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Sally Jo April 15, 2013

The effect of Diaphanous-related formin-1 (mDia1) on α-tubulin expression and vascular smooth muscle cell migration

by

Sally Jo

Kathy Griendling, PhD
Adviser

Department of Biology

Kathy Griendling, PhD
Adviser

William Kelly, PhD
Committee Member

Amanda Starnes, DVM
Committee Member

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Kathy Griendling, PhD
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Abstract

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RATIONALE: The migration of vascular smooth muscle cells (VSMCs) is essential for numerous biological processes, such as vascular development and wound healing. However, VSMC migration can also contribute to the progression of cardiovascular disease. Diaphanous-related formin-1 (mDia1), which regulates cytoskeletal assembly in response to various extracellular signals, such as reactive oxygen species, mechanical forces, and growth factors, may affect the migration of VSMCs.

OBJECTIVE: Here, we assess whether mDia1's impact on VSMC migration is realized through its regulation of α-tubulin, a building block of microtubules.

HYPOTHESIS: Previous experiments indicate that the knockout of mDia1 increases α-tubulin expression and the migration of VSMCs. Based on these results, we hypothesized that the short interfering RNA (siRNA)-induced knockdown of mDia1 would also increase VSMC migration and α-tubulin levels by promoting the expression of the α-tubulin mRNA and/or repressing the proteasomal degradation of α-tubulin.

RESULTS: Although the knockdown of mDia1 does not have a significant effect on VSMC migration, it slightly increases the cytosolic levels of α-tubulin. Our results show that the knockdown of mDia1 is unlikely to affect the mRNA levels of α-tubulin or the proteasomal degradation of α-tubulin. Instead, the knockdown of mDia1 may prevent the translocation of α-tubulin to the membrane, thereby increasing the cytosolic levels of α-tubulin.

CONCLUSION: mDia1 may promote the translocation of α-tubulin to cellular membranes. Although the exact physiological significance of this process is unknown, these membrane tubulins may regulate the growth and contraction of VSMCs by causing the activation of ion channels.

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INTRODUCTION

The walls of arteries and veins are divided into three concentric layers: the neointima, the media, and the adventitia. The neointima, which is the innermost layer, includes the endothelium and the basement membrane. The adventitia, or the outermost layer, contains connective tissue and fibroblasts. Vascular smooth muscle cells (VSMCs) embedded in elastin sheets constitute the media, or the middle layer. The migration of VSMCs is essential for various biological processes, such as vascular development and wound healing. However, their migration is also implicated in disease processes. A prime example that illustrates the role of VSMC migration in the progression of blood vessel disease is atherosclerosis, which is the thickening and stiffening of arteries and a major precursor of cardiovascular disease.

The formation of an atherosclerotic lesion, or a plaque, begins when atherogenic, or atherosclerosis-promoting, stimuli, such as cigarette smoke, oxidized low-density lipoprotein (LDL), and high blood pressure, injure the endothelium (Csordas *et al.*, 2013). Cell debris, fats, cholesterol, calcium, macrophages, and other substances collect at the site of injury. This event can induce VSMCs to migrate toward the neointima, where they proliferate and secrete connective tissue. The immediate effects of plaque formation are the narrowing of the luminal diameter and the disruption of blood flow. More serious consequences may arise when the plaque ruptures and causes a blockage in the bloodstream, thereby cutting off the oxygen supply to distal tissues and organs. Depending on its location, a blockage may cause a stroke or heart attack.

As VSMC migration contributes to the progression of heart disease, the signaling mechanisms underlying VSMC migration are of great interest. Discussed in the following

sections are the general properties of VSMCs and cellular migration, as well as the cytoskeletal elements and regulatory proteins that are involved in the migratory process.

Vascular Smooth Muscle Cells

Robert Wissler was among the first scientists to point out the phenotypic plasticity of VSMCs, a remarkable property that enables these cells to perform a multitude of tasks in their environment (Wissler, 1967). Stemming from Wissler's ideas, research in the ensuing decades has explored how the functional versatility of VSMCs impacts the short- and long-term state of blood vessels.

The majority of VSMCs in the media are *contractile*; they maintain proper blood flow by "[altering] the vessel's luminal diameter" (Rensen *et al.*, 2007). Various extracellular events, such as reactive oxygen species production, mechanical forces, and growth factor release, can induce VSMCs to adopt a *synthetic* phenotype (Owens *et al.*, 2004). These synthetic VSMCs, compared with contractile ones, show a greater potential for proliferation and migration (Rensen *et al.*, 2007). Given these characteristics, synthetic VSMCs can play a sizeable role in vessel remodeling.

While the phenotypic plasticity of VSMCs may be essential for maintaining the integrity of vessels, the same property lies at the root of our human susceptibility to the aforementioned atherogenic stimuli (Owens *et al.*, 2004). These stimuli can trigger the inappropriate movement of VSMCs, which may aggravate injuries to the vessel, rather than heal them. Thus, the capability for phenotypic switching enables VSMCs to modulate the health and disease of the cardiovascular system.

Platalet-Derived Growth Factor Signaling

Upon vascular injury, extracellular cues, such as platelet-derived growth factor (PDGF), are released into the bloodstream. These and similar extracellular signals then activate intracellular signaling pathways, which, in turn, regulate the remodeling of the cytoskeleton. PDGF is one of the most potent chemoattractants released upon vascular injury or during vascular development (Grotendorst *et al.*, 1982). Secreted by vascular endothelial cells, inflammatory cells, and VSMCs, PDGF binds to a PDGF receptor (PDGFR) on the plasma membrane of VSMCs (Raines, 2004). The binding of PDGF causes the auto-phosphorylation of PDGFR, activating a signaling cascade that affects various downstream effectors. PDGF can also indirectly stimulate migration by triggering the production of other pro-migratory signals, such as fibroblast growth factor-2 (Pintucci *et al.*, 2005). Together, these properties make PDGF a powerful stimulus for cellular migration.

Cellular Migration

Cellular migration is integral to a wide range of basic biological processes, such as embryonic gastrulation, immune surveillance, and wound healing. However, aberrant cellular migration has also been implicated in pathophysiological processes, such as cancer metastasis and the aggravation of recovering blood vessels.

Cellular migration is a cyclic process that requires the synchronized assembly and disassembly of various cytoskeletal elements. The process begins with cell polarization (San Martin *et al.*, 2010). The microtubule-organizing center (MTOC), which is the site of microtubule nucleation, positions itself between the leading edge of the cell and the nucleus. Then, a subset of microtubules is stabilized toward the direction of the cell's eventual movement.

Next, actin polymerization drives the protrusion of lamellipodia at the cell front (Small *et al.*, 2002). Meanwhile, the rear of the cell detaches from the extracellular medium, and new focal adhesions form in the lamellipodia, providing an anchor against which the cell may propel itself (San Martin *et al.*, 2010). Finally, the activity of acto-myosin motors leads to the contraction of the cell body and the forward movement of the cell (San Martin *et al.*, 2010).

During this multistep cycle, the assembly of actin filaments, microtubules, focal adhesions, and other cytoskeletal elements needs to occur in a coordinated manner. The complexity of this process is managed by Rho family GTPases.

Rho Family GTPases and mDia1

Rho family GTPases control numerous aspects of cellular migration. The key members of the family are Cdc42, Rac, and RhoA. At the protrusive edge of a migrating cell are two actin-rich structures: the lamellipodia, which contain a meshwork of actin filaments, and filopodia, which are bundles of actin filaments that probe the extracellular environment. Rac activation leads to the formation of lamellipodia and focal adhesions at the cell front (Wittmann *et al.*, 2001), and Cdc42 regulates the formation of filopodia (Kozma *et al.*, 1995). Cdc42 and Rac also regulate microtubule dynamics (Leve *et al.*, 2012), which will be discussed in the next section.

RhoA affects several downstream effectors, including Diaphanous-related formins, whose three isoforms—mDia1, mDia2, and mDia3—modulate various cytoskeletal events (T. Watanabe *et al.*, 2005). mDia is an auto-inhibited protein, whose two domains, the Diaphanous-autoregulatory domain (DAD) and the GTPase-binding domain (GBD), exert inhibitory effects on each other and keep the protein in an inactive state (Alberts, 2001). The

binding of RhoA causes a conformational change that exposes the protein's functional domains, formin homology 1 and 2, or FH1 and FH2.

Discovered in 1997 (N. Watanabe *et al.*, 1997), mDia1 has been shown to regulate actin nucleation and elongation and microtubule stabilization (T. Watanabe *et al.*, 2005). First, mDia1 can control when and where actin filaments form in the cell by instigating actin nucleation (N. Watanabe *et al.*, 2004). Subsequently, mDia1 can promote the elongation of actin filaments by binding to the barbed plus-end of an actin filament and "walking" with the growing end (Xu *et al.*, 2004). As the barbed end binds its next actin monomer, one of the two actins attached to the formin undergoes a conformational change and loses its affinity for the formin (Xu *et al.*, 2004). Partly anchored on the second actin, the formin swings its free end to latch onto the new actin monomer. Through this "stair-stepping" motion, formins allow the sequential addition of actin monomers onto the barbed end of the actin filament (Xu *et al.*, 2004). Also, as a "leaky capper," mDia1 prevents tight capping proteins from binding to the growing end of the filament and stopping elongation (N. Watanabe *et al.*, 2004).

In addition to regulating actin structures, mDia1 can promote the stabilization of microtubules, which will be discussed next.

Dynamic and Stable Microtubules

A microtubule filament is a hollow tube that consists of 13 longitudinal protofilaments, each built from α- and β-tubulin heterodimers (Kirchner *et al.*, 1985). Both the lateral interactions between neighboring protofilaments and the longitudinal interactions between adjacent heterodimers uphold the structural integrity of each microtubule filament (VanBuren *et al.*, 2002).

Microtubule filaments emanate from the MTOC in a radial manner, with the growing plus-end of each filament crossing through the cytoplasm. Unlike actin filaments, which experience momentary periods of steadiness, microtubules constantly change in length due to the dynamic instability at their plus-ends, cycling between phases of growth and catastrophe (VanBuren *et al.*, 2002). This dynamic nature of microtubules underlies their ability to participate in a wide range of cellular events that involve motion, such as chromosomal segregation and cellular migration. Both Cdc42 and Rac regulate this aspect of microtubules.

During migration, a subset of these dynamic microtubules is stabilized. Stable microtubules do not undergo cycles of dynamic instability and have much longer half-lives than regular microtubules (Schulze *et al.*, 1987). These microtubules are important for establishing cell polarity; a study by Vasiliev et al. (Vasiliev *et al.*, 2013) shows that when microtubules are destabilized with colcemid, which causes microtubules to detach from the MTOC and unravel at their unprotected minus-ends, the affected cells change their direction of movement every 30 minutes, while untreated cells maintain their "rectilinear" movement for hours. Vasiliev et al. (Vasiliev *et al.*, 2013) conclude that stable microtubules help cells "remember" the direction of their previous movement and that the loss of stable microtubules makes the cells' movement more "unpredictable." mDia1, our project's focus, promotes the stabilization of microtubules, thereby contributing to the establishment of cell polarity and the subsequent migration of cells (Yamana *et al.*, 2006).

Short Interfering RNA

To better understand mDia1's role in VSMC migration, the Griendling lab has generated smooth-muscle specific mDia1$^{-/-}$ mice. Previous experiments from the lab indicate that the

knockout of mDia1 increases α-tubulin expression and the basal and PDGF-induced migration of VSMCs (Figure 1). In this project, we used mDia1 knockdown cells instead of knockout cells to obviate any compensatory changes that may occur in response to the long-term loss of mDia1.

The knockdown of mDia1 was achieved through short interfering RNA (siRNA) transfection, which is an experimental technique that temporarily prevents the translation of a certain protein, thereby causing an acute depletion of this protein. Its mechanism of action is as follows. First, a double-stranded RNA (dsRNA) is inserted into the cell through electroporation or another method of transfection. Then, the dsRNA binds the RNA-induced silencing complex (RISC), which unwinds the two strands of the dsRNA. Once the strands separate, the siRNA-RISC complex hybridizes with the target mRNA, causing its cleavage and subsequent degradation.

We transfected wildtype VSMCs with a mDia1-targeting siRNA to knock down mDia1, while the negative control cells were transfected with a scrambled siRNA, whose sequence was demonstrated not to inhibit mDia1 expression.

HYPOTHESIS AND OBJECTIVES

mDia1, a downstream effector of RhoA GTPase, promotes actin filament nucleation and elongation, as well as microtubule stabilization. Although mDia1's role in regulating various cytoskeletal elements is well known, its impact on VSMC migration is unclear. The purpose of this project is to investigate the mechanism that ties together mDia1, α-tubulin expression, and VSMC migration. We formulated our original hypothesis based on results from previous knockout experiments, which demonstrate that the knockout of mDia1 increases α-tubulin expression and VSMC migration in both basal and PDGF conditions.

We hypothesize that the knockdown of mDia1 causes an increase in VSMC migration and α-tubulin expression by up-regulating the mRNA levels of α-tubulin and / or repressing the proteasomal degradation of α-tubulin (Figure 2). This project has three major aims: (1) to test whether the knockdown of mDia1 promotes VSMC migration; (2) to test whether the knockdown of mDia1 promotes α-tubulin mRNA expression; and (3) to test whether the knockdown of mDia1 represses a pathway leading to the proteasomal degradation of α-tubulin. To meet each objective, we performed transwell migration assays, assessed the relative mRNA levels in each siRNA group via quantitative real time polymerase chain reaction (qRT-PCR), and treated cells with a proteasome inhibitor and probed for α-tubulin content.

METHODS

Cell Culture

VSMCs isolated from WT mouse aortas were grown in Dulbecco's Modified Eagle's Medium (DMEM)-low glucose medium supplemented with fetal bovine serum, L-glutamine, and antibiotics. Cell cultures were incubated at 37^{O}C in a 5% CO_2 atmosphere. Cells between passages 9 and 12 were used in the experiments.

siRNA Transfection

To knock down mDia1, we transfected the siRNA for mDia1 into cells via electroporation using the U-25 program on the AMAXA machine. The negative control cells were transfected with the scrambled siRNA via the same method. To remove the Nucleofector solution used during siRNA transfection, which debilitates cell recuperation, the medium was replaced with new 10% FBS medium 24 hours after transfection. Then, within a few hours, the medium was replaced with serum-free medium. 48 hours after transfection, new serum-free medium was provided to the cells.

Migration Assay

To measure cell migration, we performed a modified Boyden chamber assay 60 hours after siRNA transfection. First, the porous membrane suspended in each well was coated with a mixture of rat tail collagen and acetic acid. Then, 40,000 VSMCs were added on top of each membrane in a twelve-well plate. The membranes were submerged in media with PDGF, a pro-migratory stimulant, or bovine serum albumin, the vehicle control. Four hours after seeding, we removed the membranes from the wells and cleaned the upper surface of membranes with a

cotton tip. The cells on the bottom side of the membrane, representing cells that had migrated, were fixed in ice-cold methanol and stained with a fluorescent nuclear dye, DAPI. The membranes were excised from the wells and mounted on a glass slide. The number of cells on each membrane was counted on the Zeiss Axioskop microscope and quantified on the ImageJ software. We performed this experiment four times.

Cell Lysate Preparation and Western Blot

To determine the expression of specific proteins, cells were first incubated at room temperature for 15 minutes in a lysis buffer called Buffer B, homogenized with an insulin syringe, and sonicated. Buffer B contains 50mM HEPES (pH 7.8), 50mM KCl, 300mM NaCl, 0.1mM EDTA, 0.2mM NaF, 0.2mM Na_3VO_4, 0.4mM PMSF, 10μg/mL leupeptin, 1mM DTT, 10% glycerol, and no detergent. Then, the prepared cell samples were centrifuged at 12,000 rpm for 20 minutes at 4°C. We isolated the supernatant, which contains all soluble proteins.

Microtubules in their polymerized state readily dissociate into their soluble α- and β-tubulin subunits if the cells are lysed in a buffer that lacks a microtubule-stabilizing agent, such as taxol (Minotti *et al.*, 1991). Thus, we can be confident that the microtubule filaments are being broken down into their subunits during cell lysate preparation and that we are truly isolating α-tubulin, a protein that is central to this project.

After collecting the supernatant, a Bradford protein assay was performed using bovine serum albumin as a standard (Bradford, 1976). A microplate containing the samples was read on a μ-Quant spectrophotometer. Equal amounts of protein were loaded onto a 10% gel and separated by size via SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis). Then, proteins on the gel were electro-transferred to a PVDF membrane, which was afterwards

blocked with 5% non-fat dried milk and washed with 0.1% TBS-Tween. The membrane was incubated overnight with a primary antibody against the target protein and washed with 0.1% TBS-Tween in the next morning. Then, a HRP-conjugated secondary antibody was added to the membrane. After washing off the secondary antibody, the bands were detected using chemiluminescence, and the density of each band was measured on the ImageJ software.

RNA Extraction and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

We performed qRT-PCR to quantify the relative mRNA levels of α-tubulin in each siRNA group. First, cells were transfected with the appropriate siRNA on the AMAXA machine. Sixty hours after transfection, we extracted the total RNA from cells by using the Qiagen RNeasy Mini Kit. The total RNA was transcribed into cDNA through reverse transcription with the Superscript II enzyme and random primers. cDNA samples were purified using microbiospin 30 columns. With the Platinum Taq DNA polymerase and gene-specific primers, the purified cDNA samples were amplified on the Light Cycler machine, which detects the progressive amplification of DNA by reading the level of light emitted from the SYBR Green dye intercalating with double-stranded DNA in a non-sequence-specific manner. We analyzed the qRT-PCR data using the mathematical program called MAK3, which utilizes a recursive method to deduce relative mRNA copy numbers in each siRNA group.

MG132 Proteasome Inhibitor Treatment and Cell Fractionation

To test whether mDia1 activates a pathway leading to the proteasomal degradation of α-tubulin, we used MG132, which is a peptide aldehyde that inhibits the proteolytic activity of the

proteasome enzyme (Guo *et al.*, 2013). Forty-four hours post-transfection, cells were treated with either MG132 or DMSO, the vehicle control. After MG132 treatment, cells were lysed and western blotted.

We probed for protein content in the soluble and insoluble fractions of the cell separately in order to clarify whether changes in the concentration of α-tubulin is a cell-wide phenomenon or an event that is specific to a cell fraction. We fractionated the cells through the following procedure. First, cells were lysed in Buffer B, homogenized, and sonicated. Then, the sample was centrifuged at 12,000 rpm for 20 minutes at 4°C. After centrifugation, the supernatant, containing all soluble proteins, was separated from the pellet, which contained only the insoluble proteins. The supernatant constitutes the soluble fraction.

Then, the pellet was carefully resuspended in Hunter's Buffer, which contains 25mM HEPES (pH 7.8), 1.5mM KCl, 150mM NaCl, 1mM EGTA, 0.2mM NaF, 0.1mM Na_3VO_4, 0.4mM PMSF, 10μg/mL leupeptin, 10% glycerol, and 1% Triton X-100. The key difference between Buffer B and Hunter's Buffer is that the latter contains a non-ionic detergent, Triton X-100. Detergents are amphiphatic molecules that can intercalate into lipid bilayers and solubilize membrane proteins. Thus, Hunter's Buffer can extract membrane-bound proteins that could not be isolated with Buffer B alone. Before resuspending the pellet in Hunter's Buffer, we recorded the volume of the supernatant isolated during the previous step. Then, we added the exact same volume of Hunter's Buffer to the pellet so that aliquots from different fractions would be equalized. The cell pellet solubilized by Hunter's Buffer constitutes the insoluble cell fraction.

Finally, we isolated the whole cell lysate by using just Hunter's Buffer. Without centrifugation, the lysed, homogenized, and sonicated samples, which contain both soluble and insoluble proteins, were analyzed for protein expression.

After electrophoresis, blots were probed for specific proteins using chemiluminescence. Band densities were quantified using the ImageJ software, and the results were analyzed for statistical significance with a student's t-test.

Statistical Analysis

Results were analyzed on the Prism software. Values are expressed as mean ± SEM (standard error of the mean). Migration assay results were analyzed by a two-way ANOVA, followed by post-hoc analysis. Protein levels were analyzed by a two-tailed student's t-test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

The knockdown of mDia1 does not cause a significant change in VSMC migration

Past experiments from the laboratory show that the knockout of mDia1 significantly increases the basal and PDGF-induced migration of VSMCs (Figure 1). Based on these results, we hypothesized that the knockdown of mDia1 would also increase VSMC migration. To test this hypothesis, we performed transwell migration assays with VSMCs that had been transfected with either a mDia1-targeting siRNA or a scrambled siRNA. To amplify any changes in VSMC migration, we added PDGF, a pro-migratory stimulus, to the media in half of the experimental wells. Membranes were immersed in media for 4 hours after cell seeding, and cells that migrated through the membranes were stained with DAPI, a nuclear dye, and visualized under the microscope (Figure 3A). Then, the cells were quantified on the ImageJ software. The knockdown of mDia1 was confirmed through western analyses (Figure 4).

Our results show that the knockdown of mDia1 does not cause a significant change in the basal or PDGF-induced migration of VSMCs (Figure 3B). The fold change from basal to PDGF-induced migration of WT cells, 4.97 ± 0.6376, is similar to that of mDia1 knockdown cells, 4.73 ± 0.7447 (values are means ± SEM, n=4). These results indicate that the knockdown of mDia1 has a negligible effect on VSMC migration.

The knockdown of mDia1 slightly increases α-tubulin levels in VSMCs

Although the knockdown of mDia1 does not produce a significant change in VSMC migration, it slightly increases the cytosolic levels of α-tubulin, a cytoskeletal protein that dimerizes with β-tubulins to form microtubule filaments (Figure 5). This increase in α-tubulin levels is consistent with the results from the previous mDia1 knockout experiments (Figure 1).

For the next part of the project, we explored what pathways might underlie this change in α-tubulin levels.

The effect of mDia1 knockdown on α-tubulin mRNA expression (Preliminary)

We hypothesized that mDia1 may down-regulate the expression of the α-tubulin mRNA. Then, the knockdown of mDia1 would reverse this inhibition and increase the mRNA levels of α-tubulin. This would be a pathway by which the knockdown of mDia1 would up-regulate the expression of the α-tubulin protein. To test this hypothesis, we compared the relative levels of the α-tubulin mRNA in each siRNA group using qRT-PCR.

Our results show that the mRNA levels of α-tubulin are unaltered by changes in mDia1 expression (Figure 6). These preliminary results need to be confirmed in future experiments.

The effect of mDia1 knockdown on the proteasomal degradation of α-tubulin

As our results have shown that α-tubulin levels rise with the permanent (Figure 1) and acute (Figure 5) depletion of mDia1, we hypothesized that mDia1 might cause a down-regulation of α-tubulin expression by activating a proteasomal degradation pathway. Thus, we hypothesized that the knockdown of mDia1 would prevent the proteasomal degradation of α-tubulin. To test whether mDia1 activates a pathway leading to the proteasomal degradation of α-tubulin, we treated cells with the MG132 proteasome inhibitor or DMSO, a vehicle control, and analyzed the changes in the concentration of α-tubulin through western analyses. First, we found the optimal dose of MG132 to be 1μM. This dose blocks the proteasomal degradation of ubiquitin-tagged proteins without affecting cell viability (Figure 7).

In an effort to maximize the extraction of protein from cells for use in the MG132 treatment assays, we fractionated the cells into detergent-soluble and insoluble fractions using the procedure outlined in *Methods.* This procedure also allowed us to monitor the compartmentalization of α-tubulin. Consistent with previous trends, the knockdown of mDia1 increases the concentration of α-tubulin in the cytosolic fraction of cells in DMSO (Figure 8A). Also, α-tubulin expression increases when WT cells, but not mDia1 knockdown cells, are treated with the MG132 proteasome inhibitor (Figure 8B). Such changes in α-tubulin levels are unique to the soluble fraction (Figure 8C). These data suggest that mDia1 might activate a pathway leading to the proteasomal degradation of a-tubulin. Incompatible with these results, however, are the results that show that whole-cell α-tubulin levels remain unaltered despite changes in the expression of mDia1 (Figure 9).

The knockdown of mDia1 may increase the cytosolic levels of α-tubulin in VSMCs by preventing the translocation of α-tubulin to the membrane

During the MG132 assays, we discovered that a decrease in α-tubulin levels in the *insoluble* fraction of mDia1 knockdown cells complements the increase in α-tubulin levels in the soluble fraction (Figure 10). These results suggest that mDia1 may promote the translocation of α-tubulin to cellular membranes. This may explain why cytosolic α-tubulin levels increase in mDia1 knockdown cells.

To investigate whether the α-tubulins in the insoluble cell fraction are membrane tubulins, we consulted a study by Goswami et al. (Goswami, 2012), which has shown that acetylation is one of the most common types of post-translational modification affecting membrane tubulins. Our preliminary results show that the ratio of acetylated α-tubulin over total α-tubulin is greater

in WT cells than in mDia1 knockdown cells (Figure 11). This observation suggests that mDia1 promotes the membrane translocation of α-tubulin.

In summary, our results show that the concentration of α-tubulin in the soluble fraction increases with the knockdown of mDia1, while the opposite occurs in the insoluble fraction, although we have yet to achieve statistical significance (Figure 10). Furthermore, our mDia1 knockdown experiments show that the whole-cell expression of α-tubulin is unaltered by changes in mDia1 levels (Figure 9), and that acetylation, a common characteristic of membrane tubulins, is abundant among α-tubulins in the insoluble fraction of WT cells (Figure 11). Taken together, these results imply that mDia1 may promote the localization of α-tubulin to cellular membranes.

DISCUSSION

The overall goal of this project was to investigate the effect of mDia1 on α-tubulin expression and VSMC migration. The mDia1 knockout experiments performed by Dr. Candace Adamo provided the foundation for this study. Her experiments show that the knockout of mDia1 significantly increases VSMC migration. These results are somewhat surprising, as mDia1 has been shown to promote microtubule stabilization (Yamana *et al.*, 2006), which is essential for the establishment of cell polarity and the persistent migration of a cell (Vasiliev *et al.*, 2013). However, Dr. Adamo's experiments also show that the knockout of mDia1 increases the cytosolic levels of α-tubulin in VSMCs. This suggests that mDia1 not only causes the stabilization of microtubules, but that it may also regulate the expression of α-tubulin and impact VSMC migration. Based on the results from these mDia1 knockout experiments, we hypothesized that the knockdown of mDia1 would also increase VSMC migration and α-tubulin expression. We proposed that an increase in the expression of the α-tubulin mRNA or a decrease in the proteasomal degradation of the α-tubulin protein could be possible mechanisms through which the knockdown of mDia1 enhances the cytosolic levels of α-tubulin.

First, to evaluate whether the knockdown of mDia1 increases cell migration, we performed transwell migration assays with siRNA-transfected VSMCs. Contrary to our hypothesis, our results show that the knockdown of mDia1 does not have a significant effect on the basal or PDGF-induced migration of VSMCs. These results also contradict findings from previous knockout experiments from the laboratory, which show that the knockout of mDia1 significantly increases VSMC migration. We rationalized the discrepancy between the knockdown and knockout experimental results by proposing that the knockout, or the *permanent*

depletion, of mDia1 activates a compensatory mechanism that is missing in mDia1 knockdown cells, which experience only an *acute* depletion of the protein.

Given that a number of pathways radiate from Rho family GTPases, the deletion of a key downstream effector, mDia1, may alter the strength of other related signaling pathways. mDia2, for example, is an isoform of mDia1 that also operates downstream of RhoA and helps assemble various cytoskeletal elements, including lamellipodia (Staus *et al.*, 2011; Yang *et al.*, 2007). Thus, the knockout of mDia1 may affect the activation of mDia2 by RhoA and increase VSMC migration. Another potential member of this compensatory pathway is Rho Kinase (ROCK), which is another downstream effector of RhoA. ROCK promotes the formation of acto-myosin fibers, which generate the mechanical push that is required for cell movement. The permanent depletion of mDia1 may enhance the activity of ROCK, thereby increasing VSMC migration. However, mDia2 and ROCK are only two proteins out of hundreds of other regulatory proteins that constitute the highly interweaved Rho GTPase signaling network. The exact constituents of this pathway that may compensate for the permanent loss of mDia1 are to be determined in future studies.

Although the knockdown of mDia1 does not cause a significant change in VSMC migration, it slightly increases the cytosolic levels of α-tubulin. This trend is nearly significant ($p=0.072$) and corresponds with the results from previous mDia1 knockout experiments from the laboratory. To explain this trend, we assessed the effect of the knockdown of mDia1 on α-tubulin mRNA levels using qRT-PCR. Our preliminary results suggest that the levels of the α-tubulin mRNA are unaltered by changes in mDia1 expression. However, these preliminary results need to be verified.

Seeking an alternative explanation for the increase in cytosolic α-tubulin levels with the knockdown of mDia1, we explored the effect of mDia1 on the proteasomal degradation of α-tubulin. We developed this aim in light of a recent study showing that a protein called HACE1 impairs cellular migration by promoting the proteasomal degradation of Rac, a Rho family GTPase (Castillo-Lluva *et al.*, 2012). As the ubiquitin-proteasome pathway had been demonstrated to regulate cell migration, we proposed that mDia1 might also mediate VSMC migration by affecting the proteasomal degradation of a protein—namely, α-tubulin. Specifically, we tested whether the knockdown of mDia1 affects a pathway leading to the proteasomal degradation of α-tubulin. To test this hypothesis, we treated cells with the MG132 proteasome inhibitor and measured the changes in α-tubulin levels in siRNA-transfected cells. Our results indicate that α-tubulin levels increase in response to the inhibition of the proteasome in WT cells, but not in mDia1 knockdown cells. However, whole-cell α-tubulin levels remain unaltered despite changes in mDia1 expression. These results, suggesting a change in the proteasomal degradation and a lack of change in the whole-cell content of α-tubulin, are incompatible with each other. Thus, altering the proteasomal degradation of α-tubulin seems unlikely to be the mechanism by which mDia1 impacts the level of α-tubulin in VSMCs.

Interestingly, a decrease in α-tubulin levels in the *insoluble* cell fraction complements an increase in α-tubulin levels in the soluble fraction. These patterns agree with our results, showing that whole-cell α-tubulin levels remain unaltered regardless of changes in mDia1 expression. These results suggest that mDia1 promotes the translocation of α-tubulin to plasma, nuclear, or organellular membranes. Then, we hypothesized that the knockdown of mDia1 would decrease the localization of α-tubulin to cellular membranes, thereby increasing the cytosolic concentration of α-tubulin. In support of this theory, the ratio of acetylated α-tubulins over total

α-tubulin is greater in WT cells than in mDia1 knockdown cells; acetylation has been demonstrated to be one of the most common types of post-translational modifications affecting membrane tubulins. Taken together, these results suggest that mDia1 promotes the localization of α-tubulin to the membranes. This provides a mechanism by which the knockdown of mDia1 increases α-tubulin levels in the soluble fraction of the cell.

In addition to upholding the structural integrity of a cell, microtubules can participate in signaling pathways. Membrane tubulins were first discovered around 1974 (Blitz *et al.*, 1974). Zisapel et al. (Zisapel *et al.*, 1980) found that although some membrane tubulins can interact directly with membranes, most interact with them indirectly by binding to membrane proteins. A recent study shows that membrane tubulins can regulate the opening and closing of TRPV1, an ion channel with a tubulin-binding motif (Goswami, 2012). The activation of TRPV1 in neuronal membranes may mediate pain signaling (Goswami, 2012). Interestingly, the activation of calcium channels and the subsequent influx of calcium ions are required for the contraction and growth of VSMCs. Thus, mDia1, by promoting the localization of α-tubulin to membranes and potentially regulating the activity of ion channels, may play an important physiological role in VSMCs.

Conclusions

This project has shown that the knockdown of mDia1 does not cause a significant change in VSMC migration, α-tubulin mRNA expression, or the proteasomal degradation of α-tubulin. However, our results suggest that mDia1 may promote the membrane translocation of α-tubulin, so that the knockdown of mDia1 would increase the cytosolic levels of α-tubulin. These membrane tubulins may target calcium channels, whose activity regulates the growth and

contraction of VSMCs. However, the place of membrane tubulins in the vast signaling network regulating the physiology of VSMCs is unknown. Future studies will elucidate the physiological significance of membrane tubulins in VSMCs, as well as their role in the pathophysiology of the cardiovascular system.

Future Directions

We will repeat the qRT-PCR experiments to confirm our preliminary observations regarding the effect of mDia1 on the expression of the α-tubulin mRNA. An important next step in this project will be to find the physiological implications of mDia1-mediated changes in α-tubulin expression. To elucidate the physiological and pathophysiological significance of the potential interaction between mDia1 and membrane tubulins, one may embark on a project that measures how the deficiency of mDia1 impacts the influx of calcium ions in VSMCs.

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FIGURES

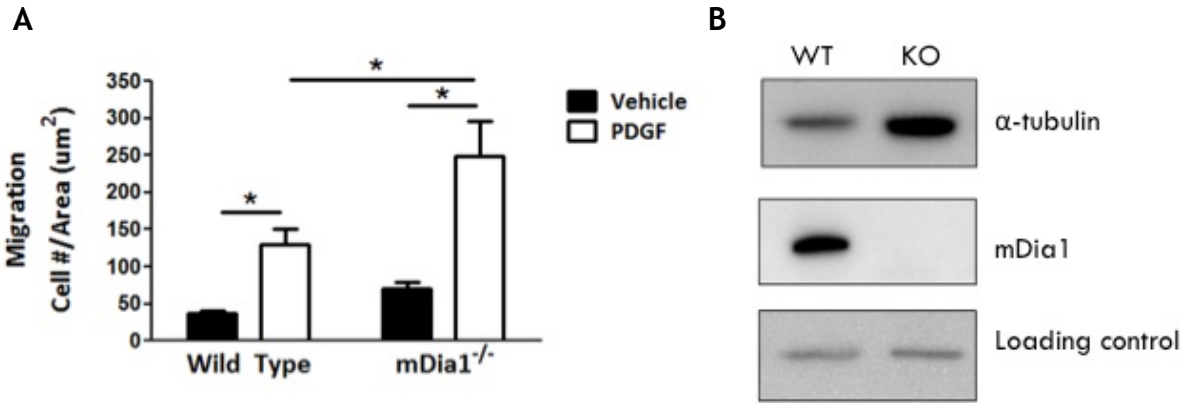

Figure 1. The knockout of mDia1 increases VSMC migration and α-tubulin expression (unpublished data, courtesy of Dr. Adamo). (A) The migration of VSMCs was analyzed using the transwell assay system. 40,000 cells were seeded on each transwell membrane suspended in a well, containing media. The media contained either vehicle control or PDGF, a pro-migratory stimulus. Cells were incubated in the media for 4 hours. Values are means ± SEM. Results were analyzed by a two-way ANOVA, followed by post-hoc test. A value of $p<0.05$ was considered to be statistically significant. N=3. (B) Protein expression in WT and mDia1 knockout cells. α-tubulin levels are shown in the top panel, and the knockout of mDia1 is shown in the middle panel.

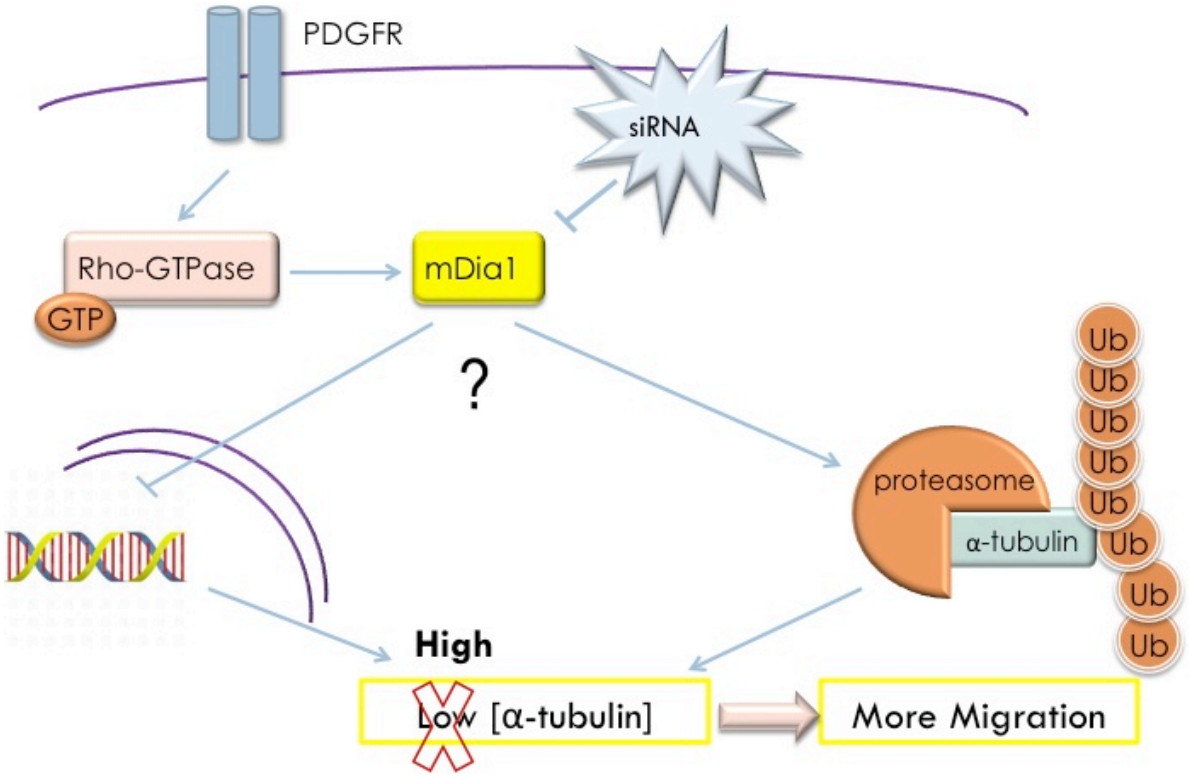

Figure 2. A model of the hypothesis. We hypothesized that the siRNA-induced knockdown of mDia1 would increase both VSMC migration and α-tubulin protein expression. We proposed that the knockdown of mDia1 might promote the expression of the α-tubulin mRNA and/or repress a pathway leading to the proteasomal degradation of α-tubulin.

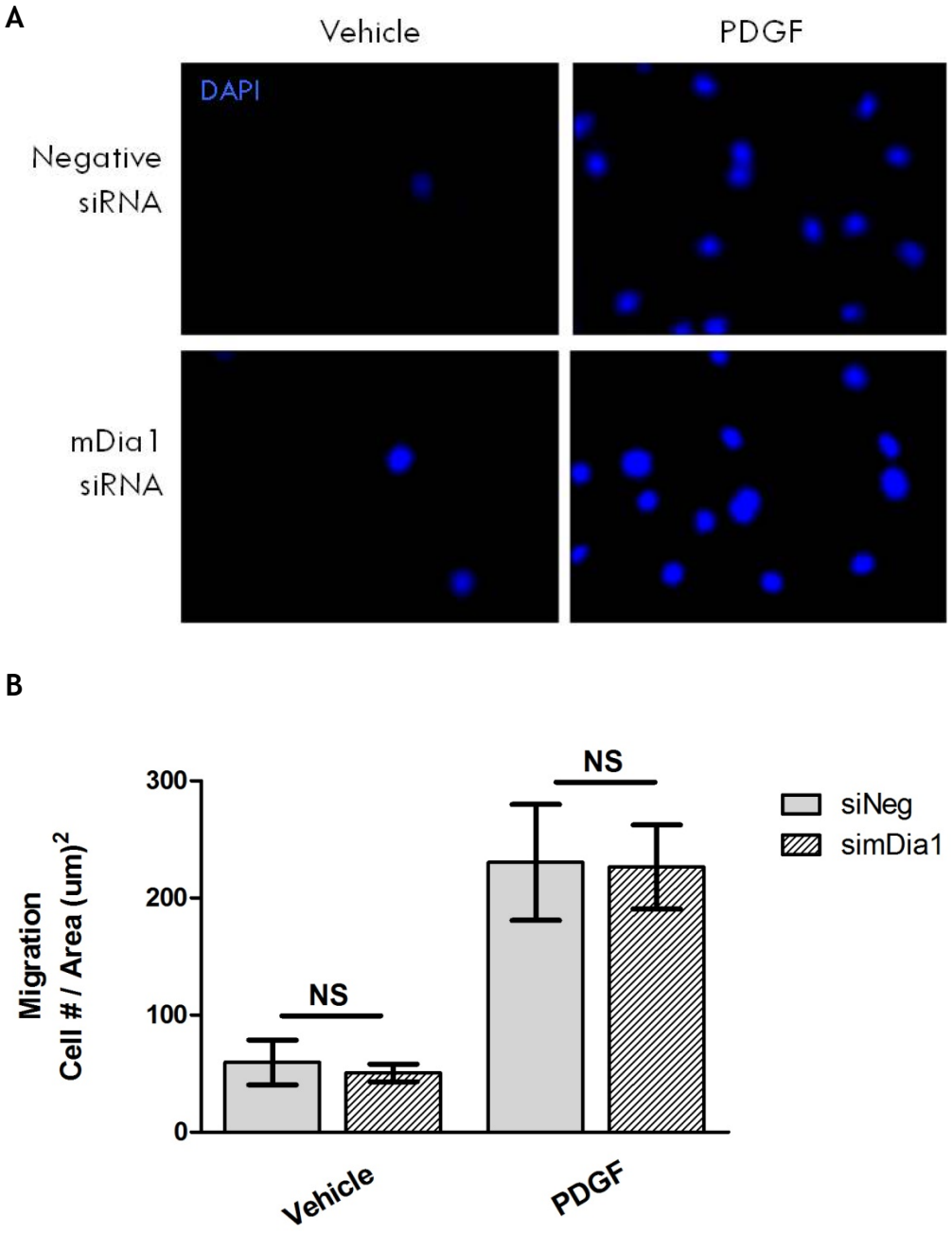

Figure 3. The knockdown of mDia1 does not alter VSMC migration. (A) Membranes showing the DAPI-stained nuclei, which are representative of cells that had migrated during the transwell migration assay. 40,000 cells were seeded on each transwell membrane. Vehicle control or PDGF, a pro-migratory stimulus, was added to media in the wells. Each migration assay was run for four hours. (B) Mean (± SEM) migration of VSMCs. Results were analyzed by a two-way ANOVA, followed by post-hoc test. A value of $p<0.05$ was considered to be statistically significant. N=4.

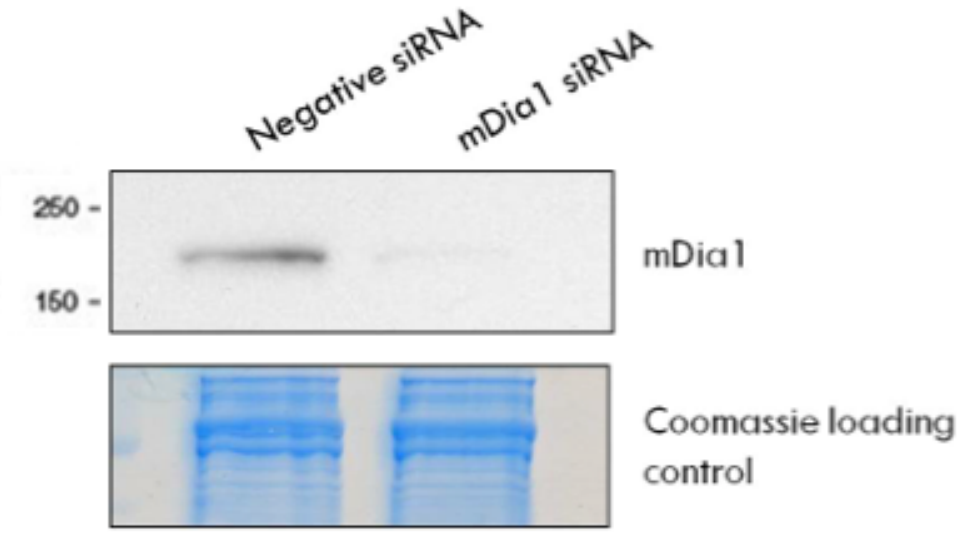

Figure 4. The knockdown of mDia1 using siRNA (top panel).
The Coomassie-stained SDS-PAGE was used as a loading control (bottom panel). N=4.

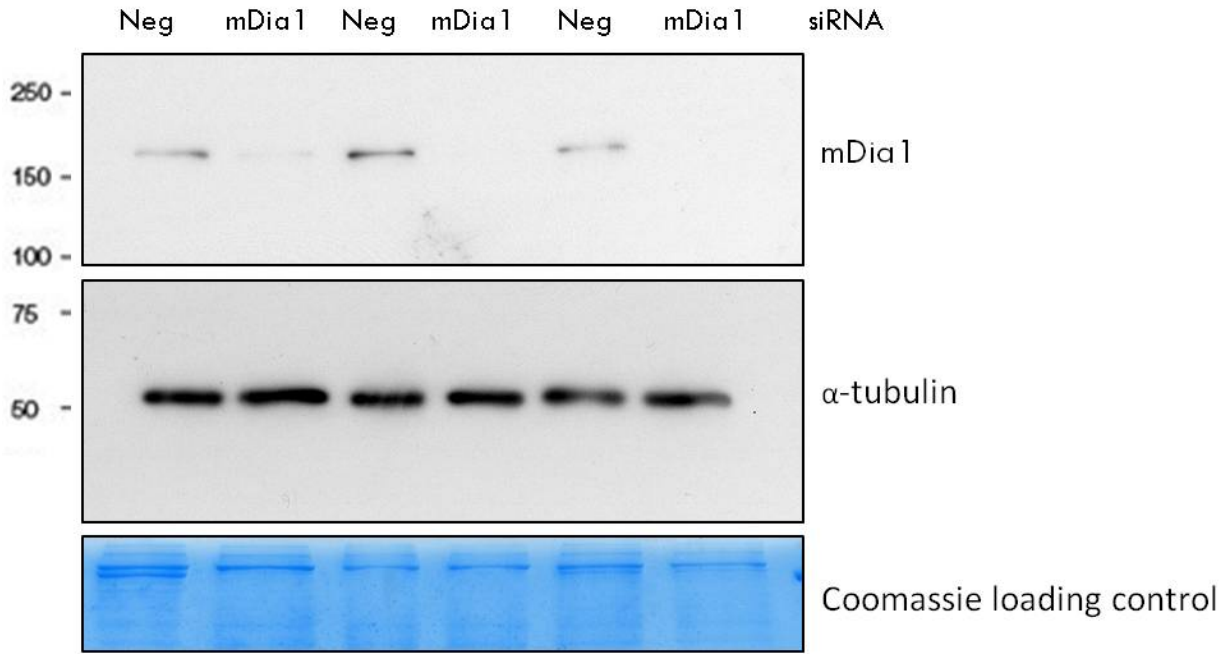

Figure 5. The knockdown of mDia1 increases the level of α-tubulin in VSMCs. The knockdown of mDia1 is shown in the top panel, and α-tubulin levels are shown in the middle panel. The Coomassie-stained SDS-PAGE was used as a loading control (bottom panel). N=3.

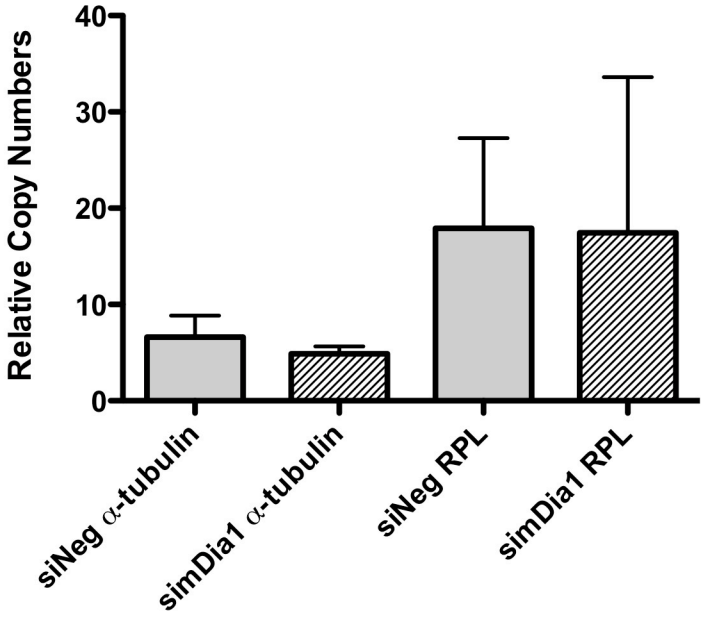

Figure 6. The mRNA levels of α-tubulin in VSMCs are unaltered by changes in mDia1 expression. Ribosomal protein L7 (RPL) was used as a housekeeping gene. VSMCs were transfected with the mDia1-targeting siRNA or the scrambled siRNA. Cells were harvested using the Qiagen RNeasy Mini Kit, and mRNA expression was analyzed by real-time PCR (qRT-PCR). The relative copy numbers of the initial cDNA in each sample was quantified using the MAK3 program. Data represent means ± SEM from one experiment.

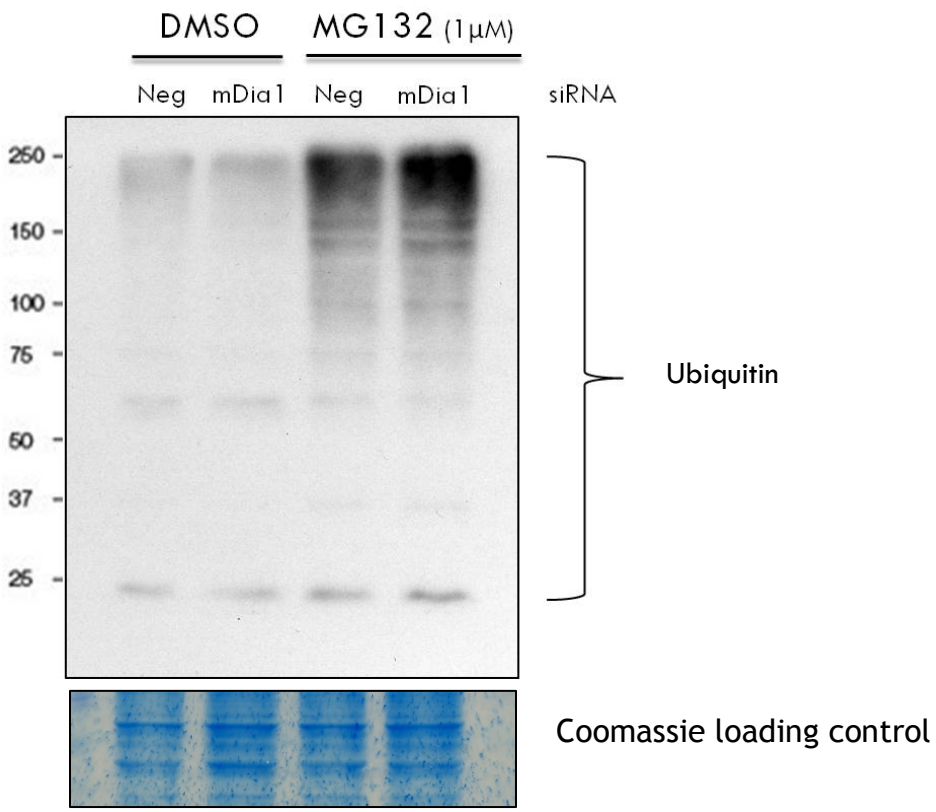

Figure 7. One μM MG132 is sufficient to prevent the proteasomal degradation of ubiquitin-tagged proteins. The concentration of DMSO was also 1μM. The Coomassie-stained SDS-PAGE was used as a loading control (bottom panel). VSMCs were incubated with either DMSO or MG132 for 16 hours and harvested with a lysis buffer.

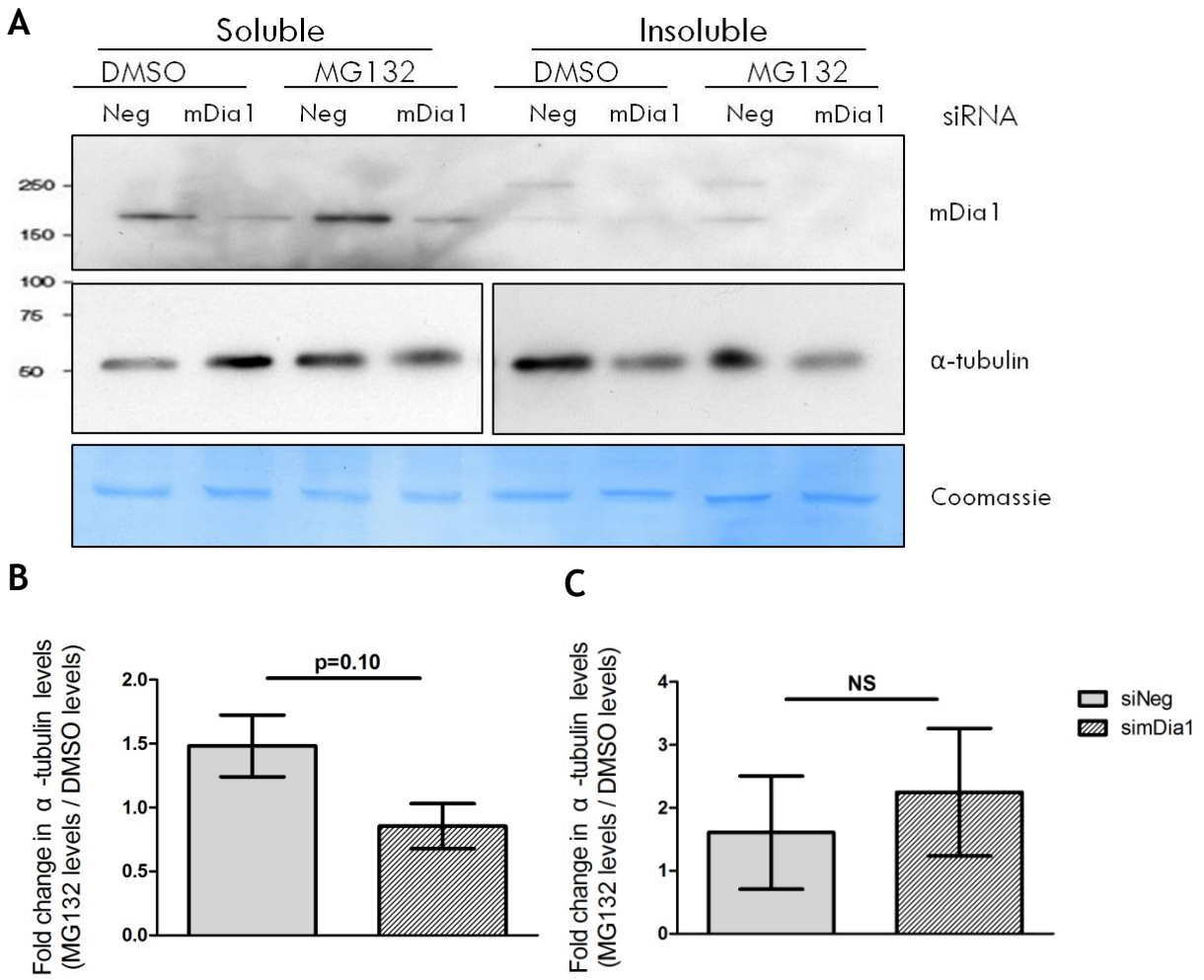

Figure 8. The treatment of VSMCs with the proteasome inhibitor increases α-tubulin levels in WT cells, but not in mDia1 knockdown cells. Cells were treated with 1μM MG132, a proteasome inhibitor, or DMSO, the vehicle control, for 16 hours. Cells were lysed in Buffer B and centrifuged at 12,000 rpm for 20 minutes. The supernatant, containing water-soluble proteins, was obtained, while the cell pellet was further solubilized in Hunter's Buffer, which contains Triton X-100, a detergent. (A) VSMC protein levels. The knockdown of mDia1 is shown in the top panel, and α-tubulin levels are shown in the middle panel. The Coomassie-stained SDS-PAGE was used as a loading control (bottom panel). (B and C) The fold change in α-tubulin levels in (B) the soluble and (C) the insoluble fraction, analyzed on the ImageJ and Prism software. The fold change was calculated by dividing the α-tubulin level in MG132-treated cells by that in DMSO-treated cells. Values are means ± SEM from three experiments. Results were analyzed by a two-tailed student's t-test. A value of $p<0.05$ was considered to be statistically significant.

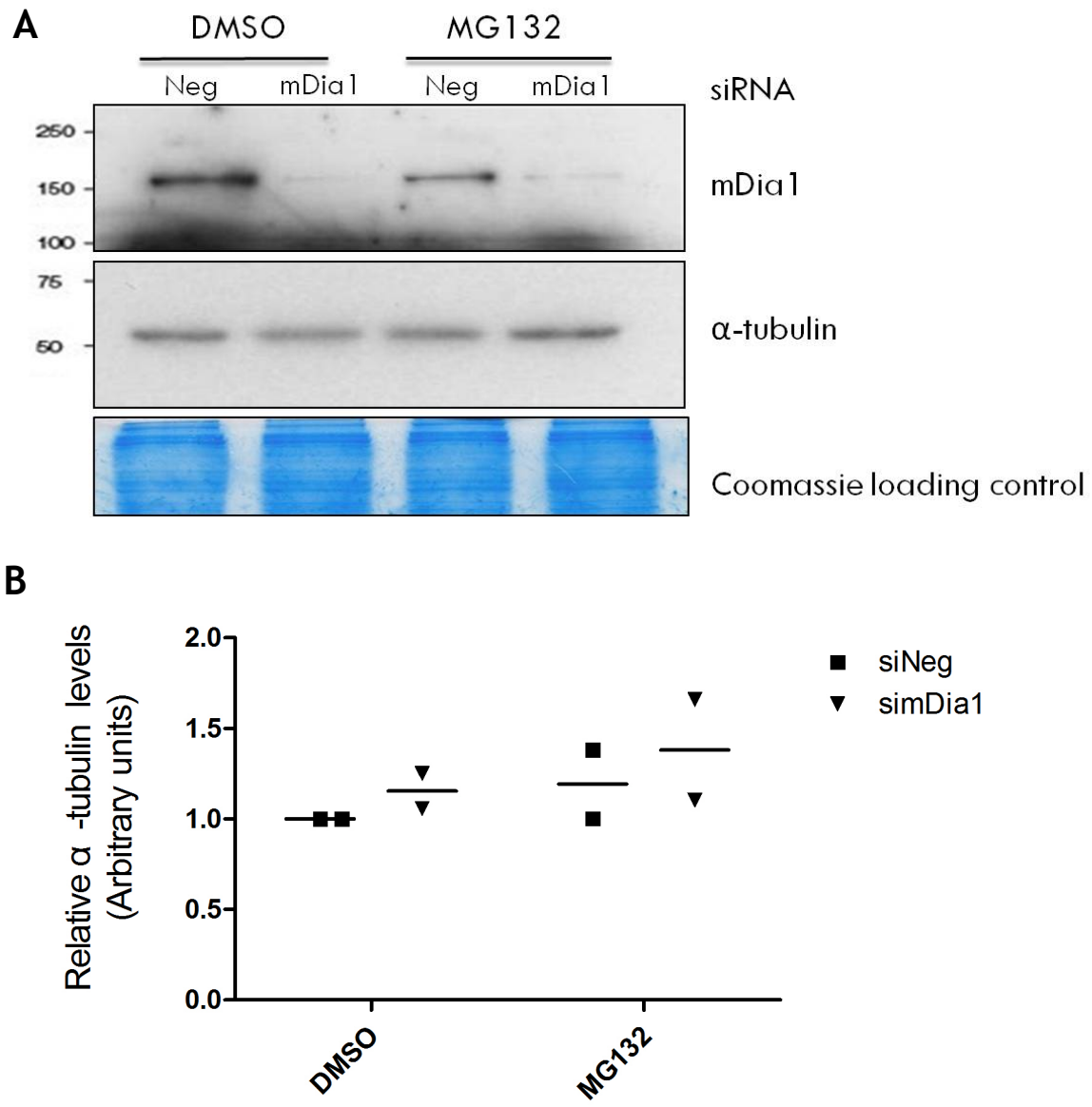

Figure 9. Whole-cell α-tubulin levels are unaltered by changes in mDia1 expression. Cells were treated with 1μM MG132, a proteasome inhibitor, or DMSO, the vehicle control, for 16 hours. Cells were lysed with Hunter's Buffer, containing Triton X-100. (A) VSMC protein levels. The knockdown of mDia1 is shown in the top panel, and α-tubulin levels are shown in the middle panel. The Coomassie-stained SDS-PAGE was used as a loading control (bottom panel). (B) A quantification of the α-tubulin bands using the ImageJ and Prism software. Data are representative of two experiments.

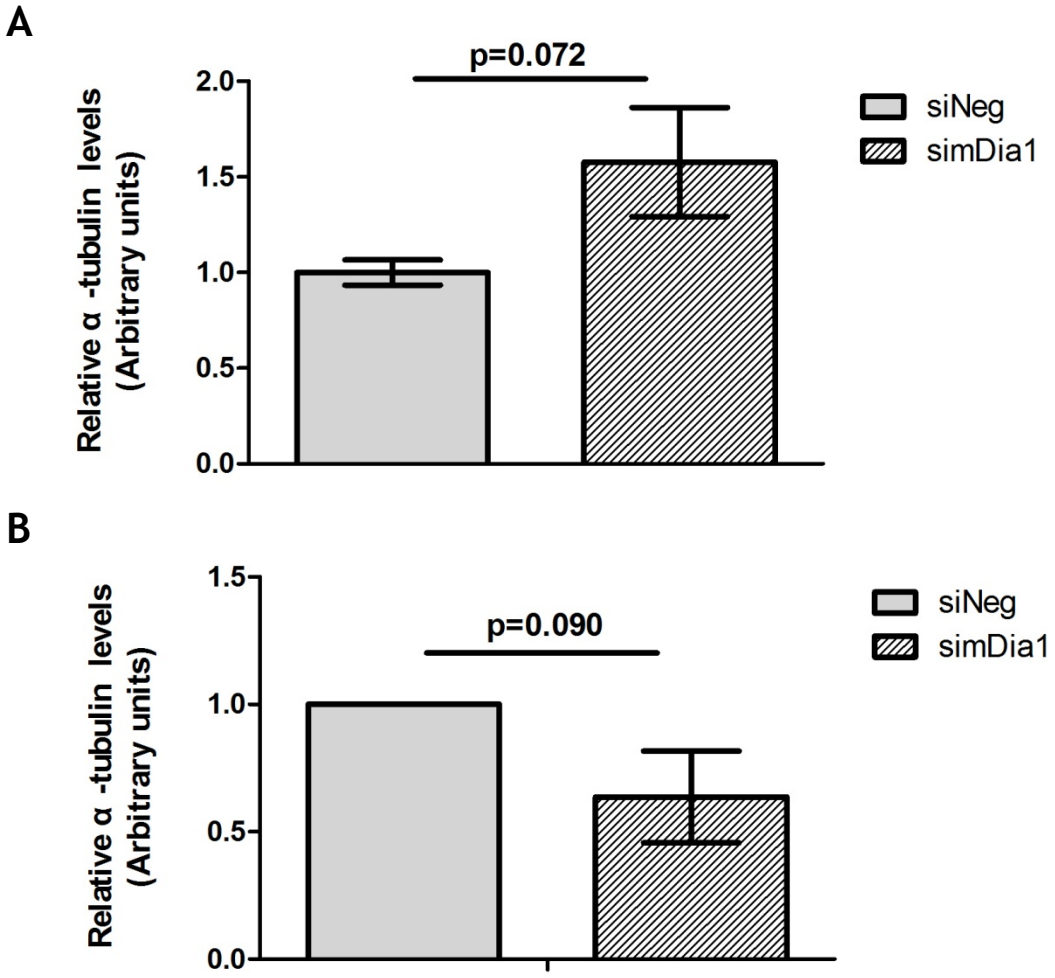

Figure 10. A decrease in the level of α-tubulin in the insoluble fraction of mDia1 knockdown cells complements its increase in the soluble fraction. A graphical representation of α-tubulin expression in (A) the soluble and (B) insoluble cell fractions. (A) P=0.072 and n=7, and (B) P=0.090 and n=3. Values are means ± SEM. Results were analyzed by a two-tailed student's t-test. A value of $p<0.05$ was considered to be statistically significant.

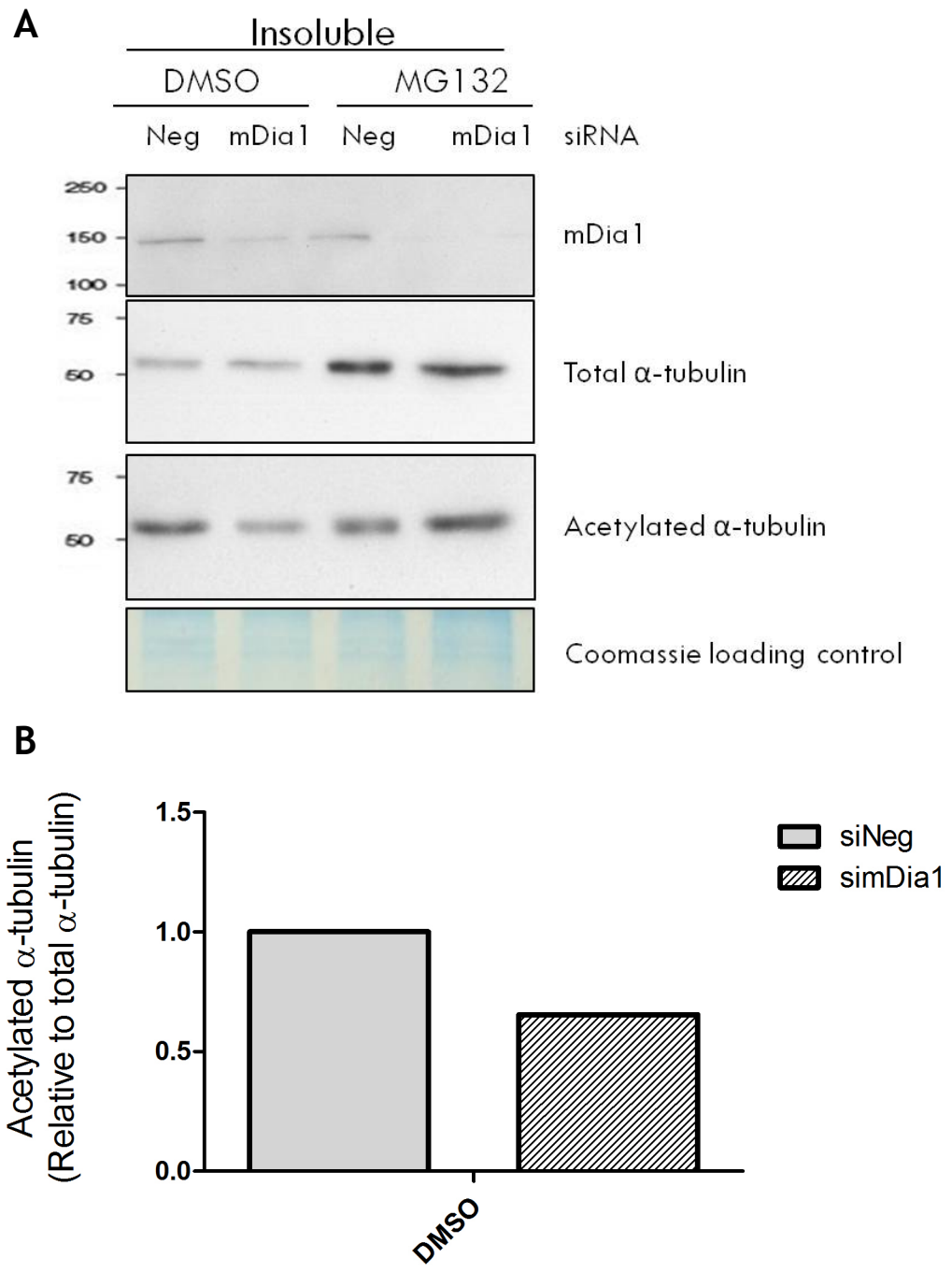

Figure 11. The ratio of acetylated α-tubulins is greater in the insoluble fraction of negative siRNA cells than in mDia1 knockdown cells. (A) Protein levels in the insoluble fraction. The top panel shows the knockdown of mDia1. The second panel shows the level of α-tubulin, and the third panel shows the level of acetylated α-tubulin. The Coomassie-stained SDS-PAGE was used as a loading control (bottom panel). (B) The ratio of acetylated α-tubulin in each siRNA group. N=1.