Mixed-Lineage Kinase 3 Deficiency Promotes Neointima Formation Through Increased Activation of the RhoA Pathway in Vascular Smooth Muscle Cells

Vidya Gadang, Eddy Konaniah, David Y. Hui, Anja Jaeschke

Objective—Mitogen-activated protein kinase pathways play an important role in neointima formation secondary to vascular injury, in part by promoting proliferation of vascular smooth muscle cells (VSMC). Mixed-lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase that activates multiple mitogen-activated protein kinase pathways and has been implicated in regulating proliferation in several cell types. However, the role of MLK3 in VSMC proliferation and neointima formation is unknown. The aim of this study was to determine the function of MLK3 in the development of neointimal hyperplasia and to elucidate the underlying mechanisms.

Approach and Results—Neointima formation was analyzed after endothelial denudation of carotid arteries from wild-type and MLK3-deficient mice. MLK3 deficiency promoted injury-induced neointima formation and increased proliferation of primary VSMC derived from aortas isolated from MLK3-deficient mice compared with wild-type mice. Furthermore, MLK3 deficiency increased the activation of p63Rho guanine nucleotide exchange factor, RhoA, and Rho kinase in VSMC, a pathway known to promote neointimal hyperplasia, and reconstitution of MLK3 expression attenuated Rho kinase activation. Furthermore, cJun NH₂-terminal kinase activation was decreased in MLK3-deficient VSMC, and proliferation of wild-type but not MLK3 knockout cells treated with a cJun NH₂-terminal kinase inhibitor was attenuated.

Conclusions—We demonstrate that MLK3 limits RhoA activation and injury-induced neointima formation by binding to and inhibiting the activation of p63Rho guanine nucleotide exchange factor, a RhoA activator. In MLK3-deficient cells, activation of p63Rho guanine nucleotide exchange factor proceeds in an unchecked manner, leading to a net increase in RhoA pathway activation. Reconstitution of MLK3 expression restores p63Rho/p63Rho guanine nucleotide exchange factor interaction, which is attenuated by feedback from activated cJun NH₂-terminal kinase. (Arterioscler Thromb Vase Biol. 2014;34:00-00.)

Key Words: neointima • Rho-associated kinases
microtubule instability, independently of JNK activation. Furthermore, ectopic overexpression of kinase-inactive MLK3 is able to rescue mitogen-induced activation of ERK in cells that have been depleted of endogenous MLK3, and MLK3 missense mutations have been identified in gastrointestinal tumors that probably affect MLK3 scaffold properties but not its kinase activity. In addition, MLK3 has been shown to limit RhoA activation by binding p63Rho guanine nucleotide exchange factor (GEF), independently of its catalytic activity. Interestingly, p63RhoGEF has been identified as a key mediator of angiotensin II–dependent RhoA activation in VSMC. RhoA is abundantly expressed in VSMC and controls a wide range of cellular functions, such as contraction, migration, and proliferation, and it is thought that many of the beneficial, nonlipid-lowering effects of statins used in the prevention and treatment of cardiovascular diseases are because of their ability to inhibit RhoA. Furthermore, inhibition of the RhoA effector protein, Rho kinase (ROCK), inhibits intimal hyperplasia, indicating that the Rho/ROCK pathway promotes neointima formation in response to injury.

The goal of this study was to determine the role of MLK3 in VSMC proliferation and neointima formation. We demonstrate that MLK3 limits RhoA activation and injury-induced neointima formation by binding to and inhibiting the activation of p63RhoGEF. In MLK3-deficient cells, activation of p63RhoGEF proceeds in an unchecked manner, leading to a net increase in RhoA pathway activation. In addition, MLK3 catalytic activity is required for JNK activation, which, through reduction of MLK3–p63RhoGEF association, provides a feedback mechanism to dampen MLK3-mediated attenuation of RhoA pathway activation and cell proliferation. Together, these studies establish an important function for MLK3 in VSMC proliferation and intimal hyperplasia in response to vascular injury.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
MLK3 Deficiency Promotes Neointima Formation In Vivo
MLK3 is an MAP kinase kinase kinase that activates multiple MAPK pathways, and MAPKs play a central role in the development of injury-induced neointimal hyperplasia. Therefore, we anticipated that Mlk3−− mice would be protected against neointimal hyperplasia after endothelial denudation. Surprisingly, we found significant injury-induced neointimal hyperplasia and thickening of the vessel wall in MLK3 knockout (KO) mice compared with minimal neointima formation in injured wild-type (WT) mice (Figure 1A). Immunohistochemical staining of smooth muscle α-actin demonstrated that VSMC are the main cellular component in the injury-induced neointimal area in Mlk3−− mice (Figure 1A, bottom). Morphometric analysis of uninjured and injured carotid arteries from 8 mice per genotype confirmed a significant neointimal area in Mlk3−− mice (Figure 1B). In addition, morphometric measurements revealed a significant increase in medial thickness in injured arteries of MLK3 KO compared with WT mice (Figure 1C). No difference in neointimal area and medial thickness was observed between uninjured control carotid arteries of WT and Mlk3−− mice (Figure 1B and 1C). Together, these data demonstrate that MLK3 deficiency promotes injury-induced neointima formation.

MLK3 Deficiency Increases VSMC Proliferation In Vitro
Previous studies have established that proliferation of VSMC is involved in neointimal hyperplasia, and we found that VSMC are the main cellular component in the injury-induced neointimal area in MLK3 KO mice. Therefore, we isolated primary VSMC from WT and Mlk3−− mice to determine the mechanism by which MLK3 deficiency exacerbates neointima formation. On injury or when established in culture, VSMC undergo a switch from a contractile to a synthetic phenotype, which is associated with changes in morphology, cytoskeleton, and synthesis and secretion of extracellular matrix components. To analyze MLK3 smooth muscle cell phenotype, we isolated primary VSMC from WT and Mlk3−− mice and serum-starved them for 48 hours. No gross morphological differences were observed in quiescent VSMC from Mlk3−− and Mlk3−− mice (data not shown). Similarly, immunoblot analysis of cytoskeletal markers, such as vinculin and calponin, revealed no differences in the expression of cytoskeletal proteins (Figure IA in the online-only Data Supplement). Furthermore, quantitative polymerase chain reaction analysis of fibronectin and collagen mRNA demonstrated comparable expression of extracellular matrix components in WT and MLK3-deficient cells (Figure IB in the online-only Data Supplement). However, we found that the proliferation rate of MLK3-deficient VSMC in medium supplemented with 10% fetal bovine serum was increased compared with WT cells (Figure 2A). Consistent with this, VSMC isolated from Mlk3−− mice displayed a dose-dependent increase in [H]thymidine incorporation into DNA with maximal 6-fold increase observed at platelet-derived growth factor (PDGF)-BB concentrations of 10 ng/mL, in contrast to the 3-fold increase observed in WT cells (Figure 2B). Together, these data show that MLK3 deficiency increases the growth rate of VSMC in response to growth factors in vitro.

MLK3 Deficiency Increases RhoA Pathway Activation
The RhoA pathway has been established as an important mediator of VSMC proliferation, and previous studies have
demonstrated that MLK3 expression attenuates RhoA activation.17,26 Therefore, to determine the underlying mechanism for increased proliferation of Mlk3−/− VSMC, we next evaluated RhoA activation. Using a GST-Rhotekin pulldown assay that specifically recognizes active, GTP-bound RhoA in combination with immunoblot analysis, we found that RhoA activation was significantly higher in MLK3-deficient compared with WT VSMC (Figure 3A). It has been well documented that activation of RhoA is associated with translocation to the membrane. Therefore, we performed subcellular fractionations to monitor the distribution of RhoA by immunoblot analysis. We observed that RhoA translocation to the membrane was increased in MLK3 KO VSMC compared with WT VSMC (Figure 3B). Because many vascular functions of RhoA are mediated by the RhoA effector protein, ROCK, we next analyzed ROCK activation. Using phosphorylation of the ROCK target protein MYPT1 as marker, we found that activation of ROCK is increased in Mlk3−/− VSMC compared with WT cells (Figure 3C). Together, these data suggest that MLK3 deficiency increases the activation of RhoA and ROCK, which may result in increased VSMC proliferation. To test this, we treated VSMC with Y27632, an inhibitor that specifically inhibits ROCK activity and has been demonstrated to inhibit VSMC proliferation in vitro and injury-induced neointima formation in vivo,38 and analyzed the growth rate of WT and MLK3-deficient VSMC in medium supplemented with 10% fetal bovine serum. Treatment with the ROCK inhibitor significantly reduced growth of WT and MLK3 KO VSMC (Figure 3D), placing MLK3 upstream of RhoA and ROCK. One downstream effect of RhoA activation is to reduce the expression of cell cycle inhibitors such as the cyclin-dependent kinase inhibitors p21Waf1/Cip1 and p27Kip1,37 and numerous studies have implicated p27Waf1 and p21Cip1 in the control of VSMC proliferation and neointima formation.38–40 Therefore, we performed immunoblot analysis of p27Kip1 and p21Cip1 in WT and Mlk3−/− VSMC. p27Kip1 expression was decreased in MLK3-deficient compared with WT cells (Figure 3E). Similarly, in agreement with the increased proliferative capacity of MLK3-deficient VSMC, we observed significantly reduced p21Cip1 levels in Mlk3−/− VSMC (Figure 3F). Together, these data indicate that MLK3 deficiency results in increased activation of the Rho/ROCK pathway and decreased p27Kip1 and p21Cip1 expression. 

MLK3 Limits p63RhoGEF Activation

Rho GTPases are activated by GEFs that catalyze the exchange of GDP for GTP. Earlier studies have suggested that MLK3 modulates RhoA activity by binding to p63RhoGEF.45 To evaluate the effect of MLK3 deficiency on p63RhoGEF activation, we used RhoA[V14] affinity chromatography, in combination with immunoblot analysis of p63RhoGEF. This assay takes advantage of a nucleotide-free mutant RhoA, with a high affinity for active GEFs.46 PDGF-BB treatment caused p63RhoGEF activation in cells of both genotypes; however, PDGF-induced p63RhoGEF activation was markedly increased.
Reconstitution of MLK3 Expression in MLK3-Deficient Cells Attenuates VSMC Proliferation

Based on our finding that MLK3 deficiency increases vascular smooth muscle cell (VSMC) proliferation in vitro, we determined the effect of MLK3 expression on the ability of PDGF (PDGF)-BB to regulate RhoA activity. For this, we coexpressed MLK3 and GST-tagged p63RhoGEF or GST control in the presence or absence of SP600125, a JNK inhibitor, to test whether JNK activity is required for VSMC proliferation. Treatment of WT cells with PDGF-BB caused increased JNK activation that was detected by immunoblot analysis using an antibody to the JNK T-loop phosphorylation site. JNK phosphorylation was markedly reduced in MLK3-deficient cells, whereas loss of MLK3 had little effect on PDGF-induced phosphorylation of ERK (Figure 6A). These data suggest that MLK3 is not required for ERK activation but contributes to PDGF-induced JNK activation in VSMC. To test whether JNK activity is required for VSMC proliferation, we treated WT and MLK3-deficient cells with SP600125, a compound that specifically inhibits JNK activity, and analyzed the growth rate of WT and MLK3-deficient VSMC in medium supplemented with 10% fetal bovine serum. Treatment with the JNK inhibitor significantly reduced growth of WT but had only minimal effects in MLK3 KO VSMC (Figure 6B), suggesting that MLK3-dependent activation of JNK contributes to VSMC proliferation. Previous studies have demonstrated that activation of JNK decreases binding of MLK3 to p63RhoGEF and thus attenuates MLK3-mediated decrease of cell proliferation. Therefore, we hypothesized that JNK inhibition increases the interaction of MLK3 with p63RhoGEF. To test this hypothesis, we coexpressed MLK3 and GST-tagged p63RhoGEF or GST control in the presence or absence of SP600125, affinity-purified proteins using glutathione sepharose, and determined MLK3 binding by immunoblot analysis using an antibody specific for MLK3. We found that MLK3 interacts with GST-p63RhoGEF but not with GST control (Figure 6C, left) and that this association is increased in the presence of the JNK inhibitor (Figure 6C, right).

Discussion

Previously, it was shown that MLK3 deficiency has either no effect or an inhibitory effect on cell proliferation. In contrast, here we find that MLK3-deficient VSMC display
accelerated proliferation in response to growth factor and serum stimulation in vitro, resulting in neointimal hyperplasia after endothelial denudation, whereas no difference in neointimal area is observed in uninjured carotid arteries of WT and Mlk3−/− mice. Comparison of carotid arteries of MLK3 KO and WT mice also reveals an increase in medial thickness in response to injury, but not in uninjured vessels.

The underlying mechanism for this is increased activation of the RhoA pathway. Genetic inactivation and pharmacological inhibition have established RhoA and ROCK as important mediators of VSMC proliferation,28,33,36 and, in agreement with previous reports,17,26 we find increased RhoA and ROCK activation in MLK3-deficient VSMC. We show that treatment of cells with the ROCK inhibitor Y27632 significantly reduced the growth of WT and MLK3−/− VSMC in medium supplemented with 10% fetal bovine serum, placing RhoA and ROCK downstream of MLK3. However, we observe no difference in the phenotype of quiescent VSMC or growth of WT and Mlk3−/− VSMC under basal conditions. Interestingly, earlier studies have demonstrated that although Rho activation is necessary for DNA synthesis, activation of this GTPase is not sufficient to induce proliferation in VSMC.24 Instead, it seems to potentiate the effects of Ras/MAPK or growth factors to stimulate cell cycle progression.37 One mechanism by which RhoA and ROCK activation controls VSMC proliferation is by modulating the expression of cell cycle inhibitors p21Waf1/Cip1 and p27Kip1.37,42,43 Consistent with this, we find decreased levels of p21Waf1/Cip1 and p27Kip1 in MLK3-deficient VSMC compared with WT cells.

Rho GTPases are regulated by Rho GEFs, which catalyze the conversion of Rho GTPases from the inactive GDP-bound to the active GTP-bound form. p63RhoGEF was originally identified as a 63-kDa Rho-GEF that specifically activates RhoA.44 Several lines of evidence indicate that p63RhoGEF specifically binds to Gαq/11, but not Gα12/13 subunits of heterotrimeric G proteins, thereby linking Gαq/11-coupled receptors to RhoA activation.45–47 Here, we show, in agreement with previous studies,26 that MLK3 associates with p63RhoGEF and inhibits p63RhoGEF-induced RhoA activation measured by GST-Rhotekin pulldown assay.
Interestingly, p63RhoGEF was shown to be a key mediator of angiotensin II–dependent signaling processes in VSMC, as well as serum-dependent RhoA activation and chemotactic migration in breast cancer cells. Because migration of VSMC from the media to the intima is a mechanism that contributes to neointima formation, future studies will focus on the role of MLK3 in VSMC migration.

Our studies also show that PDGF-induced JNK activation is attenuated in MLK3-deficient cells compared with WT VSMC. This indicates a nonredundant role for MLK3 in PDGF-induced JNK activation in VSMC in contrast to the redundant function observed in mouse embryonic fibroblasts. In addition, we do not find that MLK3 is required for ERK activation in VSMC, contrary to studies in tumor cells. These differences may be caused by redundancy of MLK3 with other MLK isoforms in specific cell types.

We demonstrate that reconstitution of MLK3-deficient VSMC with catalytically inactive MLK3 decreases cell proliferation. Similarly, pharmacological inhibition of JNK attenuates proliferation of WT but not MLK3 KO cells, indicating that JNK signals through MLK3 to regulate VSMC proliferation. We find that JNK inhibition increases binding of MLK3 to p63RhoGEF, providing a mechanism by which MLK3 regulates VSMC proliferation. How JNK modulates association

Figure 4. Interaction of mixed-lineage kinase 3 (MLK3) with p63Rho guanine nucleotide exchange factor (GEF) limits RhoA activation. A, Vascular smooth muscle cells (VSMC) from wild-type (WT) and MLK3-deficient (Mlk3−/−) mice were treated with platelet-derived growth factor (PDGF)-BB for 10 minutes. p63RhoGEF activation was determined by RhoAG17A affinity chromatography in combination with immunoblot analysis. B, GST-p63RhoGEF and MLK3 WT or a kinase-inactive mutant (MLK3 KI) was coexpressed in HEK293 cells, and RhoA activation was determined by binding to GST-Rhotekin and subsequent immunoblot analysis.

Interestingly, p63RhoGEF was shown to be a key mediator of angiotensin II–dependent signaling processes in VSMC as well as serum-dependent RhoA activation and chemotactic migration in breast cancer cells. Because migration of VSMC from the media to the intima is a mechanism that contributes to neointima formation, future studies will focus on the role of MLK3 in VSMC migration.

In summary, we show that MLK3 deficiency increases VSMC proliferation and exacerbates injury-induced neointima formation because of increased p63RhoGEF, RhoA, and ROCK activation. Furthermore, we demonstrate that loss of JNK activation, because of the expression of catalytically inactive MLK3 or pharmacological inhibition of JNK, attenuates VSMC proliferation. Furthermore, we provide evidence that inhibition of JNK increases binding of MLK3 to p63RhoGEF. Based on these data, we propose the following model for regulation of MLK3–p63RhoGEF interaction.

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proliferation. (Figure 6D). Together, these studies establish an important role for MLK3 in VSMC proliferation and neointimal hyperplasia in response to vascular injury.

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Disclosures
None.

References

Figure 6. Mixed-lineage kinase 3 (MLK3) mediates platelet-derived growth factor (PDGF)-BB–induced cJun NH2-terminal kinase (JNK) activation in vascular smooth muscle cells (VSMC). A, VSMC from wild-type (WT) and MLK3-deficient (Mlk3−/−) mice were treated with PDGF-BB for the indicated times. JNK and extracellular signal–regulated kinase (ERK) expression and phosphorylation were determined by immunoblot analysis (top). Relative phosphorylation of JNK and ERK was obtained by normalization to total JNK and ERK expression (bottom). Values are mean±SD from 3 experiments. B, VSMC from WT and Mlk3−/− mice were cultured in DMEM containing 10% fetal bovine serum in the presence of JNK inhibitor (20 μmol/L) or vehicle control, and viable cell number was determined by direct counting. Data represent ±SEM from triplicate assays from 3 independent experiments. *Significant difference from untreated cells of the same genotype at P<0.05. C, GST, GST-p63RhoGEF, and MLK3 were coexpressed in HEK293 cells and treated with SP600125 (20 μmol/L) overnight. GST-p63RhoGEF or GST control was affinity purified using glutathione sepharose, and MLK3 binding was determined by immunoblot analysis for MLK3. D, Proposed model for role of MLK3 in VSMC proliferation. MLK3 limits RhoA activation by binding to and inhibiting activation of p63RhoGEF. In addition, MLK3 mediates JNK activation, which feeds back to attenuate interaction of MLK3 and p63RhoGEF and releases inhibition of RhoA/Rho kinase (ROCK) activation by MLK3. ERK-P indicates phosphorylated extracellular signal–regulated kinase; JNK-P, phosphorylated cJun NH2-terminal kinase; and ns, statistically not significant.
Mixed-lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase that activates multiple mitogen-activated protein kinase pathways that have been implicated in neointimal hyperplasia secondary to vascular injury. However, until now, the role of MLK3 in vascular smooth muscle cells was not known. Here, we report, for the first time, that genetic deletion of MLK3 promotes neointima formation after endothelial denudation. Mechanistically, this phenotype is attributed to loss of MLK3 binding to p63RhoGEF, resulting in increased p63Rho-kinase inhibition and neointima formation.

Significance

Mixed-lineage kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase that activates multiple mitogen-activated protein kinase pathways that have been implicated in neointimal hyperplasia secondary to vascular injury. However, until now, the role of MLK3 in vascular smooth muscle cells was not known. Here, we report, for the first time, that genetic deletion of MLK3 promotes neointima formation after endothelial denudation. Mechanistically, this phenotype is attributed to loss of MLK3 binding to p63RhoGEF, resulting in increased p63Rho-kinase inhibition and neointima formation.
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Supplemental figure I: A, Expression of αSMA, vinculin, calponin, caldesmon and MLK3 in serum-starved VSMC from WT and Mlk3<sup>−/−</sup> mice was determined by immunoblot analysis. Tubulin was used as loading control. (* indicates a non-specific band). B, Expression of fibronectin and collagen-1 mRNA was measured by qPCR.
Materials and Methods

Antibodies and Other Material: MYPT1-P (#4563), JNK-P (#9255), ERK-P (#9106), ERK (4695), c-Jun (#9165), c-Jun-P (#9261), MLK3-P (#2811), and Cadherin (#4068) antibodies were purchased from Cell Signaling, MA. RhoA (sc-418), MLK3 (sc-536), MYPT1 (sc-25618), GST (sc-459), p27kip1 (sc-528), p21cip1 (sc-6246) were purchased from Santa Cruz Biotechnology, CA. Tubulin (cat#T8203), smooth muscle α actin antibodies (cat#A2547), JNK inhibitor SP600125, ROCK inhibitor Y-27632, and PDGF-BB were purchased from Sigma Aldrich, MO, GEFT (cat#51004-1-AP) antibody was purchased from Proteintech, IL, JNK (cat#554285) antibody was purchased from BD Pharmigen, CA.

Mice: Mlk3−/− mice were backcrossed 10 generations to the C57BL/6J strain (Jackson laboratories) and have been previously described. Mlk3−/− mice and C57BL/6J mice were obtained from homozygous crosses and were housed in a facility accredited by the American Association for Laboratory Animal Care. The animal studies were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

In vivo Injury by Endothelial Denudation: The procedures used for endothelial denudation, tissue preparation and evaluation of neointimal formation were performed as described by Zhu et al.2 Briefly, the left carotid arteries of Mlk3−/− and wild-type mice were injured mechanically with an epon resin probe. At 14 days after surgery, the animals were anesthetized, perfusion fixed in situ, and the entire neck was dissected from each mouse to allow evaluation of both injured and uninjured control vessels on the same section. Parallel sections were processed and subjected to hematoxylin and eosin staining as well as to Verhoeff Van-Gieson (VVG) staining of the elastic lamina as described by Zhu et al.2 The sections were also subjected to smooth muscle α-actin stain. Briefly, after blocking with horse serum for 30 min sections were incubated with anti-smooth muscle α-actin as primary antibody overnight at 4°C, washed with PBST, incubated with biotinylated secondary antibody and with streptavidin peroxidase conjugate (Vectastain ABC elite kit, Vector Labs, CA), and developed with Nova Red Substrate (Vector Nova Red substrate kit, Vector Lab, CA). The slides were lightly counterstained with hematoxylin.

Morphometric analysis: For each artery, luminal area, area inside the internal elastic lamina and the area encircled by the external elastic lamina was measured. To calculate the medial thickness for each vessel cross-section, the linear distance between internal elastic lamina and external elastic lamina was measured independently in four places, and averaged. The intimal area was calculated as internal elastic lamina-luminal area.

Vascular Smooth Muscle Cell (VSMC) Isolation: Aortic smooth muscle cells were isolated from WT and Mlk3−/− mice using enzyme dispersion method as previously described. All experiments were performed with cells between passages 3 and 5.

Proliferation Assay: VSMC cells were seeded in flat-bottom 96-well microtiter plates at 2500 cells per well. Cells were serum starved in Dulbecco’s modified Eagles Medium (DMEM) containing 0.2% fetal bovine serum (FBS) for 48 h and then stimulated with PDGF-BB (Invitrogen) at varying concentration (in quadruplets) for 16 h at 37°C. A pulse of 1 µCi [3H]
thymidine was added to the incubation medium 6 h prior to lysis with 0.025 M ammonium hydroxide. Radioactivity in the cell lysate was quantified by liquid scintillation counting.

**Growth Curve:** Growth rates were determined by seeding 10,000 cells per well in 24-well plates in 10% FBS. Trypsinized cells from each well were counted at least three times. Cells were counted by trypan blue exclusion method from three wells by trypsinization at 2, 4, 6, 8, 12, and 16 days after plating.

**Rho Activation:** RhoA activation was determined using two different assays. To measure Rho translocation to the cell membrane, PDGF-bb (10 ng/ml) treated cells were disrupted in lysis buffer (20 mM Tris pH 7.4, 300 mM sucrose, 10 mM EGTA, 5 mM EDTA, 0.3 mM PMSF, 5 mM DTT). Lysates were centrifuged at 17,000 x g for 15 min at 4°C. Supernatants were collected as the cytosolic fraction. Pellets were, re-suspended in 5xSDS sample buffer containing 1% Triton X100 for 30 min, and centrifuged at 17,000 x g for 15 min. The resulting supernatant was collected as membrane fraction. Alternatively, (GTP-bound) RhoA was affinity isolated from cell lysates after stimulation with 10% FBS for 10 min by precipitation with the Rho binding domain of the Rho effector, rhotekin according to the manufacturer’s instructions (Rho Activation Assay Kit, Cell Biolabs, Inc, San Diego, CA). For both assays, protein was subjected to 15% SDS-PAGE, followed by electrophoretic transfer and immune detection of RhoA. Total RhoA expression was measured in whole cell lysates.

**Immunoblotting:** Cell extracts were prepared using Triton lysis buffer (20 mM Tris [pH 7.4], 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and a 10-µg/ml concentration of aprotinin and leupeptin). Proteins were separated by SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore, MA). After incubating in blocking buffer (5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST)), membranes were incubated with primary antibody (1:1000 dilution) overnight at 4°C with gentle shaking. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; Dako, CA). Immune complexes were visualized by enhanced chemiluminescence (NEN).

**Quantitative PCR:** Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed using the iScript cDNA synthesis kit from Biorad and collagen and fibronectin gene expression was determined by quantitative PCR analysis using the Bio-Rad iCycler iQ real-time PCR Detection System and was normalized to the expression of actin using Taqman assays (Mm00607939_s1, Mm00801666_g1, Mm01256744_m1) (Applied Biosystems).

**Affinity purification of activated p63RhoGEF:** WT and MLK3 KO VSMC lysates were affinity purified using RhoA G17A agarose beads (Cell BioLabs, CA) according to the manufacturer’s instructions. The precipitated GEF was then detected by immunoblot analysis using an anti-p63RhoGEF antibody (ProteinTech, IL).

**Retroviral transduction of VSMC:** Retroviral vectors expressing wild type and a kinase inactive version of MLK3 (MLK3K144R) (pBabepuro-MLK3_WT and pBabepuro-MLK3_KI) were
used to reconstitute MLK3 expression in MLK3 KO VSMCs. VSMC isolated from MLK3 KO mice were seeded into 6 well plates. After reaching 75% confluence, cells were transduced with retrovirus four times per day for two days, and transduced cells were selected in medium containing 5 µg/ml puromycin for 4 days as described. MLK3 expression was determined by immunoblot analysis.

**Cloning, Transfection and Affinity purifications:** *Cloning*– p63RhoGEF was amplified by PCR from a mouse cDNA library (Panomics) using sense primer 5′-GGTGGCGGCGCAAGCCCCGACCACC-3′ and antisense primer 5′-GGTGAAGTCTTACAGCTCATCTCAGCTTG-3′ and cloned into pCR2.1-TOPO vector. The insert was digested with NotI and HindIII restriction enzymes and subcloned into a custom pRK5-GST expression vector. All constructs were checked for accuracy by DNA sequencing and for expression by transfection into HEK 293 cells. *Transfection*–Plasmids were transfected using the calcium phosphate method. *Affinity purifications*–GST-tagged proteins were purified with glutathione sepharose (Amersham-Pharmacia, NJ) and immunoblotted for MLK3.

**Statistical Analysis:** Data are reported from three independent experiments as mean ± SEM. Statistical comparison between groups was made using ANOVA method for multiple pair-wise comparisons followed by a 2-tailed Student’s t test to evaluate levels of significance at 95% confidence. The significance level was set to $P < 0.05$.