levels have been found to be elevated in breast and ovarian cancer cells (Chen et al., 2010a; Zhan et al., 2011). However, deregulated MLK3 kinase activity in tumor-derived cells has not been previously reported. We found that SKOV3 ovarian cancer cells, NCIH460 lung cancer cells and HEI193 schwannoma tumor cells have elevated basal MLK3 kinase activity (Figure 12), suggesting that MLK3 kinase activity is
deregulated in these different types of tumor cells.

The HEI193 cells are NF2 patient derived tumor cells, which lack expression of merlin. It has been reported that mutations or loss of merlin occurs in other different types of cancers, including mesotheliomas and prostate cancers (Horiguchi et al., 2008; Thurneysen et al., 2009). Merlin expression is reduced in human gliomas and reexpression of functional merlin inhibits glioma cell proliferation (Lau et al., 2008). Interestingly, we also found that SKOV3 and NCIH460 tumor cells have reduced merlin expression (Figure 12). Possibly, loss of functional merlin in these cells may also be a contributing factor for cell transformation and tumorgenesis in ovarian and lung cancer.

MLK3 interacts with B-Raf and facilitates B-Raf activation; and merlin expression disrupts the MLK3-B-Raf interaction (Chadee et al., 2006). Our recent results indicate that silencing merlin elevates B-Raf and ERK activities in normal ovarian epithelial cells (Figure 18) and overexpression of merlin blocks B-Raf and ERK activities in ovarian tumor cells (Figure 17). Possibly, loss of functional merlin promotes MLK3-B-Raf complex formation, thereby facilitating ERK activation in SKOV3 ovarian tumor cells. In addition, we observed that silencing merlin in normal T29 ovarian epithelial cells significantly elevated MLK3 and JNK kinase activities. MLK3 directly phosphorylates and activates the MAP2K, MKK4/SEK1, which in turn phosphorylates and activates JNK (Rana et al., 1996). Thus, deregulation of MLK3 activity, resulting from loss of functional merlin, may cause persistent JNK signaling which can also promote cellular transformation and tumorigenesis (Khatlani et al., 2007; Rennefahrt et
Consistent with this hypothesis, we observed that merlin suppression of cell growth and invasion is dependent on MLK3 in T29 and SW10 cells.

It has been previously reported that merlin inhibits both Rac1 and MAPK signaling through different mechanisms. For example, merlin can interact with and inhibit the expression of Grb2 protein, thereby inhibiting Ras/Rac and MAPK signaling pathways (Lim et al., 2006; Morrison et al., 2007). Our results demonstrate that merlin blocks Cdc42 binding to MLK3, and inhibits MLK3 dependent B-Raf, ERK and JNK activation; and providing a novel mechanistic link by which merlin can suppress both Rho-GTPase and MAPK signaling. We propose a model where merlin directly binds to and inhibits MLK3 by inhibiting the Cdc42-MLK3 interaction. In schwannoma and ovarian tumor cells, loss of functional merlin elevates MLK3, ERK and JNK signaling by allowing the formation of Cdc42-MLK3 and B-Raf-MLK3 signaling complexes (Figure 26).
Figure 26. Proposed model of the regulation of MLK3-dependent MAPK signaling by merlin. Merlin inhibits MLK3 kinase activity and the activation of ERK and JNK MAPK signaling pathways. Tumor cells lacking functional merlin have elevated MLK3 activity and MAPK activation.
Summary

Collectively, we demonstrated inappropriate MLK3 expression and deregulated levels of active MLK3 in ovarian tumor cell lines and tumor tissue specimens; and a novel requirement for MLK3 in MMP expression and invasion of ovarian cancer cells. Our data also implicate merlin as a critical regulator of MLK3 function. We showed that merlin regulates MLK3 activity by directly interfering with MLK3 binding to Cdc42, and that merlin suppression of cell growth and invasion are dependent, at least in part, on MLK3 expression. Hence, targeting MLK3 could be a novel approach for the development of therapies to treat tumors that lack functional merlin. We demonstrated that AA340-590 of merlin is required for the merlin-MLK3 interaction. If the minimal region of merlin that is required for binding to MLK3 is identified, then a small peptide that contains this region may be a promising candidate for medical therapies for NF2 and ovarian cancer. MLK3 has an important scaffold function in ERK activation, thus, targeting MLK3 kinase activity may not be sufficient to block MLK3-dependent signaling pathways in tumor cells. Possibly, targeting MLK3 by a mechanism that depletes the entire protein, instead of just the kinase activity, would be the better option to achieve a therapeutic effect in treating NF2 or ovarian tumors.
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