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

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Abstract

Objective—Mitogen Activated Protein Kinase (MAPK) 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 MAP kinase kinase kinase (MAP3K) that activates multiple MAPK 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 (WT) and *Mlk3*$^{-/-}$ mice. MLK3 deficiency promoted injury-induced neointima formation and increased proliferation of primary VSMC derived from aortas isolated from *Mlk3*$^{-/-}$ mice compared to WT mice. Furthermore, MLK3 deficiency increased activation of p63RhoGEF, RhoA and Rho-kinase (ROCK) in VSMC, a pathway known to promote neointimal hyperplasia, and reconstitution of MLK3 expression attenuated ROCK activation. Moreover, JNK activation was decreased in MLK3 deficient VSMC and proliferation of WT but not MLK3 KO cells treated with a JNK inhibitor was attenuated.

Conclusions—We demonstrate that MLK3 limits RhoA activation and injury-induced neointima formation, by binding to and inhibiting activation of p63RhoGEF, a RhoA activator. In MLK3 deficient cells, activation of p63RhoGEF proceeds in an unchecked manner, leading to a net increase in RhoA pathway activation. Reconstitution of MLK3 expression restores MLK3/p63RhoGEF interaction, which is attenuated by feedback from activated JNK.

Keywords

Neointima; vascular smooth muscle cell; mixed-lineage kinase; p63RhoGEF; RhoA; Rho-kinase; cJun NH_2-terminal kinase

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Disclosures
None

Introduction

Vascular response to mechanical injury comprises 2 main processes, neointimal hyperplasia and vessel remodeling.[1] In particular, release of growth factors, infiltration of inflammatory cells, and vascular smooth muscle cell (VSMC) proliferation are involved in neointimal hyperplasia[1]. Proliferation in many cell types is driven by mitogen-activated protein kinase (MAPK) activation. The three major subfamilies of MAPKs, i.e. extracellular signal-regulated kinase (ERK), c-Jun N-terminal Kinase (JNK) and p38MAPK are activated in response to stimuli such as cytokines and growth factors[2–4], and studies using both genetic models[5–7] as well as pharmacological inhibitors of MAPKs[8–11] showed significant inhibition of neointimal hyperplasia after injury, indicating that MAPKs promote neointima formation. MAPKs are regulated by a three-tiered kinase cascade in which the MAPK is phosphorylated and activated by a MAP kinase kinase, which in turn is phosphorylated and activated by a MAP kinase kinase kinase (MAP3K).[2]

Mixed-lineage kinase 3 (MLK3) is a ubiquitously expressed member of mixed-lineage kinases (MLK1-4), a family of MAP3Ks that regulates multiple MAPK pathways.[12] MLK3 function is modulated by interaction with the Rho family GTPases, Cdc42 and Rac1,[13, 14] and MLK3 has been implicated in regulating proliferation, migration and differentiation in several cell types.[15–17] Analysis of MLK3 deficient mice has demonstrated that MLK3 contributes to TNF-stimulated JNK activation in mouse embryonic fibroblasts, promotes diet-induced JNK activation in liver and adipose tissue[18–22] and regulates bone development,[23] but its role in VSMC function and vessel wall integrity has not been studied. In addition to its role as upstream activator of MAPKs, there is emerging evidence for MAP3K-independent functions of MLK3 that do not require MLK3 catalytic activity, indicating that MLK3 may also act as scaffold for protein complexes. Thus, it has been demonstrated that MLK3 is activated during G2/M phase and promotes microtubule instability, independently of JNK activation.[24] Moreover, ectopic over-expression of kinase-inactive MLK3 is able to rescue mitogen-induced activation of ERK in cells that have been depleted of endogenous MLK3,[15] and MLK3 missense mutations have been identified in gastrointestinal tumors that probably affect MLK3 scaffold properties but not its kinase activity.[25] In addition, MLK3 has been shown to limit RhoA activation by binding p63RhoGEF, independently of its catalytic activity[26]. Interestingly, p63RhoGEF has been identified as key mediator of angiotensin II-dependent RhoA activation in VSMC.[27] RhoA is abundantly expressed in VSMC and controls a wide range of cellular functions, such as contraction, migration and proliferation,[28, 29] and it is thought that many of the beneficial, non-lipid lowering effects of statins used in prevention and treatment of cardiovascular diseases are due to their ability to inhibit RhoA[30]. Moreover, inhibition of the RhoA effector protein, Rho kinase (ROCK), inhibits intimal hyperplasia,[31–33] 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 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

Material and Methods are available in the online-only Data Supplement.

Results

MLK3 Deficiency Promotes Neointima Formation *In Vivo*

MLK3 is a MAP3K that activates multiple MAPK pathways[12] and MAPKs play a central role in the development of injury-induced neointimal hyperplasia.[34] 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 KO mice compared to minimal neointima formation in injured WT mice (Fig. 1A). Immunohistochemical staining of smooth muscle α-actin (α-SMA) demonstrated that VSMC are the main cellular component in the injury-induced neointimal area in MLK3 deficient mice (Fig. 1A, bottom panel). Morphometric analysis of uninjured and injured carotid arteries from 8 mice per genotype confirmed a significant neointimal area in *Mlk3*$^{-/-}$ mice (Fig. 1B). In addition, morphometric measurements revealed a significant increase in medial thickness in injured arteries of MLK3 KO compared to WT mice (Figure 1C). No difference in neointimal area and medial thickness was observed between uninjured control carotid arteries of WT and MLK3 deficient mice (Fig. 1B,C). 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,[35] 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. Upon 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 wild-type and MLK3 deficient 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 expression of cytoskeletal proteins (Fig. IA in the online-only data Supplement). Furthermore, qPCR analysis of fibronectin and collagen mRNA demonstrated comparable expression of extracellular matrix components in wild-type and MLK3-deficient cells (Fig. 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 to WT cells (Fig. 2A). Consistent with this, VSMC isolated from *Mlk3*$^{-/-}$ mice displayed a dose-dependent increase in [^{3}H]thymidine incorporation into DNA with maximal 6-fold increase observed at PDGF-BB concentrations of 10 ng/ml, in contrast to the 3-fold increase observed in WT cells (Fig. 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 important mediator of VSMC proliferation,[28, 33] 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 to the WT VSMC (Fig. 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. Indeed, we observed that RhoA translocation to the membrane was increased in MLK3 KO VSMC when compared to WT VSMC (Fig. 3B). Since 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 to WT cells (Fig. 3C). Together, these data suggest that MLK3 deficiency increases activation of RhoA and ROCK in VSMC, 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*,[36] and analyzed 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 (Fig. 3D), placing MLK3 upstream of RhoA and ROCK.

One downstream effect of RhoA activation is to reduce expression of cell cycle inhibitors such as the cyclin-dependent kinase inhibitors p21$^{Waf1/Cip1}$ and p27^{Kip1},[37] and numerous studies have implicated p27^{Kip1} and p21^{Cip1} in the control of VSMC proliferation and neointima formation.[38–40] Therefore, we performed immunoblot analysis of p27^{Kip1} and p21^{Cip1} in WT and *Mlk3*$^{-/-}$ VSMC. p27^{Kip1} expression was decreased in MLK3 deficient compared to WT cells (Fig. 3E). Similarly, in agreement with the increased proliferative capacity of MLK3 deficient VSMC we observed significantly reduced p21^{Cip1} levels in *Mlk3*$^{-/-}$ VSMC (Fig. 3F). Together, these data indicate that MLK3 deficiency results in increased activation of the Rho/ROCK pathway and decreased p27^{Kip1} and p21^{Cip1} expression.

MLK3 limits p63RhoGEF Activation

Rho GTPases are activated by guanine nucleotide exchange factors (GEFs) which catalyze the exchange of GDP for GTP. Earlier studies have suggested that MLK3 modulates RhoA

activity by binding to p63RhoGEF.[26] In order to evaluate the effect of MLK3 deficiency on p63RhoGEF activation, we used $RhoA^{G17A}$ 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.[41] PDGF-BB treatment caused p63RhoGEF activation in cells of both genotypes, however, PDGF-induced p63RhoGEF activation was markedly increased in *Mlk3*$^{-/-}$ compared to WT VSMC (Fig. 4A), indicating that MLK3 expression suppresses p63RhoGEF activation. Next, we determined the effect of MLK3 expression on the ability of p63RhoGEF to regulate RhoA activity. For this, we co-expressed GST-tagged p63RhoGEF and wild-type MLK3 (MLK3 WT) or a kinase inactive mutant (MLK3 KI) and analyzed RhoA-GTP loading as described above. Over-expression of p63RhoGEF activated RhoA, which was inhibited by co-expression of both, MLK3 WT and KI (Fig. 4B). Together, these results show that MLK3 deficiency increases p63RhoGEF activation and interaction of MLK3 with p63RhoGEF limits RhoA-GTP loading, independently of MLK3 catalytic activity.

Reconstitution of MLK3 Expression in MLK3 Deficient Cells Attenuates VSMC proliferation

Based on our finding that MLK3 deficiency increases activation of the RhoA pathway and that interaction of MLK3 with p63RhoGEF limits RhoA activation, we hypothesized that reconstituting MLK3 expression in VSMC isolated from MLK3 deficient mice will attenuate activation of the RhoA pathway and reduce cell proliferation. To test this, *Mlk3*$^{-/-}$ VSMC were transduced with retrovirus expressing empty vector, MLK3 WT or MLK3 KI and proliferation was investigated. As expected, PDGF-induced DNA synthesis was significantly attenuated in cells reconstituted with MLK3 WT and KI compared to MLK3 KO VSMC expressing empty vector (Fig. 5A). However, surprisingly, we observed that PDGF-BB treatment of MLK3 deficient VSMC reconstituted with MLK3 WT caused a two-fold increase in the [^{3}H] thymidine incorporation into DNA, whereas PDGF-induced DNA synthesis was not significantly increased in cells reconstituted with catalytically inactive MLK3 (Fig. 5A). Similarly, we found that the proliferation rate of cells reconstituted with MLK3 in medium supplemented with 10% fetal bovine serum was decreased compared to *Mlk3*$^{-/-}$ VSMC (p=0.05 MLK3 WT vs MLK3 KO; p< 0.05 MLK3 KI vs MLK3 KO; p< 0.05 MLK3 WT vs MLK3 KI) (Fig. 5B). Together, these data indicate that MLK3 expression modulates RhoA activation, but uncover an important role for MLK3 kinase activity in the regulation of VSMC proliferation.

MLK3 Mediates PDGF-Induced JNK Activation in VSMC

Since several MAPK pathways have been implicated in the development of injury-induced neointimal hyperplasia[34] we assessed MAPK activation in WT and *Mlk3*$^{-/-}$ VSMC in order to determine which subfamily of MAPKs mediates the effect of MLK3 on 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, while loss of MLK3 had little effect on PDGF-induced phosphorylation of ERK (Fig. 6A). These data suggest that MLK3 is not required for ERK activation, but contributes to PDGF-induced JNK activation in VSMC. In order to test if 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 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 (Fig. 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[26] and thus attenuates MLK3-mediated decrease of cell proliferation. Therefore, we hypothesized that JNK inhibition increases interaction of MLK3 with p63RhoGEF. To test this hypothesis, we co-expressed 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. Indeed, we found that MLK3 interacts with GST-p63RhoGEF but not with GST control (Fig. 6C, left panel), and that this association is increased in the presence of the JNK inhibitor (Fig. 6C, right panel).

Discussion

Previously it was shown that MLK3 deficiency has either no effect or an inhibitory effect on cell proliferation.[15, 18] 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, while no difference in neointimal area is observed in uninjured carotid arteries of WT and MLK3 deficient 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 growth of WT and MLK3 KO VSMC in medium supplemented with 10% fetal bovine serum, placing Rho A 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.[28] Instead it appears to potentiate the effects of Ras/MAPK or growth factors to stimulate cell cycle progression.[37] One mechanism by which RhoA and ROCK activation control VSMC proliferation is by modulating expression of cell cycle inhibitors p21$^{Waf1/Cip1}$ and p27^{Kip1}.[37, 42, 43] Consistent with this we find decreased levels of p21$^{Waf1/Cip1}$ and p27^{Kip1} in MLK3 deficient VSMC compared to WT cells.

Rho GTPases are regulated by Rho guanine nucleotide exchange factors (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 63kDa Rho-GEF, that specifically activates RhoA.[44] Several lines of evidence indicate that p63RhoGEF specifically binds to $G\alpha_{q/11}$, but not $G\alpha_{12/13}$ subunits of heterotrimeric G proteins, thereby linking $G\alpha_{q/11}$-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,[27] as well as serum-dependent RhoA activation and chemotactic migration in breast cancer cells.[48] Since 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 to WT VSMC. This indicates a non-redundant role for MLK3 in PDGF-induced JNK activation in VSMC, in contrast to the redundant function observed in mouse embryonic fibroblasts (MEF).[18] In addition, we do not find that MLK3 is required for ERK activation in VSMC, contrary to studies in tumor cells.[15] These differences may be due to 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. Indeed, we find that JNK inhibition increases binding of MLK3 to p63RhoGEF, providing a mechanism by which MLK3 regulates VSMC proliferation. How JNK modulates association of MLK3 and p63RhoGEF is unclear. Feedback phosphorylation of MLK3 by JNK has been demonstrated to affect MLK3 localization,[49] suggesting a potential mechanism for regulation of MLK3-p63RhoGEF interaction.

In summary we show that MLK3 deficiency increases VSMC proliferation and exacerbates injury-induced neointima formation, due to increased p63RhoGEF, RhoA and ROCK activation. Moreover, we demonstrate that loss of JNK activation, due to 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 MLK3 function in VSMC: MLK3 association with p63RhoGEF decreases p63RhoGEF, RhoA and ROCK activation and reduces VSMC proliferation and neointimal hyperplasia. 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 reducing binding of MLK3 to p63RhoGEF, provides a feedback mechanism to dampen MLK3-mediated attenuation of RhoA pathway activation and cell proliferation. Loss of JNK activation, due to expression of catalytically inactive MLK3 or pharmacological inhibition of JNK, allows unrestricted interaction of MLK3 and p63RhoGEF, thus limiting availability of p63RhoGEF for RhoA pathway activation, ultimately causing a net decrease in RhoA pathway activation and cell proliferation. (Fig. 6D). Together, these studies establish an important role for MLK3 in VSMC proliferation and neointimal hyperplasia in response to vascular injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

MLK3	mixed lineage kinase 3
VSMC	vascular smooth muscle cells
ROCK	Rho Kinase
MAPK	mitogen activated protein kinase
JNK	cJun NH_2-terminal kinase
GEF	guanine nucleotide exchange factor
PDGF	platelet-derived growth factor

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Significance

MLK3 is a MAP3K that activates multiple MAPK pathways that have been implicated in neointimal hyperplasia secondary to vascular injury. However, until now, the role of MLK3 in VSMC 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 p63RhoGEF activity and subsequently activation of RhoA and ROCK. Reconstitution of MLK3 expression restores MLK3/p63RhoGEF interaction, which is attenuated by feedback from activated JNK. These data suggest that in VSMC MLK3 limits RhoA pathway activation and injury-induced neointima formation.

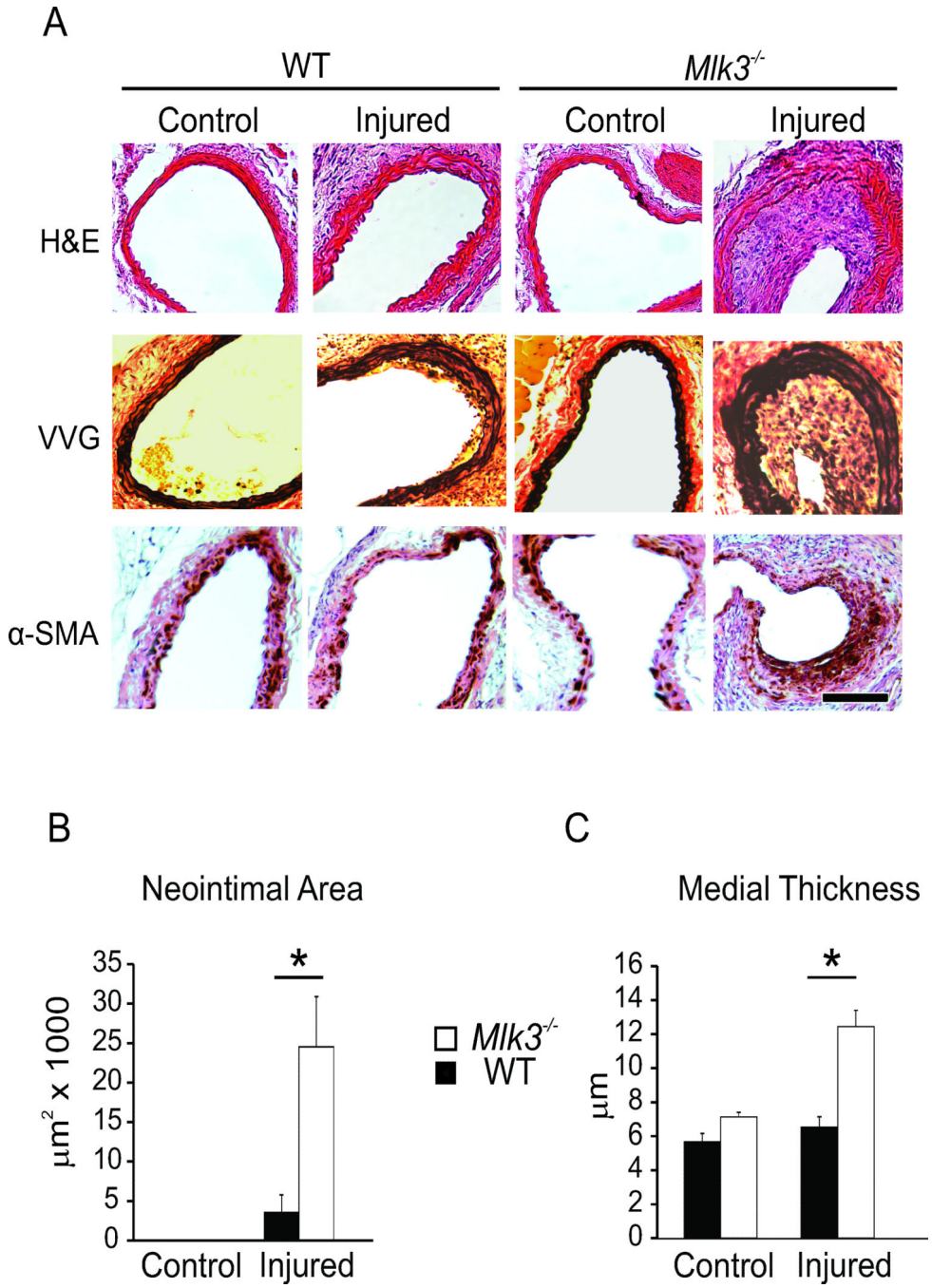

Figure 1.

Neointima formation is increased after endothelial denudation of carotid arteries in MLK3 deficient mice. **A**, Representative hematoxylin and eosin (H&E), Verhoeff-Van Gieson (VVG) or smooth muscle α-actin (α-SMA) staining of uninjured and injured aortas from wild-type (WT) and *Mlk3*$^{-/-}$ mice. Scale bar represents 100 μm. **B**, Measurements of neointima area and medial thickness. Data represent ±SEM from 8 mice in each group. *p<0.05.

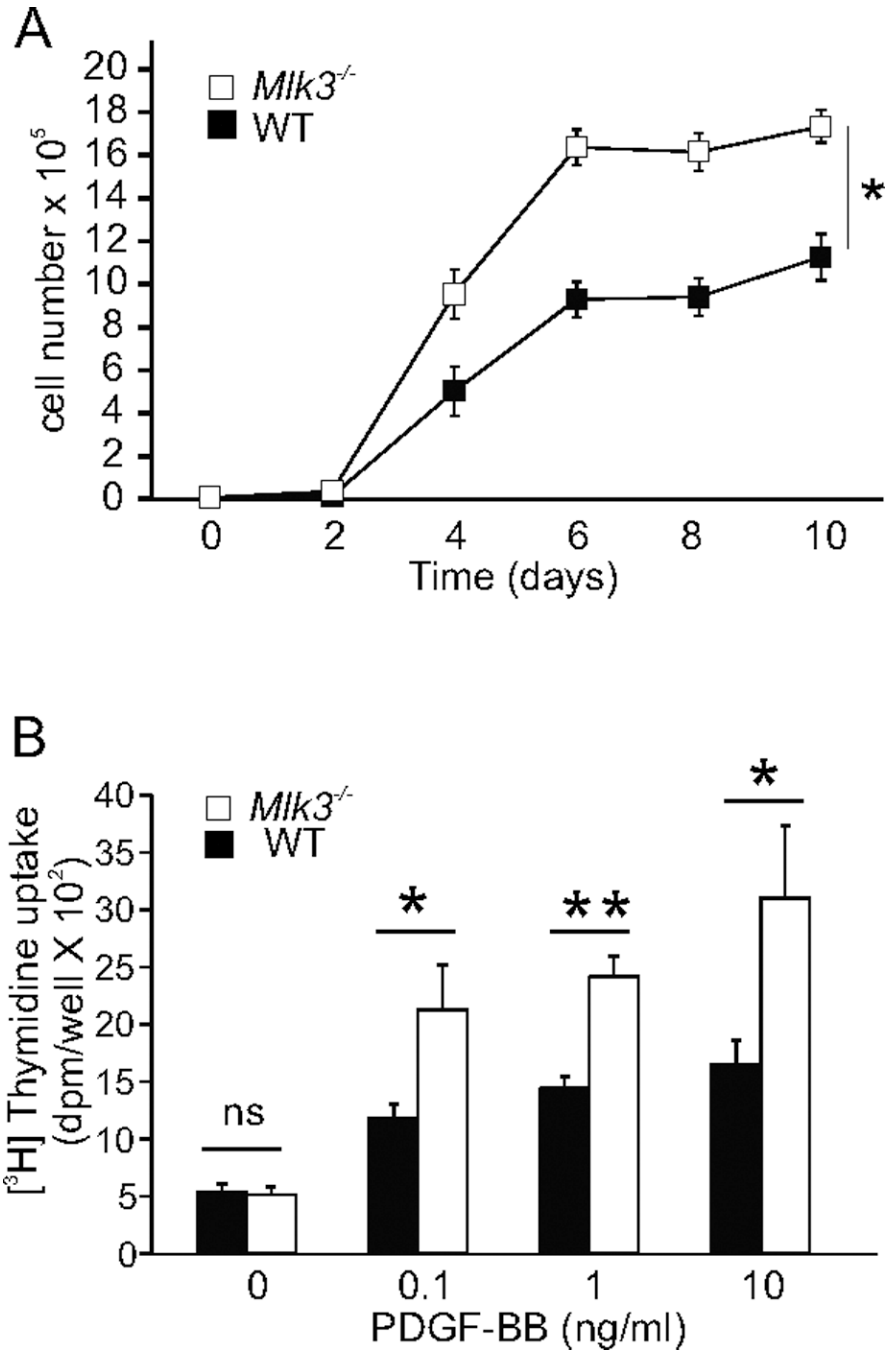

Figure 2.

MLK3 deficiency increases VSMC proliferation *in vitro*. **A**, VSMC from WT and *Mlk3*$^{-/-}$ mice were cultured in DMEM containing 10% fetal bovine serum (FBS). Viable cell number was determined by direct counting. **B**, VSMC were stimulated with the indicated amounts of PDGF-BB-BB for 18 h. Incorporation of [^{3}H] thymidine into DNA was determined after cell lysis. Data represent ±SEM from triplicate assays from 3 independent experiments. *p<0.05, **p< 0.01.

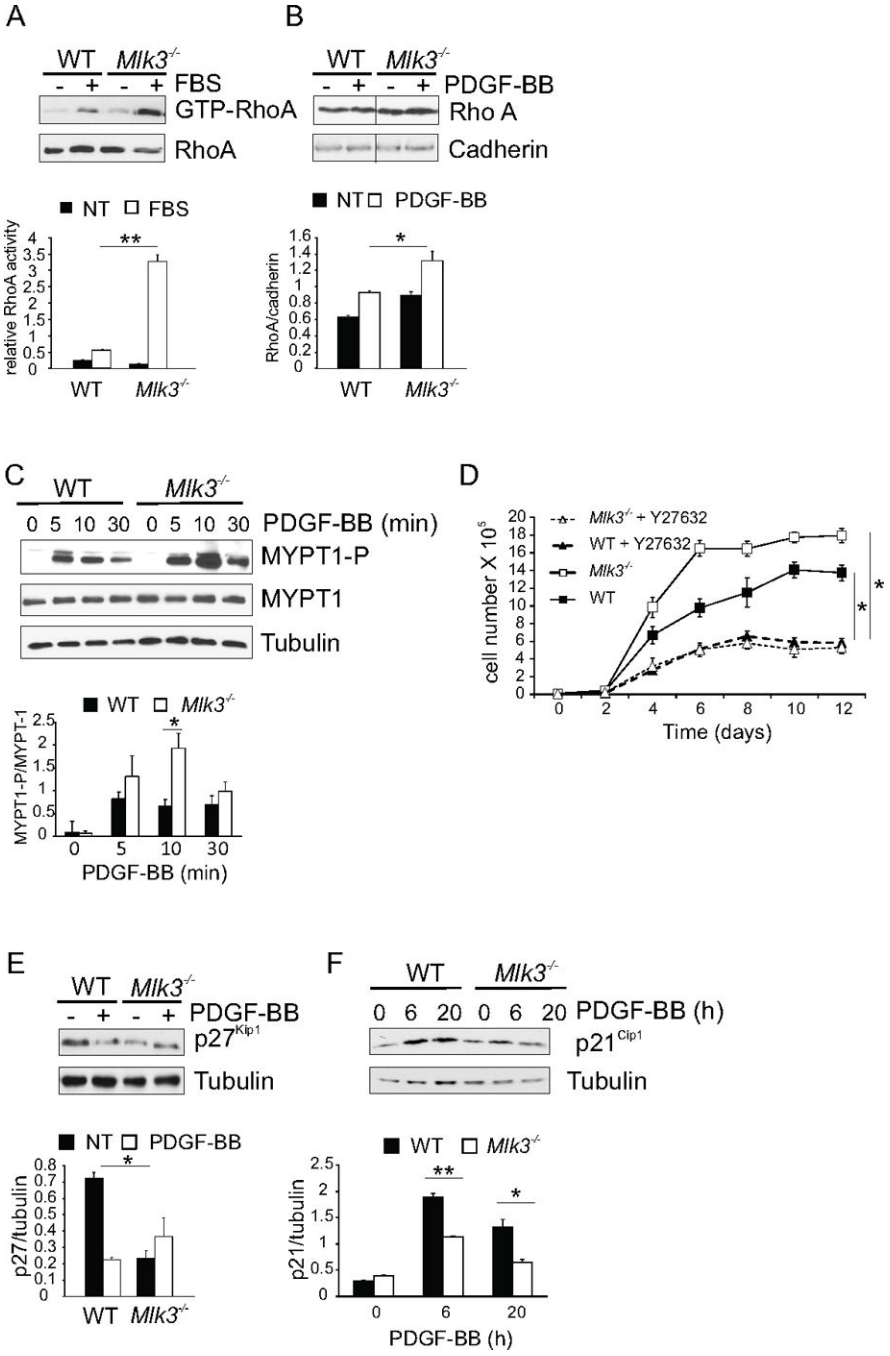

Figure 3.

MLK3 deficiency increases Rho/ROCK activation. **A**, VSMC from WT and *Mlk3*$^{-/-}$ mice were treated with 10% FBS for 10 min. RhoA activation was determined by binding to GST-Rhotekin and subsequent immunoblot analysis (top panel). Relative RhoA activity (GTP-RhoA/total RhoA) is shown in the bottom panel. **B**, VSMC from WT and *Mlk3*$^{-/-}$ mice were treated with PDGF-BB for 10 min. Cells were fractionated and the membrane fraction was analyzed for translocation of RhoA by immunoblot analysis (top panel). Relative RhoA expression was determined by normalization to cadherin (bottom panel). **C**,

VSMC from WT and *Mlk3*$^{-/-}$ mice were treated with PDGF-BB for the indicated times and MYPT1 expression and phosphorylation was determined by immunoblot analysis (top panel). Relative phosphorylation of MYPT-1 was obtained by normalization to total MYPT-1 (bottom panel). **D**, VSMC from WT and *Mlk3*$^{-/-}$ mice were cultured in DMEM containing 10% FBS in the presence of ROCK inhibitor (30 μM) or vehicle control and viable cell number was determined by direct counting. Data represent ±SEM from 3 independent experiments. * denotes significant difference from untreated cells of the same genotype at $p<0.05$. **E**, VSMC from WT and *Mlk3*$^{-/-}$ mice were treated with PDGF-BB for 20 h and p27^{Kip1} expression was determined by immunoblot analysis (top panel). **F**, VSMC from WT and *Mlk3*$^{-/-}$ mice were treated with PDGF-BB for the indicated times and p21^{Cip1} expression was determined immunoblot analysis (top panel). Relative expression of p27^{Kip1} and p21^{Cip1} was obtained by normalization to tubulin (bottom panels). Values are mean ± SD from three experiments.

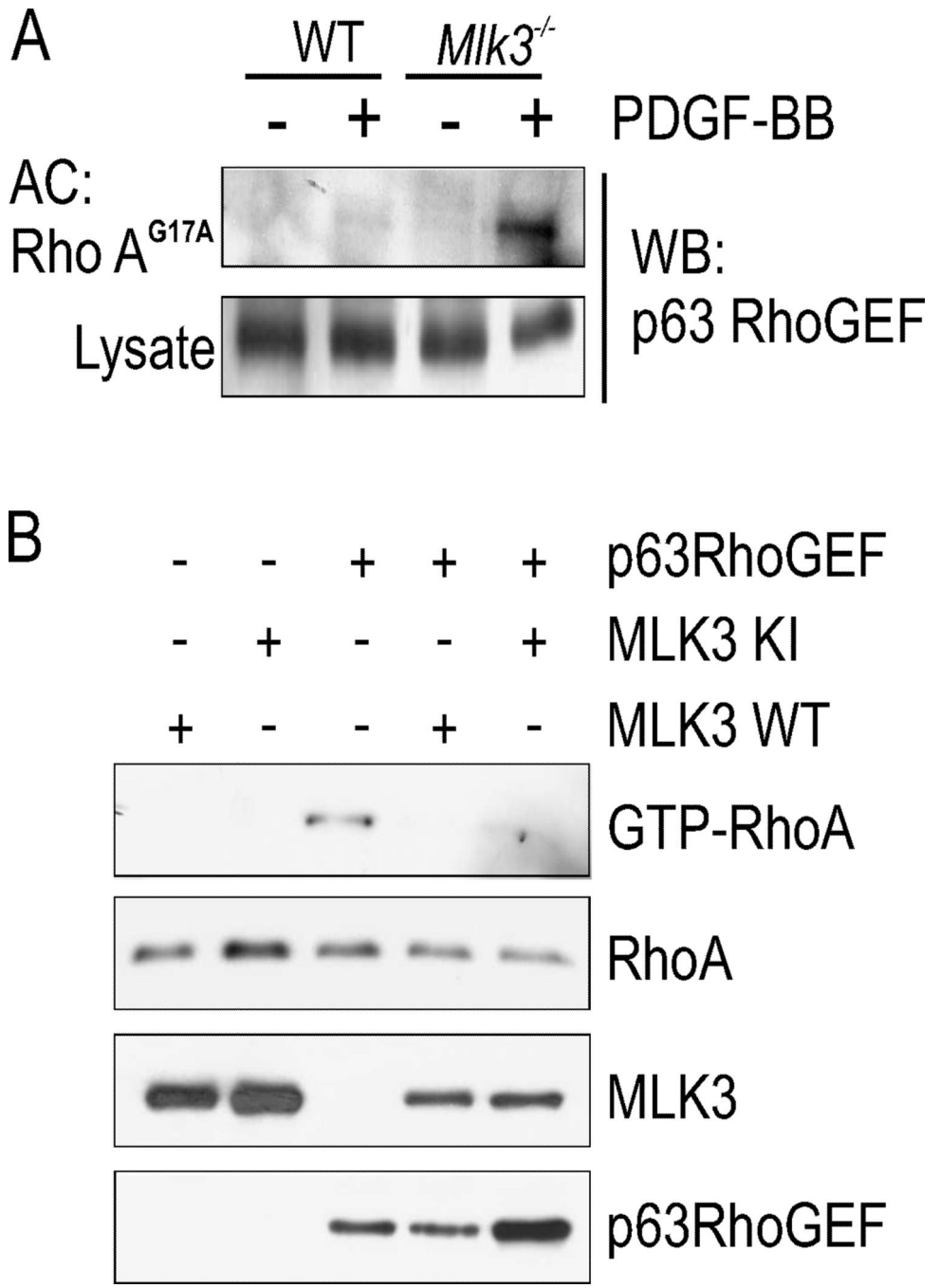

Figure 4.

Interaction of MLK3 with p63RhoGEF limits RhoA activation. **A**, VSMC from WT and *Mlk3*$^{-/-}$ mice were treated with PDGF-BB for 10 min. p63RhoGEF activation was determined by RhoA^{G17A} affinity chromatography in combination with immunoblot analysis. **B**, GST-p63RhoGEF and MLK3 WT or a MLK3 KI were co-expressed in HEK293 cells and RhoA activation was determined by binding to GST-Rhotekin and subsequent immunoblot analysis.

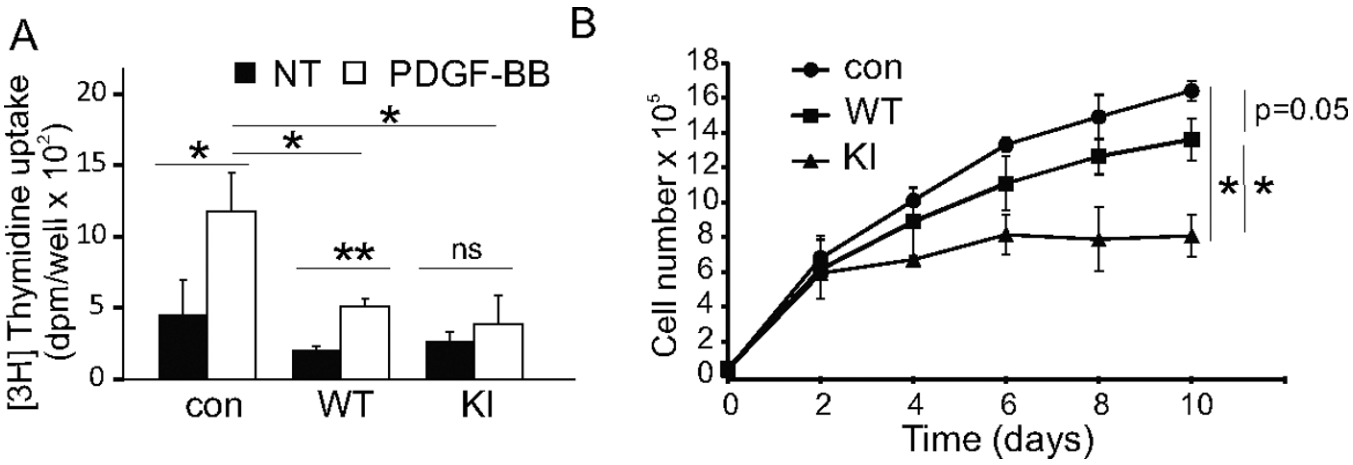

Figure 5.

Reconstitution of MLK3 expression in MLK3 deficient cells attenuates Rho/ROCK activation.h **A**, MLK3 KO cells were reconstituted with empty vector (con) or MLK3 WT or KI, treated with 10 ng/ml PDGF-BB for 18 h and incorporation of [^{3}H] thymidine into DNA was determined after cell lysis. **B**, VSMC reconstituted with empty vector (con) or MLK3 WT or KI were cultured in DMEM containing 10% FBS and viable cell number was determined by direct counting. Data represent ±SEM from triplicate assays from three independent experiments. *p<0.05, **p< 0.01.

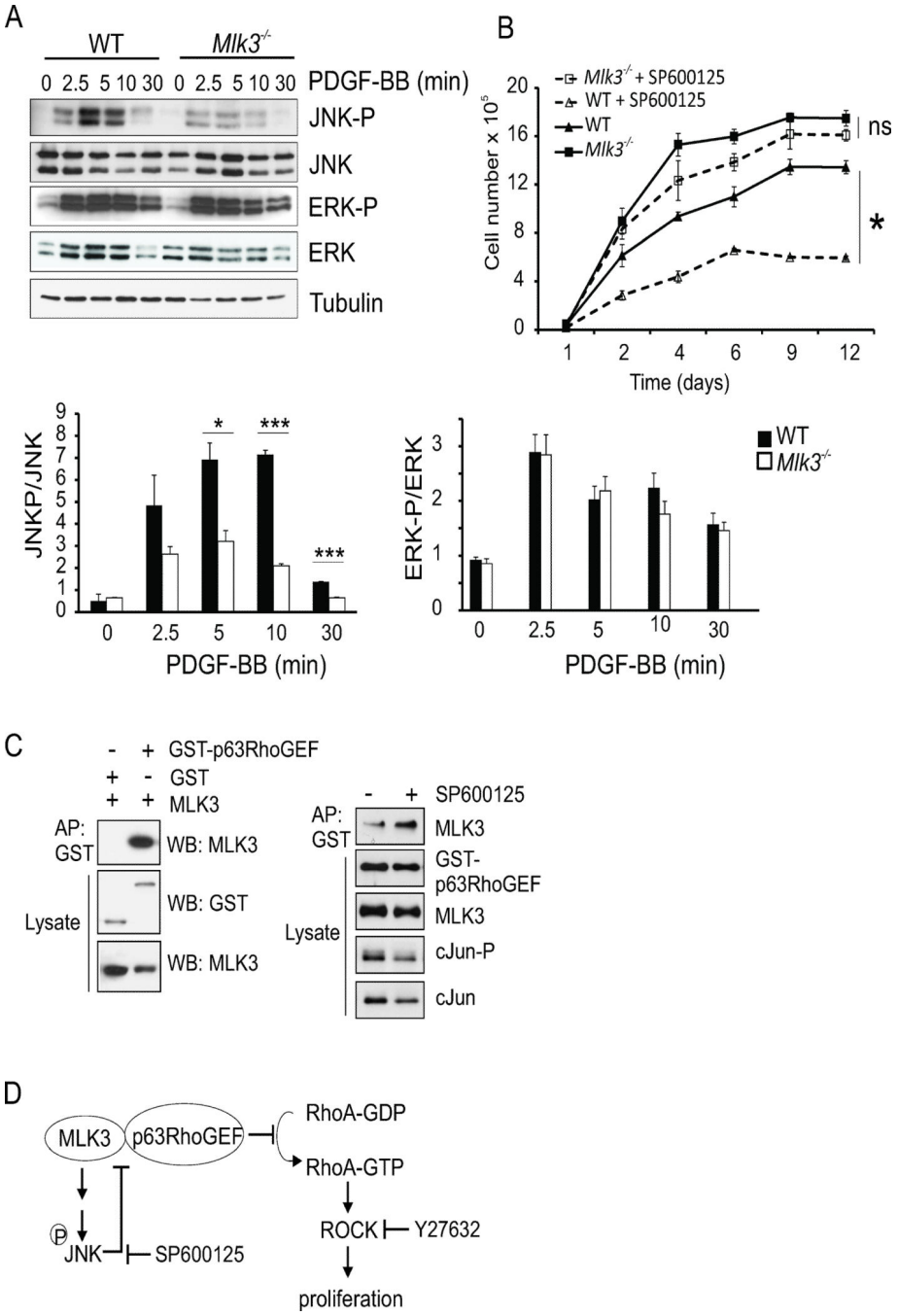

Figure 6.

MLK3 mediates PDGF-BB-induced JNK activation in VSMC. **A**, VSMC from WT and *Mlk3*$^{-/-}$ mice were treated with PDGF-BB for the indicated times. JNK and ERK expression and phosphorylation was determined by immunoblot analysis (top panel). Relative phosphorylation of JNK and ERK was obtained by normalization to total JNK and ERK expression (bottom panel). Values are mean ± SD from three experiments. **B**, VSMC from WT and *Mlk3*$^{-/-}$ mice were cultured in DMEM containing 10% FBS in the presence of JNK inhibitor (20 μM) or vehicle control and viable cell number was determined by direct

counting. Data represent ±SEM from triplicate assays from three independent experiments. * denotes significant difference from untreated cells of the same genotype at $p<0.05$, ns denotes statistically not significant. **C,** GST, GST-p63RhoGEF and MLK3 were co-expressed in HEK293 cells and treated with SP600125 (20 μM) 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/ROCK activation by MLK3.