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POLDIP2, A NOVEL REGULATOR OF NOX4 AND CYTOSKELETAL INTEGRITY IN VASCULAR SMOOTH MUSCLE CELLS

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POLDIP2, A NOVEL REGULATOR OF NOX4 AND CYTOSKELETAL INTEGRITY IN VASCULAR SMOOTH MUSCLE CELLS

by

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B.S., Georgia Institute of Technology, 2002

Advisor: Kathy K. Griendling, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

in

Molecular and Systems Pharmacology
Graduate Division of Biological and Biomedical Sciences

2009

ABSTRACT

POLDIP2, A NOVEL REGULATOR OF NOX4 AND CYTOSKELETAL INTEGRITY IN VASCULAR SMOOTH MUSCLE CELLS

By

Alicia N. Lyle

Reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet}$-) and hydrogen peroxide (H_2O_2), are implicated in the development of cardiovascular disease pathologies, including atherosclerosis and restenosis. Physiologically, ROS mediate functions including proliferation, gene expression, migration, differentiation, and cytoskeletal remodeling. One major source of ROS is the NADPH oxidase (Nox) enzymes. In vascular smooth muscle cells (VSMCs), the regulatory proteins that associate with individual Nox homologues are poorly defined. The membrane-bound Nox subunit heterodimerizes with p22phox to form the catalytic moiety and p22phox serves as the docking site for regulatory subunits. Using the cytosolic c-terminal tail of p22phox for a yeast two-hybrid screen, we identified Poldip2, polymerase (DNA-directed) delta interacting protein 2, as a novel p22phox binding partner.

Immunoprecipitation and co-localization experiments confirm the association of Poldip2 with p22phox. Poldip2 functionally associates with the Nox4/p22phox complex in a p22phox-dependent manner and significantly increases Nox4 enzymatic activity and Nox4-dependent ROS production, thus establishing Poldip2 as a novel positive modulator of Nox4. Furthermore,

functional studies indicate that Poldip2 may negatively modulate Nox1 enzymatic activity. In VSMCs, Nox1 and Nox4 are differentially regulated by agonists and exhibit distinct subcellular localization patterns. The data presented establish that Poldip2 is required for proper localization and trafficking of the Nox4/p22phox complex to focal adhesions. Activation of Nox4/p22phox by Poldip2 promotes ROS-dependent activation of RhoA, strengthens focal adhesions and increases stress fiber formation, while depletion of either Poldip2 or Nox4 results in a loss of these structures. Cell migration, which requires dynamic cytoskeletal remodeling, is impaired by either excess or insufficient Poldip2, thereby implicating Nox4/p22phox/Poldip2 in Rho-dependent cytoskeletal reorganization, focal adhesion turnover, and migration. Additionally, Poldip2 overexpression increases VSMC polyploidy by blocking cell cycle progression through G_2/M.

This is the first report of a protein that functions to positively regulate Nox4 activity and Nox4-dependent ROS production. These data altogether link ROS production by Nox4/Poldip2 to the regulation of cellular functions dependent on tight coordination of cytoskeletal regulation, such as migration and cell cycle progression. Therefore, Poldip2 may serve as a novel therapeutic target for vascular pathologies with a VSMC migratory and/or proliferative component, such as restenosis and atherosclerosis.

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ACKNOWLEDGEMENTS

I would like to thank the many people who were instrumental in helping me to complete this body of work. To my committee members Lou Ann Brown, Eddie Morgan, and Roy Sutliff, I am thankful for your guidance and feedback, which enabled me to generate a strong and interesting project. A special thanks to my exceptional mentor, Kathy K. Griendling, whose encouragement, leadership, and insight allowed me to develop strength, not only as a scientist, but as an individual. I have learned so much from your instruction. To my lab members, I would like to thank each and every one of you. It's an awesome privilege to be able to work with such a diverse group of people from all over the world, to learn about your cultures, and to form such wonderful friendships with great people, not to mention excellent scientists. To Dr. Cynthia Whitehead-LaBoo, you are an amazing person and I owe you many thanks. To the many friends who supported me before and during my graduate training, and who I know will always support me hereafter, thank you for your friendship and reassurance. A very special thanks to my loving parents Ron and Karen, my brother Eric, and my wonderful extended family. Last, but certainly not least, to my supportive and loving husband Josh, I could not have done this without you.

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LIST OF SYMBOLS AND ABBREVIATIONS

Ad	Adenovirus
Ang II	Angiotensin II
AP-1	Activator Protein-1
AS	Antisense
Cys	Cysteine
EC	Endothelial Cells
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum
ERK	Extracellular-signal Regulated Kinase
FAD	Flavin Adenine Dinucleotide
FAK	Focal Adhesion Kinase
GRP	Glucose Regulated Protein
GSH-Px	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
HA	Hemagglutinin
IL	Interleukin
JNK	c-Jun N-terminal Kinase
LIMK	LIM Kinase
LMW	Low Molecular Weight
MAPK	Mitogen Activated Protein Kinase
MCP1	Monocyte Chemotactic Protein 1

MMP	Matrix Metalloproteinase
MLC	Myosin Light Chain
NAC	N-acetyl Cysteine
NF-κB	Nuclear Factor – κB
$NO^{\bullet}$	Nitric Oxide
Nox	NADPH oxidase
NoxA1	Nox Activator 1
NoxO1	Nox Organizer 1
$O_2^{\bullet}$-	Superoxide
$ONOO^{\bullet}$-	Peroxynitrite
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
PKA	Protein Kinase A
PKC	Protein Kinase C
Prx	Peroxiredoxin
PTK	Protein Tyrosine Kinases
PTP	Protein Tyrosine Phosphatases
Ref-1	Redox Factor 1
ROS	Reactive Oxygen Species
SF	Stress Fibers
SM α-Actin	Smooth Muscle α-Actin
SM-MHC	Smooth Muscle Myosin Heavy Chain
SOD	Superoxide Dismutase

SOH	Sulfenic
SO_2H	Sulfinic
SO_3H	Sulfonic
SRF	Serum Response Factor
SSH1L	Slingshot IL Phosphatase
Trx	Thioredoxins
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VSMCs	Vascular Smooth Muscle Cells

CHAPTER 1

Introduction

1. Introduction

An increasing body of literature indicates that small amounts of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet}$-) and hydrogen peroxide (H_2O_2), play important roles as signaling molecules for normal cellular functions in vascular cells. Over the last decade, many studies have shown that a major source of ROS in the vascular wall is the nonphagocytic NADPH oxidase (Nox) family of enzymes. ROS, when produced in low and transient levels, are involved in the regulation of normal physiological processes, but, when produced in excess of natural antioxidant systems, can also contribute to the development of pathophysiological conditions, such as cardiovascular disease. What NADPH oxidases are expressed in the vasculature, the processes regulated by each of these oxidases, and how these oxidases are differentially activated and regulated by specific regulatory subunits is an area of active investigation. Additionally, how different oxidases regulate processes critical to normal cellular function, as well as understanding how Noxes mediate disease progression, are of great interest.

1.1 Reactive Oxygen Species

ROS are oxygen-based small molecules that include $O_2^{\bullet}$-, H_2O_2, and nitric oxide ($NO^{\bullet}$), all of which play important roles as signaling molecules that regulate normal cellular functions when produced in small amounts. The production of $O_2^{\bullet}$- occurs via the one electron reduction of molecular oxygen (Figure 1.1), which is a reaction that is mediated by several enzymatic systems, including NADPH oxidases. While $O_2^{\bullet}$- itself may influence signaling cascades, more

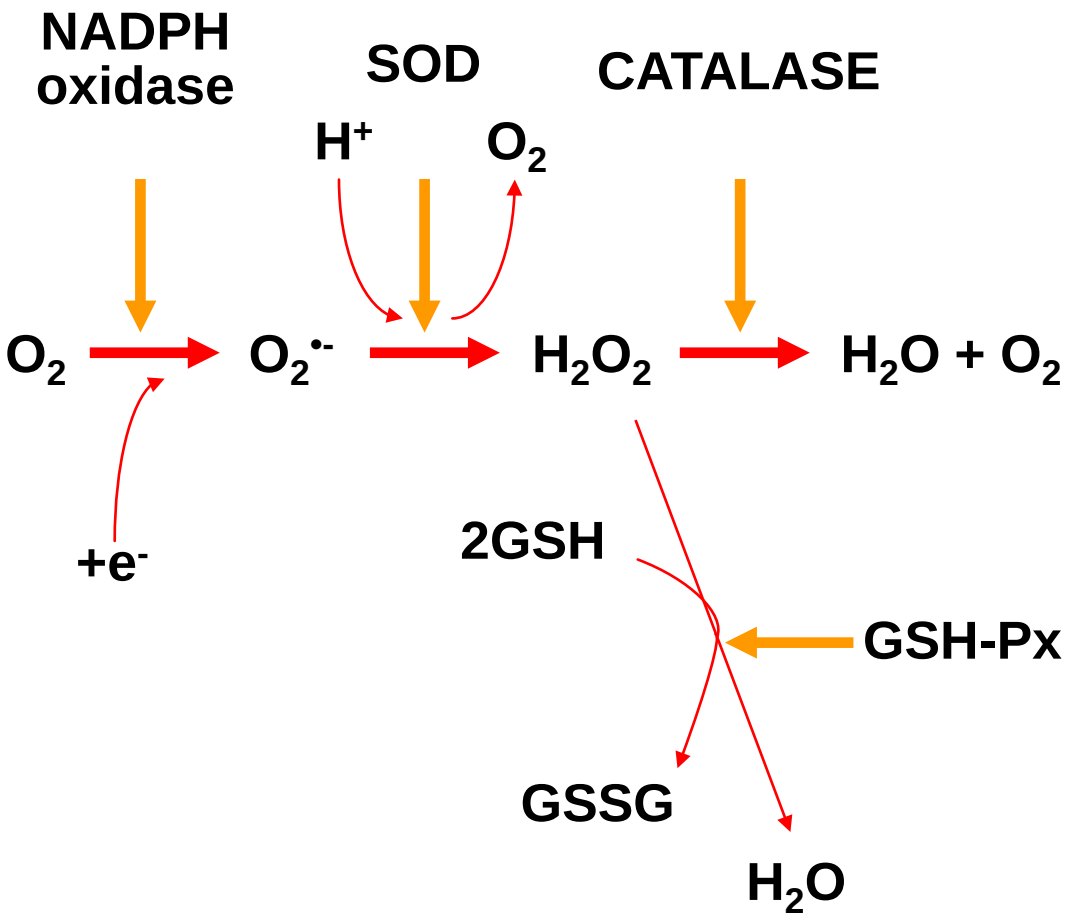


Figure 1.1. ROS Generation by Nox Enzymes and the Metabolism of ROS. Production of superoxide ($O_2^{\bullet-}$) by NADPH oxidases occurs when NADPH oxidases catalyze the one electron reduction of molecular oxygen. Superoxide is then rapidly converted to hydrogen peroxide (H_2O_2) spontaneously or by superoxide dismutase (SOD). Hydrogen peroxide can then be metabolized to water and other secondary metabolites by catalase, glutathione peroxidase (GSH-Px), and/or peroxiredoxins (Prx).

importantly, it produces other reactive species. Superoxide can be rapidly dismutated to the more stable and freely diffusible H_2O_2 either by the enzyme superoxide dismutase (SOD; $K_D = 2 \times 10^9\ M^{-1}\ s^{-1}$) or spontaneously at more acidic pHs ($K_D = 8 \times 10^4\ M^{-1}\ s^{-1}$) (Fridovich, 1983). Superoxide generated in close proximity to a primary target can serve as a signaling molecule itself before undergoing dismutation to H_2O_2 or can react with $NO^{\bullet}$ to generate the free radical peroxynitrite ($ONOO^{\bullet-}$, $K_D = 4\text{-}16 \times 10^9\ M^{-1}\ s^{-1}$), which itself has deleterious consequences and functionally decreases the bioavailability of $NO^{\bullet}$, a primary regulator of vascular relaxation and vasodilation (Ferrer-Sueta and Radi, 2009).

As second messengers, ROS production and removal is tightly regulated, enabling ROS to have a transient mode of action and to act on adjacent targets. Because these radicals have the potential to be toxic, the cell body has endogenous intra- and extra-cellular antioxidant systems that are able to scavenge them. Two enzymes responsible for the elimination of H_2O_2 in cells are catalase and glutathione peroxidase (GSH-Px), which convert H_2O_2 into water and other secondary metabolites. Peroxiredoxins (Prx) function to reduce H_2O_2 and alkylhydroperoxides by utilizing the reducing equivalents provided by thioredoxins (Trx). In contrast, GSH-Pxs utilize glutathione, while catalase requires the presence of oxygen and an iron containing heme in its active site to metabolize H_2O_2. Accumulating evidence suggests that a number of diseases are the result of an imbalance between the generation and the scavenging of these molecules, or a disruption of the redox circuits that they regulate (Bedard and Krause, 2007).

1.1.1 Physiological Functions of ROS in the vasculature

Physiologically, ROS mediate many cellular functions including host defense, gene expression, proliferation, migration, differentiation, and cytoskeletal remodeling, to name a few (Lyle and Griendling, 2006). ROS also modify the oxidative state of vascular cells, thus allowing for dynamic shifts between a pro-oxidant state and a more reductive environment. ROS are capable of influencing cellular signaling systems by both altering the intracellular redox state and by oxidative modification of proteins, thus functioning to alter specific proteins and signaling pathways that contain redox-regulated components, such as thiols, whose redox status controls the activity of the protein (Wolin et al., 2007). ROS play important roles as signaling molecules and regulate a number of normal cellular processes when produced in small amounts; therefore, it is important to fully understand how ROS potentially modify intracellular signaling.

1.1.1.1 Signaling

Currently, the best-established molecular targets directly modified by ROS in cell signaling cascades include mitogen-activated protein kinases (MAPK), protein tyrosine kinases (PTKs), and protein tyrosine phosphatases (PTPs). Protein-tyrosine phosphorylation is a major mechanism for post-translational modification of proteins and plays a critical role in regulating cell proliferation, differentiation, migration, and transformation. The level of tyrosine phosphorylation is a tightly regulated balance between PTK and PTP activity. PTPs counteract the effects of PTK activity by dephosphorylating PTK substrate

proteins, thus acting as negative regulators of a signaling process stimulated by PTK activation. Exposure of cells to low doses of oxidants or thiol-directed agents commonly induces an increase in tyrosine phosphorylation due to PTP inactivation (Heneberg and Draber, 2005; Paravicini and Touyz, 2006).

A number of MAP kinases, a family of serine/threonine kinases, are activated by growth factor stimulation. Several MAP kinases, such as c-Jun N-terminal kinases (JNK) (Schroder et al., 2007), p38MAPK (Ushio-Fukai et al., 1998), and extracellular-signal regulated kinase 1/2 (ERK 1/2) (Ranjan et al., 2006) are activated by ROS (Lassegue et al., 2001; Lyle and Griendling, 2006; Zafari et al., 1998). The activation of receptor and non-receptor PTKs can also be mediated by ROS. Examples of ROS-sensitive receptor tyrosine kinases include the epidermal growth factor receptor (EGFR) and platelet derived growth factor receptor-β (PDGFR-β), whose transactivation was shown to be mediated by ROS (Saito and Berk, 2001; Seshiah et al., 2002; Ushio-Fukai et al., 2001). The regulation of non-receptor tyrosine kinases, such as Src kinases, can also be redox-sensitive (Paravicini and Touyz, 2006). However, while these kinases were all shown to be activated by ROS, it was not established if the activation process was through direct modulation of the kinase itself; therefore, one should also consider the possibility that the modulation of substrate phosphorylation by ROS could be through the inactivation of the PTPs that normally function to dephosphorylate kinase substrates.

PTPs are critical regulators of multiple signaling pathways and, because of their structure, are susceptible to oxidation and inactivation by ROS. Most PTPs

contain a reactive and potentially redox-sensitive cysteine (Cys) residue, which can be oxidized to sulfenic acid by H_2O_2, thus rendering the PTP inactive (Figure 1.2). H_2O_2 appears to specifically target Cys residues in the thiolate form to oxidize them to cysteine sulfenic acid (Cys-SOH), which is a process that is reversible in the presence of endogenous antioxidant systems, such as thioredoxins, which function to reduce oxidized cysteine residues (Rhee et al., 2003). H_2O_2 can potentially further oxidize these residues to sulfinic acid (Cys-SO_2H) and sulfonic acid (Cys-SO_3H) forms. While the sulfenic acid and sulfinic acid reactions are reversible, the sulfonic acid reaction is not, and renders the PTP in an irreversibly inactive state. Because H_2O_2 can modify MAPKs, PTKs and PTPs, as described above, these are common targets of investigation when testing the influence of ROS on specific cell signaling pathways.

1.1.1.2 Gene Expression

A large body of evidence suggests that ROS are involved in the regulation of gene expression. Nox-dependent ROS generation, for example, induces the expression of matrix metalloproteinases (MMPs) and inflammatory genes, such as interleukin 6 (IL-6) and vascular cell adhesion molecule (VCAM)-1, to name a few (Paravicini and Touyz, 2006). Many studies suggest that the mechanism by which ROS regulate gene expression is through redox-sensitive second messenger systems, such as alteration of MAP kinases (Lyle and Griendling, 2006). Other studies suggest that ROS may regulate gene expression through modulation of specific transcription factors, such as activator protein (AP)-1 and nuclear factor (NF)-κB, that contain redox-sensitive Cys residues in their DNA

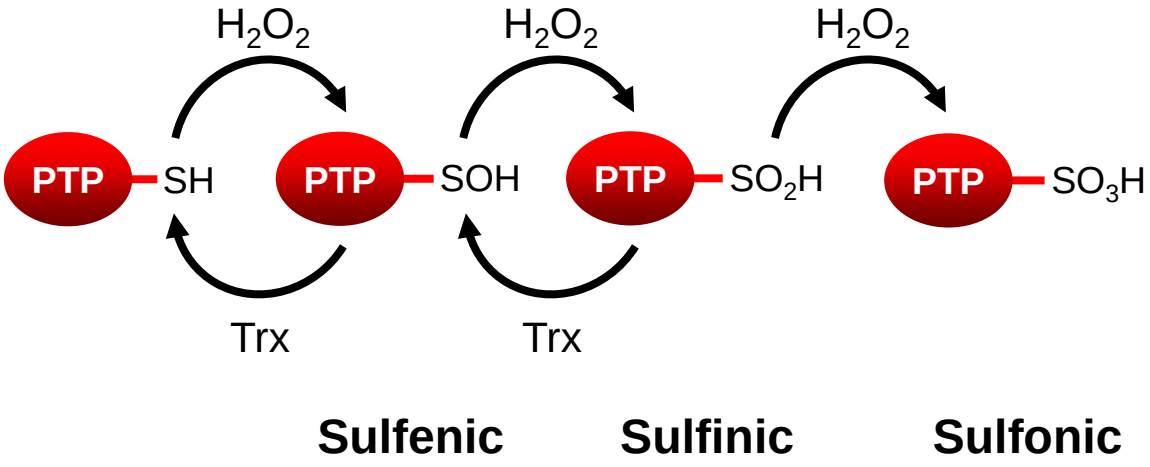


Figure 1.2. Oxidation and Reduction of Protein Tyrosine Phosphatases. Protein tyrosine phosphatases (PTPs) contain a reactive and, potentially, redox-sensitive cysteine (Cys) residue. These cystine residues can be oxidized by H_2O_2 to sulfenic acid (Cys-SOH). H_2O_2 can further oxidize these Cys residues to sulfinic (Cys-SO_2H) and sulfonic (Cys-SO_3H) forms. While the oxidation of sulfenic and sulfinic reactions are reversible, the sulfonic reaction renders the PTP irreversibly inactivated.

binding domains (Brasier et al., 2000; Sun and Oberley, 1996). Additional mechanisms by which ROS may mediate its effects on gene expression may be through alterations in mRNA stability (Chong et al., 2000) or the regulation of proteins responsible for the chemical modifications of DNA, such as histone deacetylases, and thus chromatin structure changes necessary for altered gene expression (Noh et al., 2009).

1.1.1.3 Proliferation and Growth

De-differentiated VSMCs are prominent in cardiovascular diseases such as hypertension, atherosclerosis, and restenosis after balloon angioplasty. These phenotypically altered VSMCs are programmed to grow and migrate, and ROS production is intimately involved in many of the cellular processes that promote the proliferation and hypertrophy of these cells (Paravicini and Touyz, 2006). The regulation of growth-related signaling by ROS occurs through modulation of multiple growth factors and hormones, but one of the best studied is platelet derived growth factor (PDGF).

PDGF, a potent proliferative signal for VSMCs, exerts its effects by binding to the PDGF receptor (PDGFR), which is a receptor tyrosine kinase. This receptor consists of two single transmembrane domain proteins that dimerize to form a functional receptor, allowing for the cross-phosphorylation of tyrosine sites in the cytoplasmic tail regions. Weber, et al. (Weber et al., 2004) previously demonstrated that phosphorylation of the PDGFR occurs in an ROS-independent manner. Prx II, a cellular peroxidase that eliminates endogenous H_2O_2 produced in response to growth factors such as PDGF, negatively

regulates PDGF signaling via its association with the PDGFR and thus functions to suppress PTP inactivation, suggesting a role for ROS in PDGF signaling (Choi et al., 2005). Interestingly, cellular deficiencies in Prx II result in increased production of H_2O_2 and enhanced activation of the PDGFR (Choi et al., 2005).

It has also been shown activation of the PDGFR by PDGF activates Nox1 in a p47phox-dependent manner in VSMCs (Lassegue et al., 2001; Lavigne et al., 2001; Lee et al., 2009; Marumo et al., 1997). This activation increases ROS production, which is then capable of modulating additional signaling proteins downstream of the PDGFR activation. For example, PDGFR activation stimulates the association of Shc/Grb2/Sos/Ras with the receptor (Rao, 1996), where Ras is activated by oxidative modification (Sundaresan et al., 1995), leading to activation of Raf kinase, an upstream kinase for ERK1/2 (Berk et al., 1990; Berk et al., 1991). ERK1/2 phosphorylation is blocked by catalase, implicating H_2O_2 in this response (Sundaresan et al., 1995). ERK1/2 has multiple targets, but is quickly translocated to the nucleus, where it activates transcription factors such as AP-1 and Elk-1, leading to upregulation of additional genes that increase VSMC proliferation and growth. Of importance, the activity of the transcription factor AP-1 is dependent on redox factor-1 (Ref-1), a multifunctional DNA base excision repair and redox regulation enzyme (Hirota et al., 1997). PDGF activates Ref-1 by changing its redox status, thereby promoting the reduction of specific Cys residues of AP-1 and thus enhancing the activity of AP-1 and transcriptional upregulation of pro-growth genes (Abate et al., 1990). Additional PDGF-induced proliferative pathways in VSMCs are known to be

modulated at several points by ROS and this is reviewed in detail in multiple articles (Lee and Griendling, 2008; Lyle and Griendling, 2006; Paravicini and Touyz, 2006).

1.1.1.4 Differentiation

Differentiated VSMCs are primarily localized in the medial layer of the vessel wall and their differentiated phenotype allows them to contract to regulate the vessel diameter. These differentiated VSMCs highly express a number of pro-contractile proteins that include smooth muscle myosin heavy chain (SM-MHC), smooth muscle alpha actin (SM α-actin), and heavy-caldesmon (H-caldesmon) and differ in both signaling and function from their de-differentiated, pro-growth VSMC counterparts found in regions of cardiovascular injury and disease. The mechanisms required for the maintenance of the VSMC differentiated phenotype remain poorly understood; however, previous studies linked basal ROS, attributable to Nox4 in VSMCs, to differentiation marker gene expression (Szocs et al., 2002; Sorescu et al., 2002), and a study in fibroblasts linked Nox4 to the process of fibroblast differentiation into myofibroblasts (Cucoranu et al., 2005). Interestingly, VSMC differentiation, specifically the expression of differentiation marker gene expression, was recently shown to be mediated by ROS derived from Nox4 (Clempus et al., 2007). The mechanisms by which these ROS regulate differentiation marker gene expression is an area of ongoing investigation; however, the data from Clempus et al. (Clempus et al., 2007) suggest that ROS may function to control the expression of this subset of genes through the regulation of a common transcription factor linked to

differentiation marker gene expression, such as the serum response factor (SRF), whose expression is significantly reduced in siNox4-treated VSMCs. The potential involvement of additional transcription factors in the maintenance of the differentiated phenotype by ROS and Nox4 remains poorly understood and requires further investigation.

1.1.1.5 Cytoskeletal Remodeling and Migration

VSMC migration is critical for a number of physiological processes, including embryonic development, inflammatory responses, and the response to injury (Horwitz and Parsons, 1999). ROS are implicated in VSMC migration in response to phenylephrine and vascular endothelial growth factor (VEGF), which is inhibited by treatment with catalase or the antioxidants N-acetyl cysteine (NAC) and pyrrolidine dithiocarbamate (Nishio and Watanabe, 1997; Wang et al., 2001b). Thrombin-stimulated migration is inhibited by the Nox inhibitor apocynin and the flavin-containing oxidase inhibitor diphenylene iodonium (DPI), specifically implicating Nox-derived ROS in this migratory response (Wang et al., 2004). Wang et al. (Wang et al., 2001b) showed that VEGF treatment of human VSMCs increased intracellular ROS, NF-κB activation, IL-6 expression, and migration, all of which were blocked by antioxidants. Monocyte chemotactic protein-1 (MCP-1) acts as a chemoattractant for VSMCs and MCP-1-stimulated migration requires both ROS production and ERK 1/2 activation in a positive activation loop, which may contribute to the atherogenic effects of MCP-1 (Lo et al., 2005).

A number of signal transduction pathways regulate actin polymerization and contractility in cells, two processes integral to cell migration. However, the regulation of the actin cytoskeleton and the involvement of ROS in this regulation with respect to VSMC migration is not well understood. To begin to understand the role of ROS in VSMC motility, migration pathways delineated in fibroblasts will be used as a model (Figure 1.3).

The process of cell migration can be essentially divided into distinct processes (Horwitz and Parsons, 1999). Migration begins with an initial protrusion or extension of the plasma membrane at the front/leading edge of the cell, known as a lamellipodium. The formation of these protrusions requires the polymerization of a network of cytoskeletal actin filaments and is stabilized through the formation of adhesive complexes within the protrusion. Next, as the cell migrates, the focal complexes at the front of the cell strengthen into larger, more organized focal adhesions that serve as points of traction over which the cell body moves. For the cell to make forward progression, it must release its rear adhesions to allow a net forward displacement.

1.1.1.5.1 Extension of the Plasma Membrane at the Cell's Leading Edge

To establish a leading edge, the cell must first sense a chemoattractant gradient and establish polarity, which in turn allows the plasma membrane to extend a lamellipodium in the direction of eventual movement (Lauffenburger and Horwitz, 1996; Nobes and Hall, 1995a). In vascular injury, a gradient is commonly established by platelet release of PDGF, such that VSMCs migrate

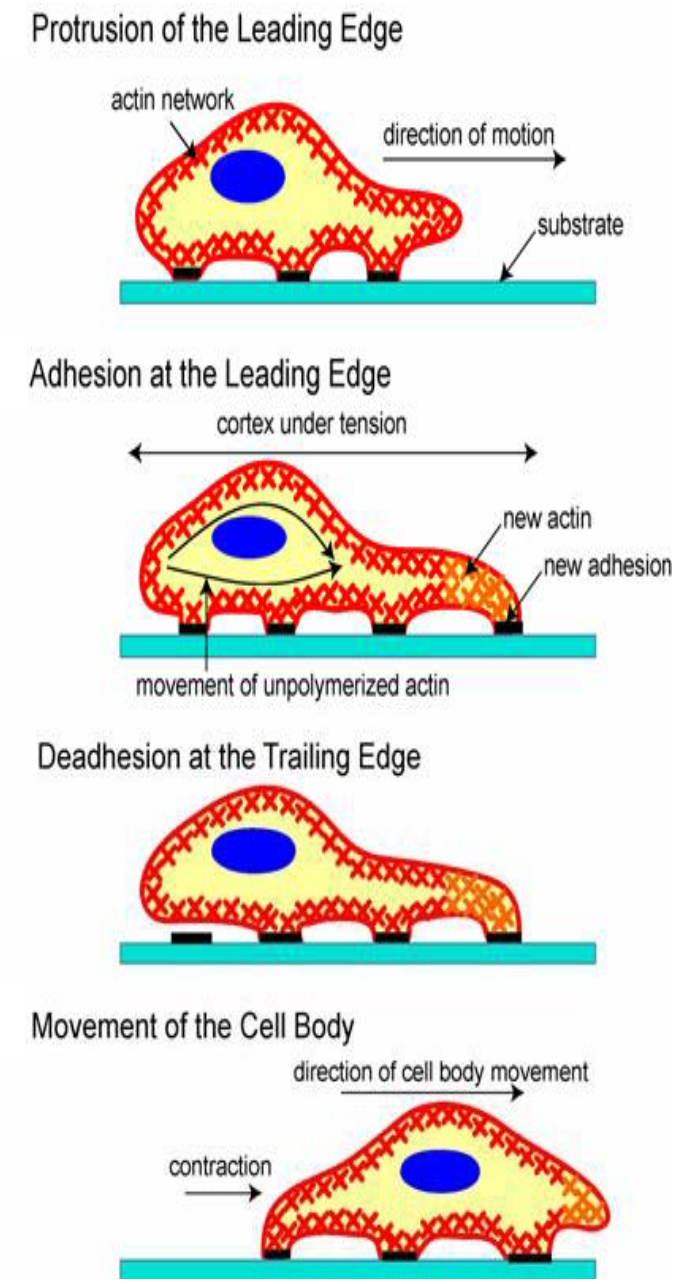


Image adapted with permission from:
Ananthakrishnan R, Ehrlicher A. The Forces Behind Cell Movement. *Int J Biol Sci* 2007; 3:303-317.

Figure 1.3. Regulation of Cell Migration. Migration begins with an initial protrusion or extension of the plasma membrane at the front/leading edge of the cell. The formation of these protrusions requires the polymerization of a network of cytoskeletal actin filaments. The protrusion is then stabilized through the formation of new adhesive complexes within the protrusion. Next, as the cell migrates, the focal complexes at the front of the cell strengthen into larger, more organized focal adhesions that serve as points of traction over which the cell body moves. For the cell to make forward progression, it must release its rear adhesions to allow a net forward displacement.

toward the lumen of the vessel (Chen et al., 2006; Lauffenburger and Horwitz, 1996). Generation of filopodia and/or lamellipodia is driven by actin assembly, which is a process regulated by Rac (Nobes and Hall, 1995a). Rac stimulates actin polymerization via several mechanisms, including nucleation of new actin filaments (Machesky and Insall, 1998; Miki et al., 1998), extension of existing filaments (Hartwig et al., 1995), and activation of LIM kinase (LIMK), which phosphorylates and inactivates the actin-capping protein cofilin, thus preventing actin depolymerization (Arber et al., 1998; Yang et al., 1998). The ROS-sensitivity of actin dynamics, while not fully understood, has become an area of recent interest and investigation. In endothelial cells (ECs), inhibition of Noxes or the use of SOD mimetics reduces actin monomer incorporation at the fast-growing barbed ends of filaments (Ikeda et al., 2005). Additionally, glutathiolation of G-actin increases the rate of actin polymerization (Wang et al., 2001a).

It has also been demonstrated that the small molecular weight G-protein Rac is required for the activation of Noxes (Cheng et al., 2006), specifically Nox1. Therefore, the involvement of Rac and Nox activity in mediating the formation of lamellipodia in VSMCs has recently become an area of active investigation. Recent studies demonstrate that in Nox1 knockout mice, wire injury-induced neointimal formation, a process that requires VSMC migration, is significantly blunted compared to wild-type mice. Interestingly, Nox1 knockout cells show increased cofilin phosphorylation, which causes decreased cofilin activity (Lee et al., 2009). This increase in cofilin phosphorylation (decreased activity) occurs

through a decrease in Slingshot (SSH) IL phosphatase activity (San Martin et al., 2008), leading to significantly decreased VSMC migration in response to PDGF (Lee et al., 2009; San Martin et al., 2008). These studies directly link the NADPH oxidase Nox1 to lamellipodia formation in VSMC migration. The involvement of additional Noxes in VSMC migration remains to be investigated.

1.1.1.5.2 Formation of Focal Complexes and Adhesions for Cell Movement

As described above, stabilization of the lamellipodia protrusions at the leading edge of the cell occurs through the formation of adhesive complexes (Carpenter, 2000). These complexes are regions of the plasma membrane where integrin receptors, actin filaments, and other proteins cluster together, activate, and then bind to extracellular matrix components (Nobes and Hall, 1995a; Nobes and Hall, 1995b). These focal complexes, which contain proteins such as focal adhesion kinase (FAK), vinculin, paxillin, p130Cas, and a number of PTPs (Zaidel-Bar et al., 2003), mature, grow and strengthen into larger, more organized focal adhesions that serve as points of traction over which the cell body moves during the process of migration (Nobes and Hall, 1995a; Nobes and Hall, 1995b). The precise mechanisms regulating the conversion of focal complexes to focal adhesions are unclear, but appear to require the activation of RhoA (Nobes and Hall, 1995a). Stimulation of actin-myosin contractility by Rho/ROCK leads to the bundling of actin fibers to generate stress fibers and the clustering of integrins to mature focal complexes into focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996). In aorta, ROS activate the Rho pathway (Jin et al., 2004), but the relationship of this activation to focal adhesions

formation is poorly defined. While recent work shows that Rho-GTPases contain a conserved redox-sensitive motif that appears to be critical for guanine nucleotide dissociation (Heo and Campbell, 2005) and thus raises the possibility of direct regulation of Rho-GTPases by ROS, the NADPH oxidase responsible for the regulation of Rho-GTPases remains to be investigated.

Additional proteins that may serve as distinct targets of ROS and as potential ROS-sensitive regulators of cytoskeletal dynamics are PTPs. A number of PTPs localize to focal adhesions and maintain their stability. Of these, the low molecular weight (LMW)-PTP and PTP-PEST are regulated by ROS (Angers-Loustau et al., 1999; Chiarugi et al., 2000a; Chiarugi et al., 2000b). Of interest, PTP-PEST is strongly associated with focal adhesion turnover and migration in other cell types, modulates a number of signaling proteins upstream of RhoA, such as FAK (Angers-Loustau et al., 1999) and, therefore, may serve as a potential target for ROS mediated regulation of VSMC migration.

1.1.1.5.3 Generation of Force and Release of Rear Adhesions for Forward Cell Progression

The next step in migration is the generation of force to initiate forward progression of the cell. The GTPases cdc42 and Rho are proposed to regulate contractile forces by influencing myosin light chain (MLC) phosphorylation. MLC phosphorylation promotes dimerization and interaction with actin to drive contraction (Horwitz and Parsons, 1999). Rho kinase, activated by Rho and ROS (Jin et al., 2004), functions to inhibit the myosin phosphatase, allowing

MLCs to remain in a contractile (phosphorylated) state (Horwitz and Parsons, 1999).

The release of rear adhesions and the continued forward progression of the migrating cell depends on focal adhesion turnover, which is critical for the continued reorganization of adhesion contacts during cell migration (Lauffenburger and Horwitz, 1996; Regen and Horwitz, 1992). Cells lacking Src family kinases, FAK, and calpain exhibit migratory defects that appear to reflect an inhibition of focal adhesion turnover; however, focal adhesion formation is not impaired (Huttenlocher et al., 1997; Klinghoffer et al., 1999; Sieg et al., 1999). Little is known about the process of focal adhesion turnover in VSMCs and the involvement of Nox-derived ROS.

1.2 The NADPH Oxidase Enzyme Family

One major source of ROS in vascular cells is the NADPH oxidase family of enzymes. The catalytic subunits of Noxes all share common and highly conserved structural characteristics that include: 1) an NADPH binding site, 2) a flavin adenine dinucleotide (FAD) binding region, 3) six transmembrane domains, and 4) four highly conserved heme-binding histidine residues. Most of these enzymes have multiple regulatory subunits.

1.2.1 Nox2, the Classical Neutrophil NADPH Oxidase

The classical Nox2-based oxidase, also known as gp91phox, consists of five subunits. Together, the membrane proteins Nox2 and p22phox comprise the cytochrome b558 membrane complex, which is localized in sub-membranous vesicles and at the plasma membrane (Borregaard et al., 1983). Nox2 is the

catalytic subunit of the phagocyte cytochrome and binds one FAD and two heme molecules (Bedard and Krause, 2007). p47phox, p67phox, and the small molecular weight G-protein Rac are cytosolically localized and do not interact with the cytochrome in resting phagocytic cells. Upon agonist stimulation, p47phox is phosphorylated on 8-9 serines by either proline-directed kinases or protein kinase C (PKC) (El-Benna et al., 2009). S359 and S370 are phosphorylated first, followed by S379, which exposes an SH3 binding site and allows p47phox to interact with the proline-rich region of p22phox, facilitating translocation to the membrane (Johnson et al., 1998). Finally, S303 and S304 are phosphorylated, leading to full catalytic activity (Inanami et al., 1998). p67phox then binds to the translocated p47phox, providing a binding site for activated Rac and forming the functional enzyme capable of producing $O_2^{\bullet}$- (Diebold and Bokoch, 2001; Han et al., 1998).

1.2.2 The Nox Family Members

The catalytic moieties of Nox enzymes are homologues of the flavin- and NADPH-binding protein gp91phox (Nox2) termed Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2. Most cell types express multiple Nox enzymes that are differentially regulated and have distinct sub-cellular localizations, suggesting that these oxidases serve unique roles. For example, VSMCs from large vessels predominantly express the Nox1 and Nox4 homologues. In this cell type, as in other cell types that express multiple Nox enzymes, each of these Noxes differs in their mode of regulation, what agonists stimulate their expression and activation, and where they are localized within the cell. Because the best

understood oxidase is the classical Nox2-based oxidase, all other Noxes will be related back to this primary Nox.

1.2.2.1 Nox1

Nox1 was the first homologue of Nox2 to be described and shares ~60% sequence similarity to Nox2 (Suh et al., 1999). In the vasculature, Nox1 is expressed in both VSMCs and ECs (Lassegue and Clempus, 2003). Nox1 mRNA and protein expression is induced in VSMCs primarily by growth factors, such as PDGF and angiotensin II (Ang II) (Lassegue et al., 2001). Nox1 expression is also upregulated in the neointima of injured carotid arteries (Lee et al., 2009).

In VSMCs, the primary subcellular distribution of Nox1 corresponds to that of a caveolar localization (Hilenski et al., 2004). Like Nox2, activation and ROS generation by Nox1 is dependent on cytosolic regulatory subunits, specifically p47phox, NoxA1 (Nox activator 1), a p67phox homologue, and Rac in VSMCs (Ambasta et al., 2006; Kim et al., 2007; Miyano and Sumimoto, 2007; Ueyama et al., 2006). Unlike Nox2, whose primary function in phagocytic cells is host defense and microbicidal activity, Nox1 seems to primarily function in VSMCs to regulate growth and migration in response to agonist stimulation.

1.2.2.2 Nox3

Nox3 is the most recently described homologue of Nox2 and shares ~56% sequence similarity to that of Nox2. Nox3 has not been detected within any cell type contained in the vascular wall; however, Nox3 is expressed in the inner ear and other tissues, such as fetal kidney, the brain and the lung (Banfi et al.,

2004a; Cheng et al., 2001). Like Nox2 and Nox1, Nox3 is a p22phox-dependent enzyme (Cheng et al., 2004); however, the requirement of additional cytosolic subunits for the activity of Nox3 is unclear. Recent studies indicate a weak constitutive activity when Nox3 is co-expressed with p22phox, while full activation of Nox3 requires Rac and additional cytosolic subunits in various combinations (Miyano et al., 2009; Miyano and Sumimoto, 2007; Ueyama et al., 2006). Interestingly, mice deficient in either Nox3 or the regulatory subunit known as Nox organizer 1 (NoxO1), a p47phox homolog, exhibit a head tilt phenotype, strongly suggesting that NoxO1 is one of the primary functional components necessary for Nox3 in the inner ear (Kiss et al., 2006). Because multiple combinations of cytosolic components are capable of activating Nox3 to various degrees (Cheng et al., 2004), the precise molecular composition of the Nox3 complex may be tissue- or cell-type specific.

1.2.2.3 Nox4

Originally identified as a Nox homolog highly expressed in the kidney, Nox4 shares ~39% sequence similarity to Nox2. In the vasculature, Nox4 is expressed in ECs, VSMCs, and fibroblasts (Lassegue and Clempus, 2003). Nox4, in VSMCs, is necessary for regulation of differentiation (Clempus et al., 2007), as well as fundamental cellular processes necessary for VSMC function.

Nox4 mRNA expression increases in response to a number of stimuli including endoplasmic reticulum (ER) stress, shear stress, carotid artery injury, TGFβ, TNFα, and 7-ketocholesterol (Bedard and Krause, 2007; Lassegue and Clempus, 2003). Interestingly, a number of stimuli that are known to induce

Nox1 expression at the mRNA level, such as IL-1, thrombin, Ang II, BMP4, and PDGF, promote the downregulation of Nox4 at the mRNA level, suggesting that Nox1 and Nox4 are antagonistically regulated. There are very few studies published focusing on the promoter structure of Nox4. One such study was recently published examining the transcriptional regulation of Nox4 expression. In this study, the authors mapped the Nox4 transcriptional initiation site and identified the transcription factor E2F1 as a positive regulator of Nox4 mRNA expression in rodent VSMCs (Zhang et al., 2008); however, more studies are needed to determine if there are additional transcription factors that promote or suppress transcriptional regulation of Nox4. In addition to regulation at the mRNA level, evidence suggests that Nox4 is also regulated at the post-translational level. Peshavariya, et. al. (Peshavariya et al., 2009) showed that increases and decreases in Nox4 mRNA did not directly correlate with increased or decreased Nox4 protein levels and propose that Nox4 mRNA levels are influenced by a translation-initiated mRNA destabilization program.

Another potential factor that may regulate Nox4 activity is its localization in the cell. Nox4 localization seems to differ depending on the cell type examined. In VSMCs, endogenous Nox4 localizes to focal adhesions, along stress fibers, and the nucleus (Clempus et al., 2007; Hilenski et al., 2004; Lyle et al., 2009). In transfected and transduced endothelial cell lines, Nox4 localization is primarily observed in the ER, where Nox4 was shown to regulate PTP1B activity and, in turn, EGFR trafficking (Chen et al., 2008). Additional functional roles for Nox4 in the ER are possible and remain to be investigated; however, such a localization

pattern in an overexpression system may more likely be due to inadequate protein folding and processing or may potentially represent an accumulation of Nox4 at its site of synthesis. It is possible that by increasing the trafficking of Nox4 to its proper compartment in a respective cell type, there would be a concurrent increase in Nox4 activation and Nox4-dependent ROS production.

Nox4 enzymatic activity is p22phox-dependent, as shown by functional studies, and Nox4 both co-immunoprecipitates and co-localizes with p22phox (Lyle et al., 2009; von Lohneysen et al., 2008). Recent investigations into the structural elements of p22phox essential for Nox4-dependent ROS generation and localization confirmed the requirement of p22phox for Nox4 maturation, localization, and activity (von Lohneysen et al., 2008). Additionally, this study suggested that the N-terminus of p22phox was required for Nox4/p22phox localization and that some regions of the c-terminus of p22phox may not be required for Nox4-dependent ROS production. However, whether Nox4 requires cytosolic subunits for its enzymatic activity remains controversial. When Nox4 is heterologously expressed in a reconstituted system, the data suggest that Nox4 may be a constitutively active enzyme and that none of the presently known cytosolic regulatory subunits are required for Nox4 activation in these reconstituted systems (Martyn et al., 2006); however, it is currently impossible to know if other potential regulatory proteins for Nox4 are endogenously expressed in these cell lines. Additional studies suggest that the principal mechanism of Nox4 regulation may be induction at the mRNA level, rather than assembly of an enzyme complex or post-translational protein modifications (Serrander et al.,

2007). While it is known that Nox4 requires p22phox, no systematic search for proteins that bind to the Nox4/p22phox complex has been performed.

Recent data from reconstituted systems support the idea that H_2O_2, and not $O_2^{\bullet}$-, is the principal product generated by the Nox4 enzyme; however, upon closer examination of the methods used, $O_2^{\bullet}$- production was measured in intact cells, which would limit any detectable $O_2^{\bullet}$- to that generated extracellularly (Martyn et al., 2006). Another possible explanation for the detection of H_2O_2 could be the result of the subcellular localization of Nox4, where this localization would result in the release of $O_2^{\bullet}$-; however, the $O_2^{\bullet}$- produced by Nox4 may be so rapidly converted to H_2O_2 that the release of $O_2^{\bullet}$- from this enzyme is almost undetectable (Dikalov et al., 2008; Serrander et al., 2007). While the primary form of ROS generated by Nox4 remains controversial, it is believed that H_2O_2 is responsible for the downstream signaling events mediated by Nox4.

1.2.2.4 Nox5

Nox5, another recently identified Nox2 homologue, is the most distant member of the Nox1-4 family. Specifically, Nox5 differs from other Noxes by the presence of a longer intracellular NH_2 terminus containing a Ca^{2+}-binding EF-hand region (Banfi et al., 2001). Nox5 is not expressed in rodents, but is expressed in human VSMCs (Banfi et al., 2001)**;** however, very little is known about the subcellular distribution of Nox5. There is also very little known about the Nox5 promoter and how expression is regulated for the different Nox5 isoforms. Interestingly, Nox5 does not require p22phox for its enzymatic activity (Kawahara et al., 2005), nor does it require the classically known cytosolic

regulatory subunits for its activation, since activation appears to be dependent on cellular increases in cytoplasmic Ca^{2+} concentrations (Banfi et al., 2004b). Additional studies have also indicated that PMA stimulation increases Nox5 phosphorylation on residues T494 and S498, which increases the sensitivity of Nox5 to Ca^{2+}, and thereby further increases Nox5 enzymatic activity (Jagnandan et al., 2007). Interestingly, Nox5 activity was shown to be influenced by subcellular localization where, upon the binding of PIP_2 to N-terminal polybasic region of Nox5, Nox5 localization changes from internal membranes to the plasma membrane (Kawahara and Lambeth, 2008). In addition, PDGF was identified as an agonist that stimulates Nox5 enzymatic activity in human VSMCs and links Nox5 regulation to PDGF-induced human VSMC proliferation (Jay et al., 2008), making this a potential target for vascular pathologies accompanied by an increase in VSMC proliferation.

1.2.3 Classical Nox Regulatory Proteins

1.2.3.1 p22phox

p22phox has two principal functions: 1) it binds to Nox proteins to promote Nox protein stabilization, and 2) it serves as the binding site of other cytosolic subunits that function to regulate NADPH oxidase activity. p22phox associates with Nox1, Nox2, Nox3, and Nox4 (Brown and Griendling, 2009). When p22phox is present and associates with a Nox subunit in a heterodimer complex, it stabilizes that particular Nox protein (DeLeo et al., 2000). p22phox down regulation leads to decreased function of all of the Noxes that require p22phox for activity (Kawahara et al., 2005; Martyn et al., 2006). As mentioned, the

cytosolic C-terminal tail of p22phox also, classically, serves as a docking site for the other cytosolic regulatory proteins that function to activate Nox activity. Association of p22phox with organizer subunits is required for proper activation of Nox1, Nox2, and Nox3; however, the requirement of the cytosolic C-terminus region for proper Nox4 activation was unclear until recently. While some studies conclude that a portion of the C-terminal region of p22phox, specifically amino acids 130-195 of human p22phox, is not required for Nox4 activity (Kawahara et al., 2005; von Lohneysen et al., 2008), the experimental evidence shown herein suggests that a portion of the C-terminal region used for the studies described in this body of work, specifically amino acids 106-129, may be required for subunit association and proper Nox4 activation. This 24 amino acid domain (aa 106-129) is highly conserved across species and may serve as an additional site on p22phox required for subunit association in addition to the proline rich region shown to be necessary for activation of some Nox enzymes by the classical cytosolic subunits.

1.2.3.2 p47phox and NoxO1

p47phox and its recently identified homologue, NoxO1 (Banfi et al., 2003; Takeya et al., 2003), are known as the organizing cytosolic subunits. While these two proteins only share ~25% sequence homology, they share a significant degree of functional similarities. Both of these proteins contain phox homology (PX) domains and contain two SH3 domains that allow them to dock with the proline-rich region of the cytosolic C-terminal tail of p22phox (Leto et al., 1994). However these two organizer proteins differ in that p47phox requires

phosphorylation to release its autoinhibitory domain, whereas NoxO1 does not require phosphorylation due to a lack of this autoinhibitory domain (Takeya et al., 2003). These subunits are known as organizing subunits due to their ability to, upon activation, bind to and promote the translocation of the activator subunits to the membrane to allow for Nox activation.

1.2.3.3 p67phox and NoxA1

p67phox and its recently identified homologue, NoxA1 (Banfi et al., 2003; Takeya et al., 2003), are known as the activating cytosolic subunits. p67phox and NoxA1 only share ~28% sequence homology; however, these two proteins are functionally quite similar. Both of these cytosolic activating subunits contain a carboxy-terminal SH3 domain, which allows them to associate with the organizer subunits for translocation to the membrane, as well as a critical activation domain, which allows for the activation of the Nox enzymes (Ambasta et al., 2006; Banfi et al., 2003; Takeya et al., 2003; Valente et al., 2007). Previous studies have shown that when cAMP is increased and protein kinase A (PKA) is activated downstream, there is a subsequent inhibition of some Noxes, such as Nox2. Recent evidence indicates that this PKA-induced inhibition of ROS is through the increased phosphorylation on Ser 172 and Ser 461 of NoxA1 by PKA, which results in an increased association between NoxA1and 14-3-3, which in turn leads to inhibition of Nox1 activity (Kim et al., 2007). Interestingly, studies demonstrate that the organizer subunits, p47phox and NoxO1, are capable of interacting interchangeably with the two activator subunits, p67phox and NoxA1, to promote Nox activation (Takeya et al., 2003), suggesting that

each tissue or cell type may have functional Nox complexes of unique molecular composition.

1.2.3.4 Small Molecular Weight G-Protein, Rac

While the principal function of the Rac family of GTPases is regulation of the cytoskeleton, these proteins also serve additional cellular functions, including the association and activation of NADPH oxidases. Rac-GTPases are involved in the activation of both Nox1 and Nox2, while activation of Nox3 is Rac-independent (Hordijk, 2006). In a reconstituted system, the activation of Nox4 was unaffected by the knockdown of Rac, supporting the concept that Nox4 activity is Rac-independent (Martyn et al., 2006). A large number of the studies designed to determine whether specific Noxes are or are not Rac-dependent were performed in reconstituted systems; however, whether this Rac-dependence or -independence translates directly to cells that endogenously express Nox enzymes remains to be studied.

1.2.4 Vascular Smooth Muscle NADPH Oxidases

Most cell types that comprise the vessel wall express multiple Nox homologues, including gp91phox (aka Nox2), as well as Nox1, Nox4, and Nox5 as well as their regulatory cytosolic subunits described above (Figure 1.4). As noted above, classical subunits and novel homologues are capable of interacting with one another to regulate Nox activity and recent studies suggest that each cell type may have Noxes of unique molecular compositions. The primary oxidases expressed in VSMCs, Nox1 and Nox4, require p22phox for oxidase function (Hanna et al., 2004; Lassegue and Clempus, 2003). While the

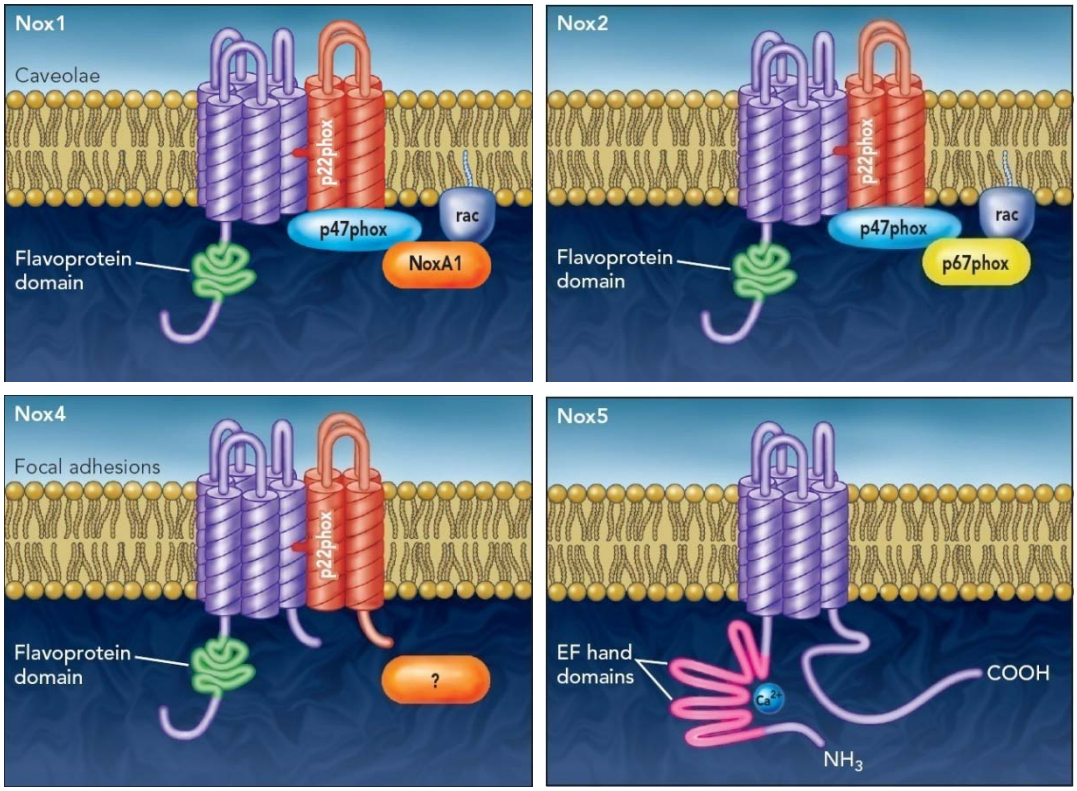


Image used with permission from: Lyle AN and Griendling KK (2006) Modulation of vascular smooth muscle signaling by reactive oxygen species. *Physiology (Bethesda, MD)* 21:269-280.

Figure 1.4. Structure and Assembly of Vascular NADPH Oxidases. The gp91phox homologues expressed in vascular smooth muscle cells (VSMCs) include Nox1, Nox2, Nox4, and Nox5. Each Nox subunit consists of six transmembrane domains. Nox1, Nox2 and Nox4 require the subunit p22phox for stabilization and to form functional ROS-generating enzymes. In contrast, Nox5 does not require p22phox or additional cytosolic subunits. The subcellular distribution of Nox1 and Nox4 differ in VSMC, where Nox1 is localized to caveolae and Nox4 is localized in focal adhesions. The subcellular distribution of Nox2 and Nox5 in VSMC is unknown. Nox1 associates with two cytosolic factors, p47phox and NoxA1, and the small molecular weight G-protein Rac to form a functional enzyme complex. Nox2 is regulated by p47phox and p67phox, while Nox4 presently has no known regulatory subunits. Nox5 is contains 4 EF-hand domains, at the N-terminus that serve as Ca^{2+} binding domains.

regulatory proteins required for Nox1 activation in VSMCs were identified as p47phox, NoxA1, and Rac (Ambasta et al., 2006; Brandes et al., 2002; Lavigne et al., 2001), whether Nox4 has unique regulatory partners remains to be determined.

The subcellular localizations of Nox1 and Nox4 are distinct in VSMCs, which may suggest specific cellular functions. It was recently reported that Nox1 co-localizes with caveolin in punctate patches on the surface and along the cellular margins, whereas Nox4 is found in the nucleus and co-localized with vinculin in the focal adhesions (Hilenski et al., 2004). p22phox co-localizes in similar patterns with both Nox1 and Nox4 (Ambasta et al., 2004; Hilenski et al., 2004), supporting the notion that p22phox is required for Nox1 and Nox4 function. In addition to their distinct subcellular localizations, Nox1 and Nox4 also seem to serve distinct roles in VSMCs and are differentially coupled to agonists. Recent studies link Nox1 to Ang-II mediated hypertension, hypertrophy, and proliferation in VSMCs (Lassegue and Clempus, 2003). Ellmark et al. showed that in VSMCs, Nox4 is responsible for basal $O_2^{\bullet}$-production (Ellmark et al., 2005) and functional studies implicate Nox4 in the regulation of basic cellular processes, such as oxygen sensing and senescence (Geiszt et al., 2000; Shiose et al., 2001), apoptosis (Pedruzzi et al., 2004), and survival (Vaquero et al., 2004) and differentiation (Clempus et al., 2007; Cucoranu et al., 2005; Li et al., 2006). Additionally, Nox4 has recently been implicated in the regulation of fundamental cellular processes such as cell cycle

progression (McCrann et al., 2009a; McCrann et al., 2009b; Yamaura et al., 2009) and cytoskeletal modulation (Meng et al., 2008).

1.3 NADPH Oxidases and Cytoskeletal Dynamics

1.3.1 Trafficking of NADPH Oxidases

For many proteins to function properly on their downstream targets, they must localize to their proper subcellular compartments. After undergoing proper protein folding in the ER, a protein can traffic either 1) from the ER directly to their subcellular compartment or 2) if further post-translational modifications are required, such as glycosylation, can be transported from the ER to the Golgi where these modifications are made prior to protein exit via the trans-face of the Golgi to traffic to its subcellular compartment (Marie et al., 2008). Very little is understood or known about the trafficking of Nox proteins. It is assumed, however, that one could potentially modulate Nox activity by altering a protein that affects the biosynthesis or targeting of the Nox/p22phox complex. For example, recent studies demonstrate that other Nox homologues, namely Duox1 and Duox2, require the co-expression of a maturation factor for proper processing (Grasberger et al., 2007; Grasberger and Refetoff, 2006). Duoxes are Nox homologues highly expressed in the thyroid that function in the iodination of thyroid hormones, which is a process catalyzed by the thyroid peroxidase and utilizes Duox-derived H_2O_2 (Bedard and Krause, 2007). For other Nox homologues, such as Nox2 and Nox3, heme incorporation and interaction with p22phox during transit through the ER are required for complex stabilization and full enzyme activity (DeLeo et al., 2000; Nakano et al., 2007);

however, it is not well understood if Noxes require additional proteins for proper maturation and subcellular targeting. In the case of Duoxes, studies in which these proteins were overexpressed showed that the Duox protein was retained in the ER. Interestingly, upon co-expression of DuoxA2, an ER resident protein that mediates ER-to-Golgi transition of Duoxes, the ER retained Duox protein was able to undergo maturation and proper trafficking to the plasma membrane (Grasberger et al., 2007; Grasberger and Refetoff, 2006; Zamproni et al., 2008). Interestingly, when Nox4 is overexpressed it accumulates in the ER, which is reported to occur in ECs, HEK 293 cells, and VSMCs (Martyn et al., 2006; Van Buul et al., 2005; von Lohneysen et al., 2008), suggesting that Nox4 may require a maturation factor or additional co-factor for proper processing and/or trafficking to other subcellular compartments from the ER; however, this requires further investigation.

Protein transport to specific subcellular compartments not only requires proper protein processing and maturation, but also requires proper trafficking and a “highway” on which to travel. Both microtubules and stress fibers function not only as the skeleton of the cell, but also as the two major highway systems by which proteins travel to their respective compartments within the cell. Improper protein processing or delayed trafficking of proteins from the ER results in an increase in ER stress and initiation of the unfolded protein response, commonly indicted by an upregulation in the ER chaperone known as glucose regulated protein (GRP) 78 (Ron and Walter, 2007). The improper folding or trafficking of Noxes could potentially increase ER stress and initiate the unfolded protein

response (Santos et al., 2009). Therefore, understanding how individual Noxes traffic to their subcellular compartments and what modifications specifically promote differential subcellular localization, as in the case of Nox1 localization to caveolae and Nox4 localization to focal adhesions in VSMCs (Hilenski et al., 2004), is of interest and remains to be investigated.

1.3.2 Nox Proteins and Focal Adhesion Dynamics

The differential regulation and localization of Nox1 and Nox4 in VSMCs has tremendous potential implications for the function of these enzymes. Nox1 is expressed in caveolae (or lipid rafts), which function as reservoirs for many growth-related signaling molecules, and may function to initiate signaling in response to growth factors. In contrast, Nox4 is localized in focal adhesions, a subcellular compartment intimately linked to the actin cytoskeleton, making it a potential regulator of PTKs and PTPs integral to cytoskeletal remodeling and focal adhesion dynamics.

Of interest, cytoskeletal remodeling is highly regulated by ROS. Studies in migrating ECs showed that G-actin incorporation into the barbed ends of actin filaments is blocked by the flavin containing oxidase inhibitor DPI (Moldovan et al., 2000). LDL-induced reorganization of the actin cytoskeleton has also been shown to be mediated by ROS (Holland et al., 2001). Additional evidence demonstrates that focal adhesion proteins in lamellipodia undergo oxidative modification when TRAF4 and p47phox are overexpressed (Wu, 2005). As mentioned previously, Nox1 is involved in the formation of lamellipodia (Lee et al., 2009; San Martin et al., 2008), while Nox4 is linked to stress fiber formation

and is localized to focal adhesion structures (Clempus et al., 2007; Hilenski et al., 2004). Together, these observations support the concept that the cytoskeleton, and in particular focal adhesions, is a major target of Noxes.

Focal adhesions are sites of cell attachment to the extracellular matrix where transmembrane integrins link the matrix to the actin cytoskeleton. Focal adhesions also represent key centers of signal integration and the assembly and disassembly of these structures regulate various signal transduction pathways. Among the numerous proteins localized to focal adhesions are the multifunctional Src family kinases, FAK, and the serine/threonine kinase p21-activated kinase (PAK), as well as the scaffolding proteins paxillin and p130Cas, and the actin binding proteins vinculin and talin. Additional focal adhesion residents include a number of PTPs, of which LMW-PTP and PTP-PEST are ROS-sensitive and are linked to focal adhesion turnover. The dynamic turnover of focal adhesions is critical in the modulation of growth and survival, as well as responses involving cell motility, such as migration and mitosis. Gaining insight into the mechanisms regulating the assembly and disassembly of focal adhesions is thus central to understanding the control of many important cellular functions.

1.3.3 Cell Cycle Regulation by ROS in Vascular Smooth Muscle Cells

The cell cycle is one of the essential biological processes of life. The process of cell-cycle progression is highly ordered and ensures the replication of a cell's genome and subsequent division into two identical daughter cells. A growing body of evidence indicates that ROS produced by Noxes play critical roles in proliferation, differentiation, and cell cycle regulation in VSMCs. Cell

cycle arrest occurs in response to high levels of ROS (Ishida et al., 1997; Sarkar et al., 1997), while moderate levels of ROS function to coordinate a number of crucial events that promote growth (Sarsour et al., 2009). Previous studies in VSMCs indicate that H_2O_2 both promotes growth and induces apoptosis, and several cell cycle regulatory proteins that control translation initiation and gene expression are ROS sensitive (Deshpande et al., 2002). ROS generation was shown recently to occur during cell cycle progression at both the G_1/S and G_2/M checkpoints (McCrann et al., 2009b; Menon et al., 2003). Recently, Nox1-dependent ROS production was shown to specifically modulate cyclin D1 and thereby regulate G_1/S transition (Ranjan et al., 2006), whereas Nox4-dependent ROS production was shown to specifically alter the PTP cdc25, and thereby modulate cyclin B activation to regulate G_2/M transition and cell polyploidy (Yamaura et al., 2009). Because the cell cycle is such a critical and fundamental cellular function, gaining further insight into how ROS specifically function to regulate the cellular processes required for cell cycle control will prove critical to multiple pathologies in which cell proliferation is a key component.

1.4 Objectives of This Dissertation

Based on the aforementioned studies detailing the activation of Noxes (section 1.2.2) by cytosolic subunits (section 1.2.3), with a specific emphasis on the activation and regulation of VSMC NADPH oxidases (section 1.2.4), this dissertation describes a new Nox4 regulatory protein discovered during a systematic search for additional Nox regulatory proteins. The regulatory protein

that is characterized in this body of work was discovered when we conducted a yeast two-hybrid screen to search for proteins that associate with the proline-rich cytosolic C-terminal tail region of p22phox. The initial objective of this dissertation was to characterize one of the proteins pulled out of this yeast two-hybrid screen, Poldip2, and to determine what other oxidase subunits, if any, Poldip2 physically associated with in VSMCs. We then sought to determine if Poldip2 functions as a regulator of Nox activity in VSMCs. Functional studies conducted to determine the effects of Poldip2 manipulation in VSMCs revealed that Poldip2 alters the cytoskeleton in VSMCs, so we investigated the specific cytoskeletal targets of Poldip2. Finally, because of its effects on the cytoskeleton, we investigated if and how Poldip2 influences other cellular functions that are dependent on Nox4 and proper cytoskeletal regulation, such as cell cycle regulation and proper protein trafficking.

CHAPTER 2

Identification of Poldip2, a Novel Binding Partner for p22phox in Vascular Smooth Muscle Cells

2.1 Introduction

The mechanism by which Nox4 is regulated remains controversial. Some studies suggest that the principal mechanism of Nox4 regulation may be induction at the mRNA level, rather than assembly of an enzyme complex or post-translational protein modifications (Serrander et al., 2007). While it is known that Nox4 requires p22phox for its activity, there has been no systematic search for regulatory proteins that bind to the Nox4/p22phox complex. Based on the aforementioned studies detailing the activation of different Noxes (section 1.2.2) by cytosolic subunits (section 1.2.3), with a specific emphasis on the activation and regulation of VSMC Noxes (section 1.2.4), we sought to conduct a systematic search to determine if there are any additional Nox regulatory proteins not yet discovered. To do this, we conducted a yeast two-hybrid screen to specifically look for proteins that associate with the proline-rich cytosolic C-terminal tail region of p22phox, because the cytosolic C-terminal tail of p22phox classically serves as a docking site for the other cytosolic regulatory proteins that function to activate Nox activity.

2.2 Methods

2.2.1 Cell Culture

VSMCs from rat thoracic aorta were grown in Dulbecco's Modified Eagle's Media (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin, all of which were obtained from Invitrogen (Carlsbad, CA). Human aortic smooth muscle cells (HASMCs) were from Lonza (Basel, Switzerland) and were grown in

Smooth Muscle Growth Medium (Sm-GM; Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum (Lonza, Basel, Switzerland). Rat aortic VSMCs stably transfected with empty vector (Vector) or antisense (AS) p22phox (p22AS) vector were generated and cultured in 10% calf serum DMEM supplemented with 400 µg/mL G418 (Mediatech, Inc., Herndon, VA). Cells at passages 6 to 12 were used after 48 hours of quiescence in serum free DMEM for all experiments, unless otherwise noted. HEK293 cells (Clontech, Mountain View, CA) were cultured in DMEM with 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA).

2.2.2 Antibodies

p22phox antibodies were kindly provided by Dr. Mark Quinn (Montana State University) or were purchased from Santa Cruz, Nox4 antibodies were provided by Dr. David Lambeth (Emory University) or custom made by Sigma (St. Louis, MO). Poldip2 goat antibody was custom made by GenScript Corporation (Piscataway, NJ) against the peptide sequence NPAGHGSKEVKGKTC. When available, commercial antibodies were used: Nox1 and RhoA antibodies were purchased from Santa Cruz (Santa Cruz, CA), the vinculin antibody was from Sigma (St. Louis, MO), the paxillin antibody was from BD Biosciences (Bedford, MA), the V5-tag antibody was from MBL International (Woburn, MA), the HA-tag antibody for immnocytochemistry was obtained from Abcam (Cambridge, MA), the HA-tag antibody for immunoblotting and the Myc-tag antibodies were purchased from Cell Signaling (Danvers, MA).

2.2.3 Yeast Two-Hybrid Assay

We utilized the Matchmaker LexA yeast two-hybrid system (Clontech, Mountain View, CA) and a VSMC cDNA library constructed in pB42AD. The hydrophilic cytosolic tail of rat p22phox (nt 360-579 in accession number FJ515740, corresponding to the C-terminal 73 amino acids), used as bait, was inserted into pLexA vector such that it is expressed as a fusion protein containing an N-terminal DNA binding domain that binds to the LexA operator sequence. The plasmid was co-transformed with the pB42AD vector and a vector (p8op-lacZ) containing the lacZ reporter gene into yeast (strain EGY48). Co-transformants containing all three plasmids (pLexA-BD-p22phox, pB42AD-cDNA, and p8opLacZ) were amplified on minimal medium (-His/-Trp/-Ura). Colonies were scraped and replated on Leu induction medium to further screen for colonies transformed with all three plasmids. X-gal was included in this selection medium to confirm expression of hybrid proteins by assaying β-galactosidase activity conferred by the LacZ reporter and to help to eliminate false positives. False positives were further removed by mating independent positive clones with YM4271 yeast bearing the pLexA containing the binding domain (BD) alone.

2.2.4 Constructs

HA-tagged Nox1 (Nox1-HA) and V5-tagged p22phox (V5-p22phox) were prepared, as described previously (Hanna et al., 2004). To prepare an adenovirus (Ad) for overexpression of N-terminal Myc-tagged rat Poldip2, the coding region of Poldip2 was first amplified by PCR and subcloned into the pAdTrackCMV vector at Kpn I and Xba I sites.

2.2.5 Adenoviruses

The AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA) was used to prepare viruses with either no insert (AdGFP), hemagglutinin (HA)-tagged Nox1 (AdNox1HA), or Myc-tagged Poldip2 (AdPoldip2). VSMCs were transduced with recombinant adenoviruses for 2 h at 37°C in serum-free DMEM, followed by incubation for 48 h-3 days in serum-free DMEM without virus.

2.2.6 GST-Pulldown

VSMCs were transfected with AdGFP or with AdPoldip2 and labeled with ^{35}S-methionine (20 µCi; 3 h). Lysates were incubated with GST fusion proteins (GST-vector or GST-p22phox) prepared using the TNT T7 Quick coupled transcription/translation system (Promega, Madison, WI). Binding partners were detected by autoradiography.

2.2.7 Immunoblotting and Immunoprecipitation

VSMCs were lysed in standard lysis buffer (50 mmol/L HEPES, 50 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L Na-pyrophosphate, 50 mmol/L NaF, 1 mmol/L Na-orthovanadate, 1% Triton X 100, and protease inhibitors) for Fig. 1b, or in Hunter's buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 0.1 mmol/L Na-orthovanadate, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, and protease inhibitors) for all experiments. Cells were rocked with end-over-end rotation at 4°C for 20 minutes to allow for cell lysis prior to sonication. Cell samples were sonicated on ice at 10 watts for 10 x 1 second pulses to further disrupt the cell membrane using a Microson Ultrasonic Cell Disruptor XL (Misonix, Inc., Farmingdale, NY). Whole cell lysates were utilized for Western

blot (WB) and immunoprecipitation (IP) experiments. For all IP experiments, lysis buffer (LB) alone was incubated with the primary IP antibody and protein-conjugated agarose beads and serves as a negative control to show what bands can be attributed to possible cross-reactivity between the IP antibody and the antibody used for western analysis. Cell lysate incubated with normal IgGs of the same species as the primary antibody of interest and protein-conjugated agarose beads serves as an additional negative control to verify specificity of the primary antibody being used for IP. After co-IP or for WB analysis, proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with appropriate primary antibodies. Primary antibodies were used at the following dilutions for immunoblotting: 1) Myc at 1:1000, 2) V5 at 1:2000, 3) Poldip2 at 1:2000, 4) p22phox at 1:2000, 5) Nox4 at 1:2500, 6) Nox1 at 1:1000, and 7) HA at 1:500. Proteins were detected by ECL (GE Healthcare). Band intensity was quantified by densitometry using ImageJ 1.38 software.

2.2.8 RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA), per the manufacturer's recommendations. Superscript II (Invitrogen, Mountain View, CA) and random primers were used for reverse transcription. Message expression of poldip2 (primer sequences for rat: GTATGAGACGGGACAGCTATTTCTCCA and CTGACATAGTCCAAGCCTGGGATG) and 18S rRNA were measured by amplification of rat VSMC cDNA using the LightCycler (Roche, Basel,

Switzerland) real-time thermocycler and SYBR green dye. Specific rat poldip2 primers were used to measure mRNA and normalized to 18S rRNA. Copy number was calculated by the instrument software from standard curves of genuine templates.

2.2.9 Immunocytochemistry and Confocal Microscopy

VSMCs were plated on collagen-coated glass coverslips (BD Biosciences, Bedford, MA) and were either: 1) serum deprived for 48 h prior to labeling experiments conducted in untreated cells, 2) transiently transfected with siRNA for 72 h in OPTI-MEM prior to labeling in all knockdown experiments, or 3) grown to 50-60% confluence before transducing cells with adenovirus for 48-72 h prior to labeling in all overexpression experiments. All VSMCs were plated onto 22-mm diameter round glass coverslips. Cells were rinsed quickly in ice-cold PBS, fixed in freshly prepared 4% formaldehyde (Invitrogen, Carlsbad, CA) in PBS for 10 min at room temperature, permeabilized in 0.075% Triton X-100 in PBS for 7.5 min, and rinsed in PBS prior to quenching in 50 mmol/L NH_4Cl for 10 min. After incubation for 1 h in blocking buffer (3-5% bovine serum albumin in PBS), the cells were incubated with primary antibodies for 1 h, rinsed in PBS/bovine serum albumin, and then incubated for 1 h with the appropriate species-specific secondary antibodies conjugated to either FITC, Rhodamine Red X, or Cy5. The primary antibodies used for immunocytochemistry experiments include: 1) Poldip2 at 1:50, 2) Nox4 at 1:50, 3) p22phox Rb5554 at 1:50, 4) Myc-tag at 1:50, and 5) DAPI (nuclei) at 1:1000. Cells were always incubated with primary antibody for 1 hour at room temperature. Cells were then incubated for 1 hour at

room temperature with fluorophore-conjugated secondary antibodies purchased from The Jackson Laboratory (Bar Harbor, Maine). Nuclei were labeled with DAPI for 10 minutes at room temperature. Cells on coverslips were mounted onto glass slides in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and examined with a confocal microscope. Images were acquired with a Zeiss LSM 510 META Laser Scanning Confocal Microscope System using a Plan-Apo 420782-9900 63x oil objective lens (numerical aperture: 1.40) and either Zeiss LSM 510 or Zeiss ZEN acquisition software. Controls consisted of omission of primary antibody or the use of normal IgGs of the same species in place of the primary antibody. Controls were performed in all single and multiple labeling experiments and were used to subtract out negligible bleed-through and non-specific labeling. When comparing cells from different treatment groups, all image threshold settings of the confocal microscope remained constant.

2.2.10 Immunohistochemistry

Tissues from rats were harvested and prepared for immunohistochemistry, as described previously (San Martin et al., 2007). The Poldip2 staining patterns were visualized by the streptavadin-catalyzed color reaction and 3', 3'-diaminobenzidine (DAB).

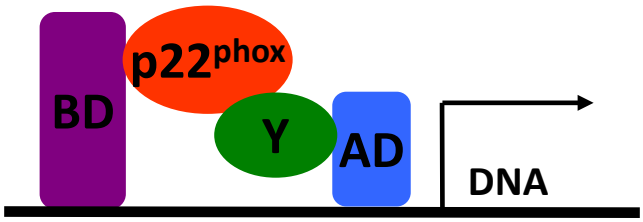
2.3 Experimental Results

2.3.1 Identification of Poldip2 as a Novel p22phox-Interacting Partner

2.3.1.1 Yeast Two-Hybrid

Using the proline-rich region of the cytosolic C-terminal tail of p22phox as bait, a likely binding site for regulatory molecules, we performed a yeast two-hybrid screen on a cDNA library from rat VSMCs, a cell line with high Nox4 expression. We utilized the Matchmaker LexA yeast two-hybrid system and a VSMC cDNA library constructed in pB42AD (Figure 2.1). The hydrophilic cytosolic tail of rat p22phox (nt 360-579 in accession number FJ515740, corresponding to the C-terminal 73 amino acids), used as bait, was inserted into pLexA vector such that it is expressed as a fusion protein containing an N-terminal DNA binding domain that binds to the LexA operator sequence. The plasmid was co-transformed with the pB42AD vector and a vector (p8op-lacZ) containing the lacZ reporter gene into yeast (strain EGY48). Co-transformants containing all three plasmids (pLexA-BD-p22phox, pB42AD-cDNA, and p8opLacZ) were amplified on minimal medium (-His/-Trp/-Ura). Colonies were scraped and replated on Leu induction medium. X-gal was included in this selection medium to confirm expression of hybrid proteins by assaying β-galactosidase activity conferred by the LacZ reporter and to help to eliminate false positives. False positives were further removed by mating independent positive clones with YM4271 yeast bearing the pLexA containing the BD domain alone.

After stringent elimination of false positives, we isolated the cDNA of a protein that interacted with p22phox, obtained its full-length sequence using RT-PCR, and identified the clone as Poldip2 (GenBank accession #FJ515740). Poldip2 bears no homology to the classical Nox cytosolic regulatory subunits



Transcription is activated only if the binding domain coupled to p22phox binds Y to recruit the activation domain (AD) to the appropriate DNA binding site.

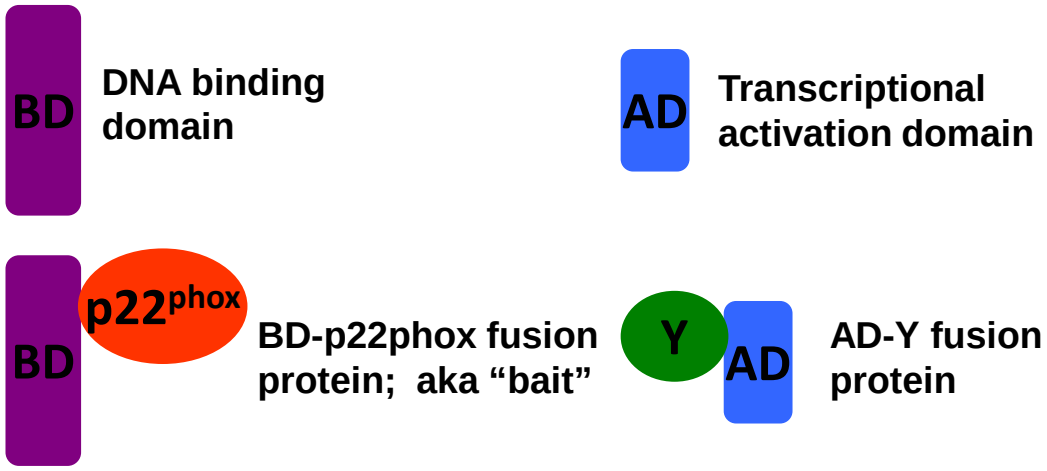


Figure 2.1. Yeast Two-Hybrid Method. A yeast two-hybrid screen was performed on a VSMC cDNA library in which the cytosolic C-terminal tail of rat p22phox was used as bait by cloning it into a vector to make a pLexA-BD-p22phox construct (containing the DNA binding domain). The VSMC cDNA library was inserted into the pB42AD vector, containing the activator domain, such that if one of the AD-Y fusion proteins from the cDNA library binds to the BD-p22phox construct, transcription is activated and drives the expression of a marker used to screen colonies.

p47phox and p67phox or their homologues NoxO1 and NoxA1. It consists of 368 amino acids (aa) and has a predicted molecular weight of approximately 42 kDa, with a potential signal peptide cleavage site after the first N-terminal 48 residues, which would result in a protein with a predicted molecular weight of 37 kDa. Using the Swiss Institute for Bioinformatics Expert Protein Analysis System (ExPASy) proteomics server and the PROSITE database, we scanned the full length Poldip2 sequence for predicted features. The predicted features found included: 1) an ApaG domain, which is a 125 amino acid domain common to proteins that associate with F-box proteins and the anaphase promoting complex, 2) a phox homology (PX) domain, which binds to the headgroups of phosphatidylinositols present in the membrane and is a common feature of Nox cytosolic regulatory proteins, 3) N-Myristoylation sites, which are fatty acid modifications that serves as a targeting signal, allow for membrane tethering, and mediate protein-protein interactions, 4) phospho-Serine/Threonine sites, which may be phosphorylated and confer Poldip2 activity, and 5) Tyrosine sulfation sites, which modulate protein-protein interactions.

To further characterize the association of p22phox and Poldip2, we generated an antibody raised against an epitope region of Poldip2 corresponding to aa 117-130 (Figure 2.2, a). To evaluate the specificity of the Poldip2 antibody, we performed a western blot analysis using antibody alone or antibody incubated with blocking peptide (Figure 2.2, b), where Poldip2 was detected as a 37kD protein. To determine if the Poldip2 antibody was suitable for immunocytochemsitry (ICC), we performed a characterization experiment in

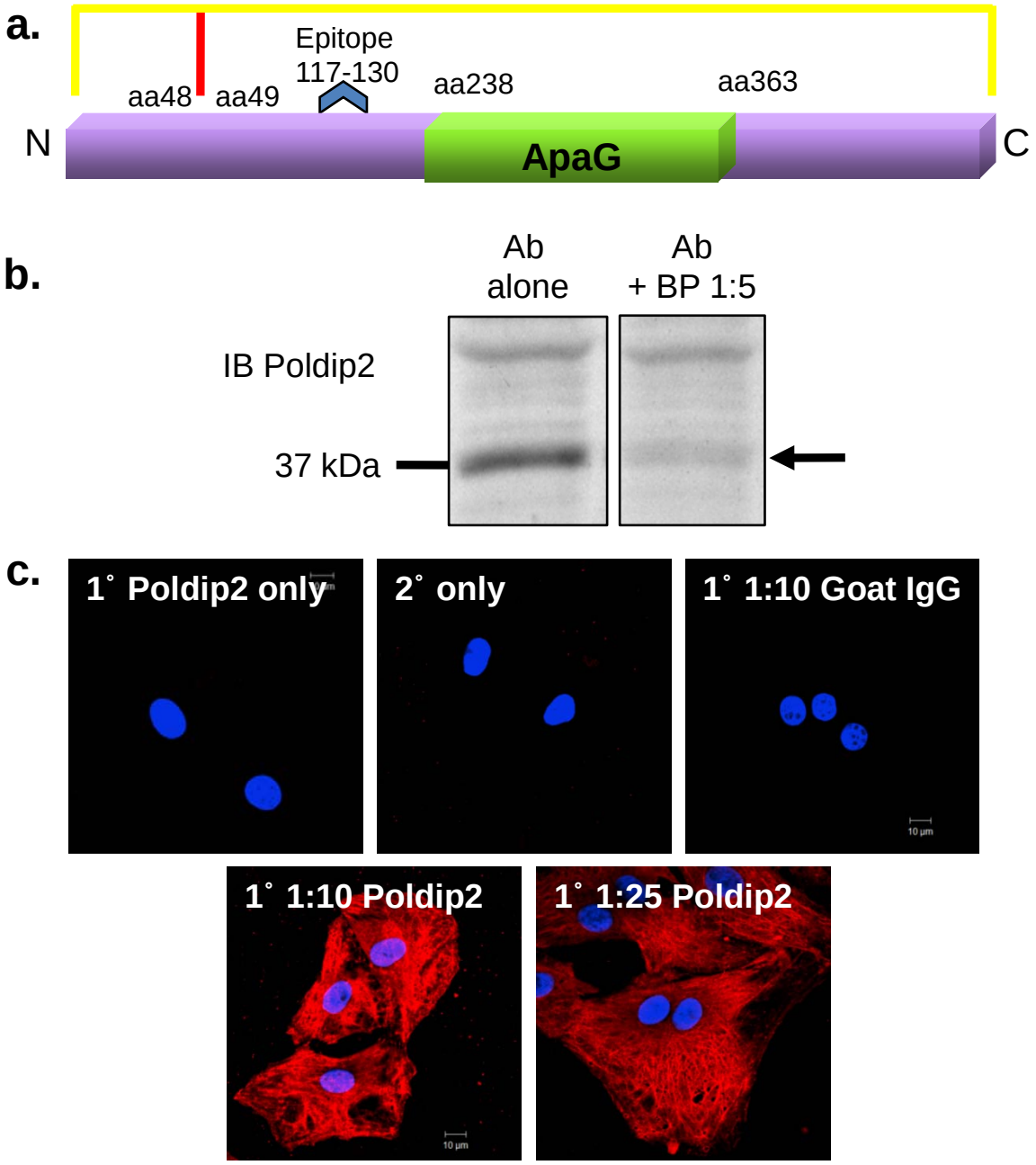


Figure 2.2. Poldip2 Antibody Characterization. a, The Poldip2 goat antibody (Ab) was raised against amino acids 117-130 (peptide sequence NPAGHGSKEVKGKTC). **b**, Western analysis of Poldip2 protein expression in rat VSMC lysate immunoblotted with goat Poldip2 Ab alone or 1:5 ratio of goat anti-Poldip2 Ab to blocking peptide (BP). **c,** Immunocytochemistry of rat VSMCs single labeled with Poldip2 Ab (red), or normal IgG, or 2˚ Ab alone as negative controls. Images acquired at the focal adhesion plane are shown.

which the staining pattern of the Poldip2 antibody was verified to be specific when compared to normal goat IgGs alone (negative control) or to secondary antibody alone (negative control) (Figure 2.2, c). Poldip2 appears to localize primarily to focal adhesions, stress fibers, and in the nucleus of VSMCs, which are compartments where p22phox has previously been reported to localize in VSMCs (Hilenski et al., 2004).

2.3.1.2 Association of p22phox and Poldip2

To further validate the association of Poldip2 and p22phox, we co-transfected HEK293 cells with V5-tagged p22phox (V5-p22phox) and either empty vector control or N-terminally Myc-tagged Poldip2 (Myc-Poldip2). Using an antibody against the V5 tag of p22phox, we performed a co-immunoprecipitation. An antibody against the Myc tag of Poldip2 confirmed that Myc-Poldip2 co-immunoprecipitates with V5-p22phox in co-transfected HEK 293 cells (Figure 2.3).

To verify the interaction between Poldip2 and p22phox, we used a GST-pulldown assay. GST fusion proteins were generated containing either empty vector (GST-vector) or p22phox (GST-p22phox) and were used to perform a pull-down on lysates from cells transfected with either Poldip2 or empty vector and labeled with ^{35}S-methionine. Indeed, ^{35}S-Poldip2 pulls down with GST-p22phox (Figure 2.4), confirming the association of these two proteins.

Because the above described experiments were conducted using tagged proteins, we next utilized the Poldip2 antibody we developed to confirm an association between endogenous Poldip2 and p22phox. To do this, we

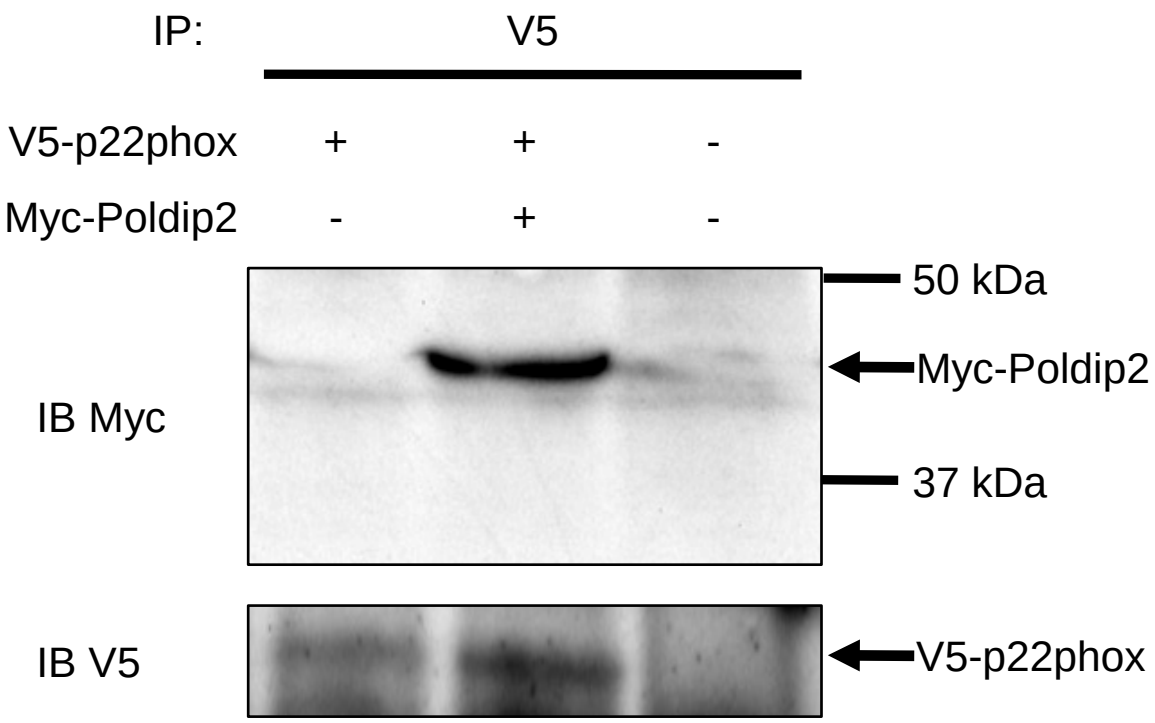


Figure 2.3. Association of p22phox and Myc-Poldip2 in Co-Transfected HEK 293 cells. HEK 293 cells were co-transfected with V5-tagged p22phox (V5-p22phox) and either empty vector (-) or vector expressing Myc-tagged Poldip2 (+; Myc-Poldip2). Cells were immunoprecipitated (IP) with V5 antibody and immunoblotted (IB) with a Myc (*upper*) or V5 (*lower*) antibody.

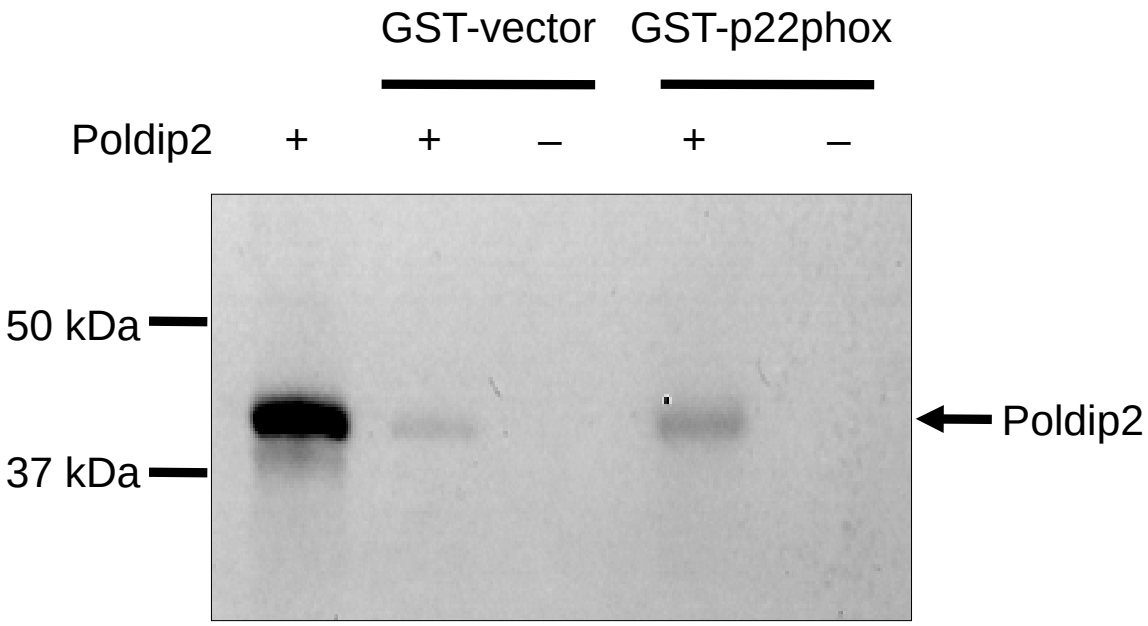


Figure 2.4. GST-p22phox Pulls Down Poldip2 in Vascular Smooth Muscle Cells. Rat VSMCs were transfected with vector control (-) or Poldip2 (+), labeled with ^{35}S-Methionine, and used in a GST-pulldown assay. Binding partners for GST-vector control and GST-p22phox were detected by autoradiography. Positive control: in vitro translated Poldip2 (*lane 1*). We thank Yoshihiro Taniyama, M.D. and Pingfeng Du, M.D. for kindly providing this figure.

performed a co-immunoprecipitation on VSMC lysates using an anti-p22phox antibody. As expected from our observations with tagged proteins, endogenous Poldip2 co-immunopreciptated with p22phox (Figure 2.5)

2.3.1.3 Poldip2 co-localizes with p22phox in VSMCs

To further investigate Poldip2, we generated an adenovirus (Ad) that expressed the Myc-tagged version of full-length Poldip2 (AdPoldip2). To verify that we could successfully overexpress Poldip2 using the adenovirus we generated, VSMCs were transduced with control adenovirus (AdGFP) or with an equal amount of AdPoldip2. Western blot analysis of these lysates showed successful overexpression of Myc-Poldip2 in VSMCs, which was detected as a 42 kD band with an anti-Myc antibody (Figure 2.6, a). We then utilized the Poldip2 adenovirus to overexpress Myc-Poldip2 in VSMCs to determine if Myc-Poldip2 co-localizes with p22phox. As shown in Figure 2.6 b, Myc-Poldip2 co-localizes with p22phox in focal adhesions of VSMCs. To determine if endogenous Poldip2 co-localizes with p22phox in VSMCs, we used the anti-Poldip2 antibody and an anti-p22phox antibody. Poldip2 co-localizes with p22phox in focal adhesions, along stress fibers, and in the nucleus of VSMCs (Figure 2.7).

Previously published data showed that both Nox1 and Nox4 co-localize with and co-immunoprecipitate with p22phox in VSMCs (Ambasta et al., 2004; Hilenski et al., 2004); however, other Nox homologues are not detectable in aortic VSMCs. Therefore, we sought to investigate if there is a potential

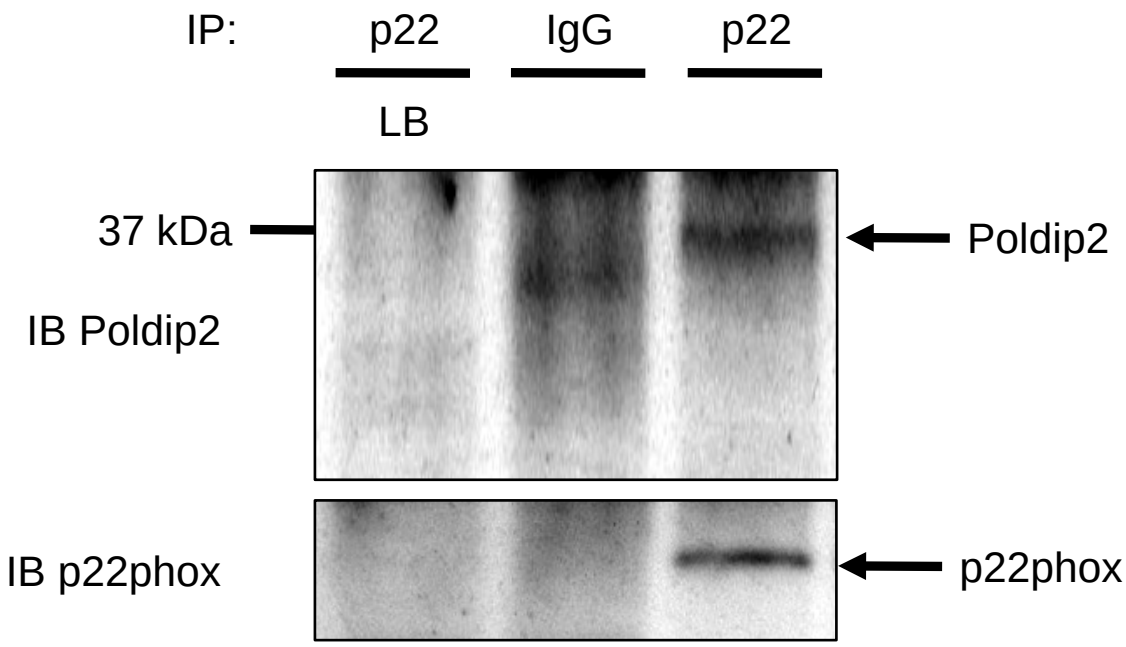


Figure 2.5. **Association of p22phox with Endogenous Poldip2 in Vascular Smooth Muscle Cells.** Human VSMC lysates were immunoprecipitated (IP) with rabbit IgGs (IgG) or p22phox antibody and immunoblotted (IB) with a Poldip2 (*upper*) or p22phox (*lower*) antibody. Lysis buffer (LB), to which the 1˚ IP antibody and protein-conjugated agarose beads are added, was used as a negative control in all IP experiments to show what bands are attributable to possible cross-reactivity between the IP antibody and IB antibody. Poldip2 is detected as a 37 kDa band; IgGs interfere with resolution of the 42 kDa band.

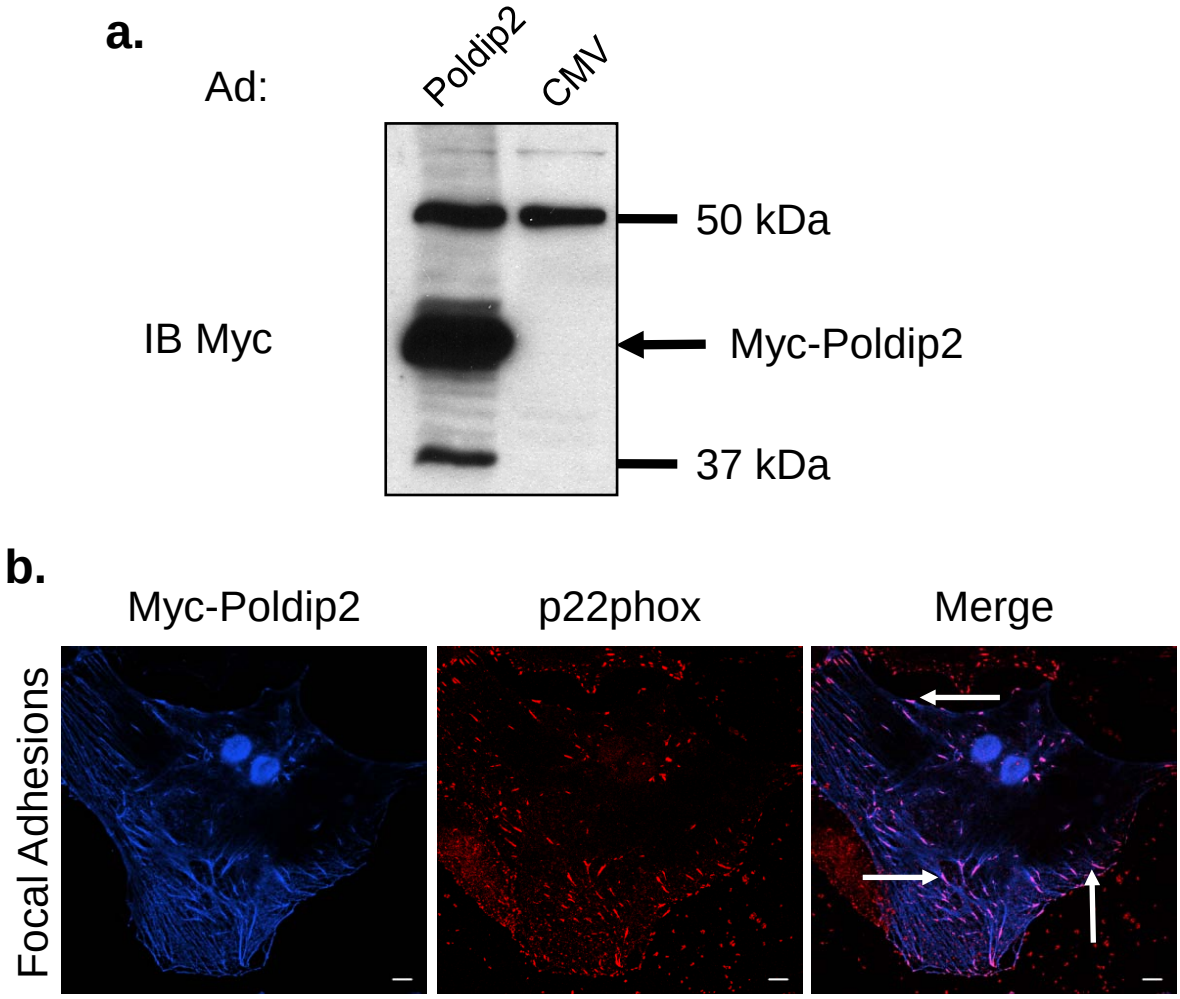


Figure 2.6. a, Expression of Myc-Poldip2 in Vascular Smooth Muscle Cells using Adenovirus. Rat VSMCs transduced with either control adenovirus (AdGFP) or adenovirus to express Myc-tagged Poldip2 (AdPoldip2) for 72 h were immunoblotted (IB) with a Myc antibody to verify expression of Myc-Poldip2. Myc-Poldip2 is detected at 42 kDa and 37 kDa. **b, Myc-Poldip2 Co-localizes with p22phox in Vascular Smooth Muscle Cells.** Rat VSMCs transduced with AdPoldip2 were double labeled with Myc (pseudo-colored blue) and p22phox (red) antibodies. Arrows indicate areas of co-localization (purple) in the merge. Scale bars, 10 µm.

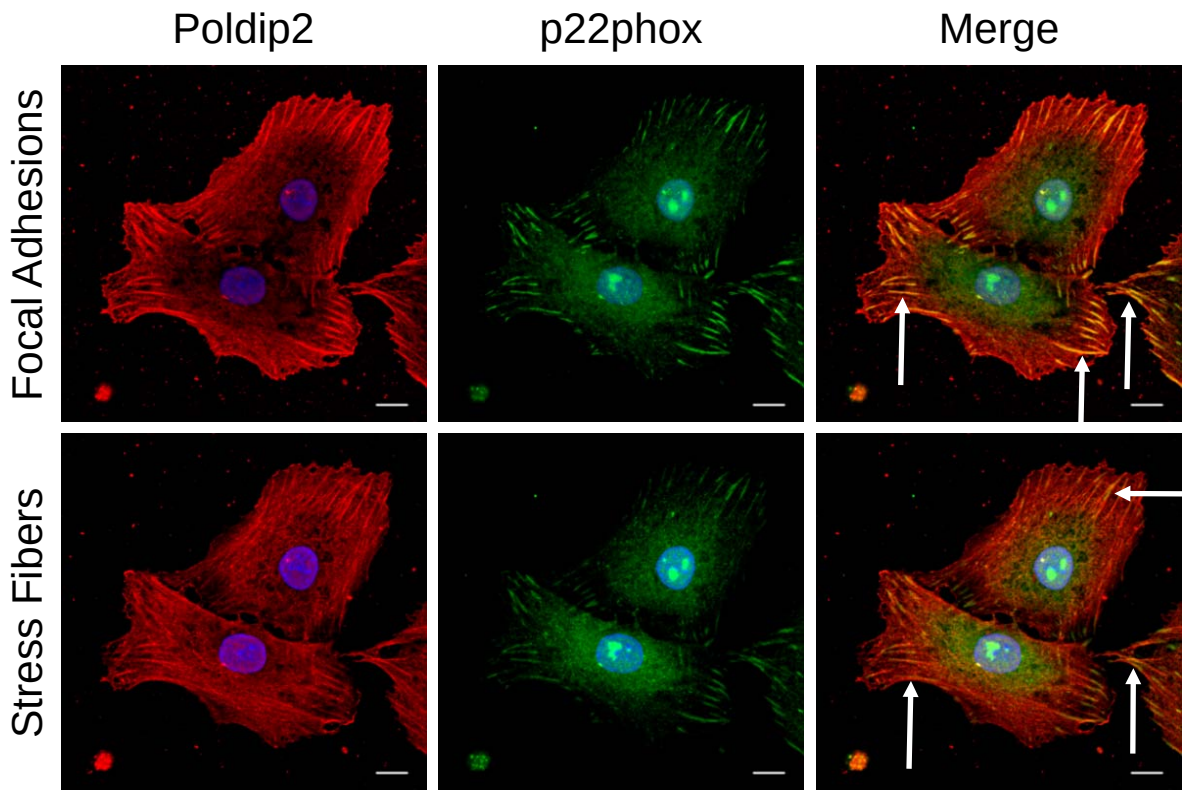


Figure 2.7. Endogenous Poldip2 Co-Localizes with p22phox in Vascular Smooth Muscle Cells. Rat VSMCs were double labeled with Poldip2 (red) and p22phox (green) antibodies. Arrows indicate areas of co-localization (yellow) in the merge. Nuclei are labeled with DAPI (blue). Images acquired at the focal adhesion (*upper panel*) and stress fiber (*lower panel*) planes are depicted. Scale bars, 10 µm.

association of Poldip2 with either Nox1 or Nox4, both of which require p22phox for their activity. We next investigated if Poldip2 associates with Nox1 or Nox4.

2.3.2 Poldip2 Association with other NADPH Oxidase Subunits

2.3.2.1 Association of Poldip2 with Nox4 and Nox1 in VSMCs

To investigate if Poldip2 and Nox4 associate, VSMCs were transduced with adenovirus to overexpress Myc-Poldip2 (AdPoldip2) or AdGFP. Lysates were immunoprecipitated with an anti-Myc antibody and immunoblotted for Nox4. Endogenous Nox4 associates with Myc-Poldip2 in VSMCs (Figure 2.8). Interestingly, when VSMCs are transduced with increasing amounts of AdPoldip2 and are immunoprecipitated with an anti-Myc antibody, increasing amounts of Nox4 co-immunoprecipitate with increasing Myc-Poldip2 expression; however, we were unable to detect co-immunoprecipitation of endogenous Nox1 with Poldip2 in these samples (Figure 2.9).

To assess if the association of Poldip2 with Nox4 is dependent on p22phox, we utilized a rat VSMC line stably transfected with antisense p22phox (p22AS) in which p22phox expression is ablated (Figure 2.10). Poldip2 co-immunoprecipitates with Nox4 in vector-transfected cells, but not in cells lacking p22phox (Figure 2.11), suggesting that Poldip2 requires p22phox to associate with Nox4.

Since we were unable to detect the association of endogenous Nox1, which is expressed at low levels in serum deprived VSMCs, with Poldip2, as shown in Figure 2.9, we chose to repeat this experiment in VSMCs kept in serum containing media, which increases Nox1 expression. Additionally, we

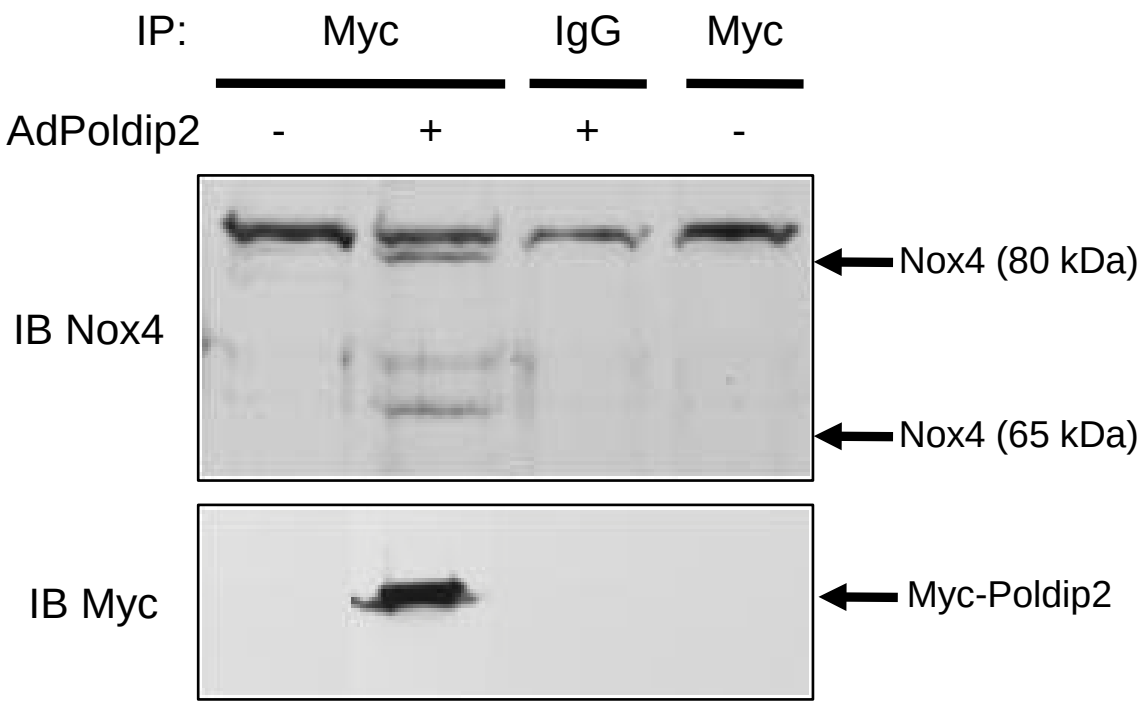


Figure 2.8. Association of Myc-Poldip2 with Nox4 in Vascular Smooth Muscle Cells. Rat VSMCs were transduced with 125 µL/dish of control adenovirus (-) or Myc-tagged Poldip2 adenovirus (+ , AdPoldip2). Lysates were immunoprecipitated (IP) with IgGs or a Myc antibody and immunoblotted (IB) with a Nox4 (*upper) or Myc* (*lower)* antibody. Nox4 is detected as an 80 kDa and 65 kDa band.

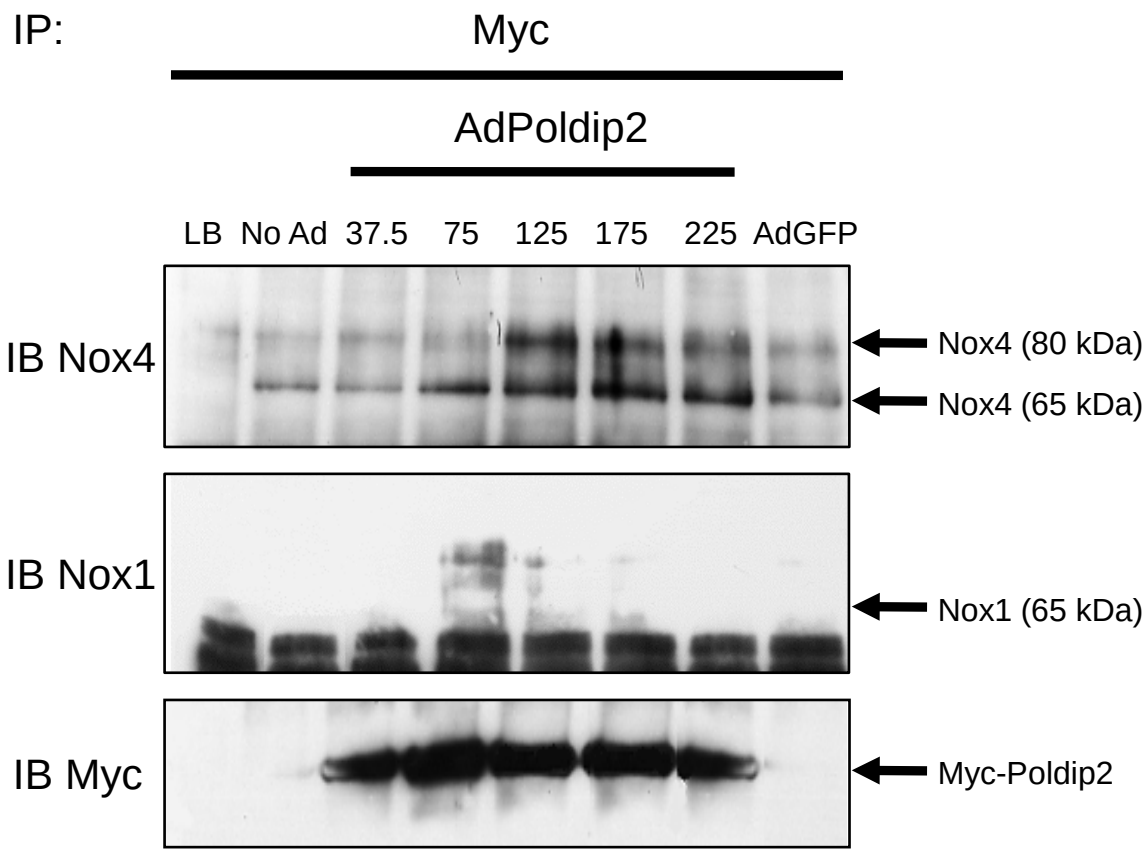


Figure 2.9. Increasing Amounts of Nox4 Associate with Myc-Poldip2 in Vascular Smooth Muscle Cells. Rat VSMCs were transduced with no adenovirus (No Ad), 125 µL/dish of control adenovirus (AdGFP) or increasing amounts of Myc-tagged Poldip2 adenovirus (AdPoldip2). Lysates were immunoprecipitated (IP) with a Myc antibody and immunoblotted (IB) with a Nox4 (*upper*), Nox1 (*middle*), or Myc (*lower*) antibody. Lysis buffer (LB) was used as a negative control.

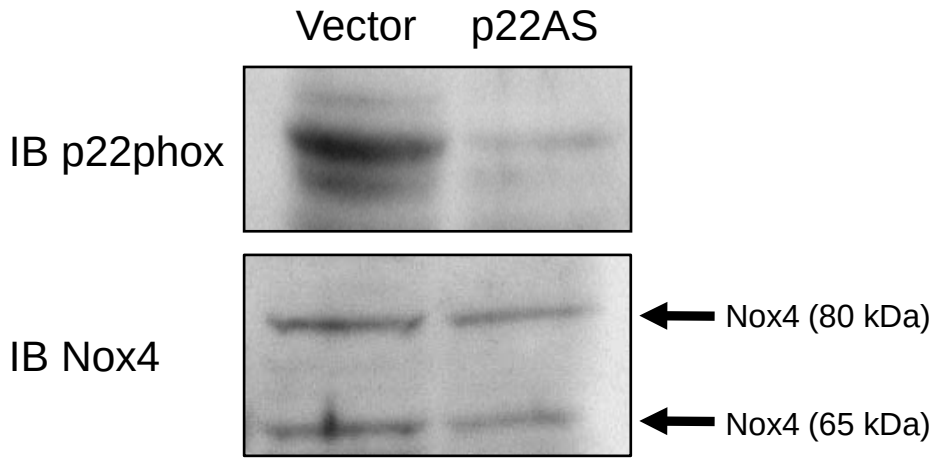


Figure 2.10. Expression of p22phox and Nox4 in Vector Control and p22phox Antisense Vascular Smooth Muscle Cells. Western analysis of rat VSMCs stably transfected with antisense p22phox (p22AS) or vector control (vector) to verify knockdown of p22phox (upper) and to determine the expression levels of Nox4 (lower).

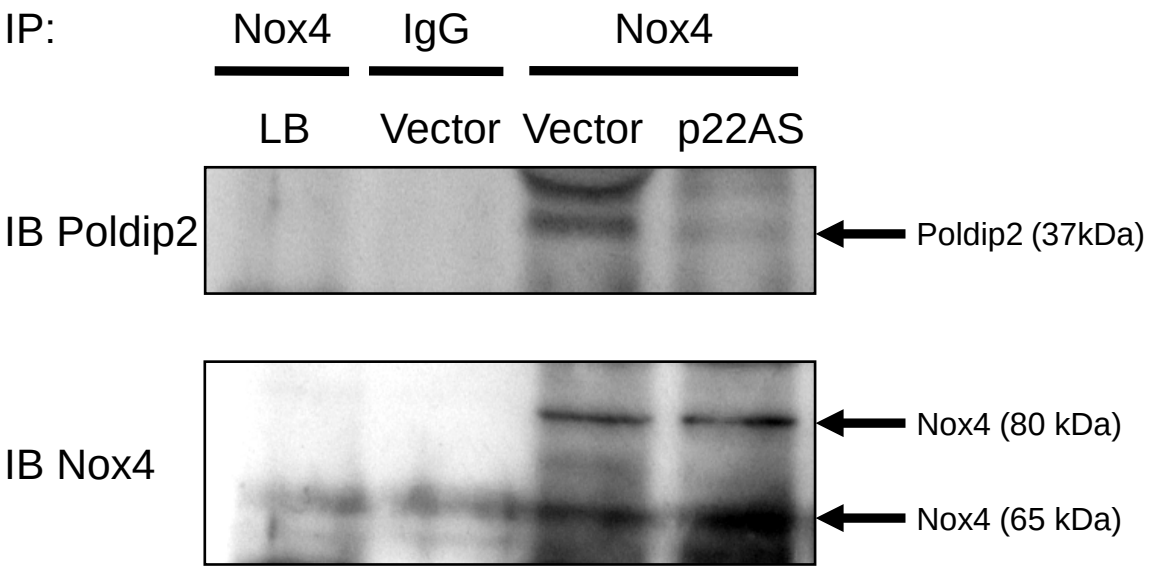


Figure 2.11. Association of Endogenous Poldip2 with Nox4 in Vascular Smooth Muscle Cells Requires p22phox. Rat VSMCs stably transfected with empty vector (Vector) or antisense p22phox (p22AS) vector were immunoprecipitated (IP) with rabbit IgGs or a Nox4 antibody and immunoblotted (IB) with a Poldip2 (*upper*) or Nox4 (*lower*) antibody. Lysis buffer (LB) was used as a negative control.

overexpressed an HA-tagged Nox1 in VSMCs using an adenovirus and either left these cells in serum containing media or stimulated the cells with 100 nmol/L of Ang II for 4 hours, which is known to activate Nox1 (Lassegue et al., 2001), to determine if the association of Poldip2 with Nox1 is, perhaps, agonist dependent. An association between Poldip2 and endogenous Nox1 in serum treated VSMCs was not detected in the co-immunoprecipitation shown in Figure 2.12 (lane 3, from left); however, Poldip2 did co-immunoprecipitate with HA-tagged Nox1 (Figure 2.12, lane 4). Agonist stimulation with Ang II for 4 hours decreased the association between Poldip2 and HA-tagged Nox1 (Figure 2.12, lane 5), suggesting that Poldip2 does not associate with the active Nox1 complex, but may associate with the inactive Nox1 complex in VSMCs.

2.3.2.2 Poldip2 co-localizes with Nox4 in VSMCs in focal adhesions and along stress fibers

In VSMCs, Nox4 is detected in comparable locations to those depicted in the p22phox immunocytochemistry experiments. Because of this, we used the Poldip2 adenovirus to overexpress Myc-Poldip2 in VSMCs and sought to determine if Myc-Poldip2 co-localizes with Nox4. Indeed, co-localization experiments show that Myc-Poldip2 co-localizes with Nox4 in VSMCs in focal adhesions, along stress fibers, and in the nucleus of VSMCs (Figure 2.13). To determine if endogenous Poldip2 co-localizes with Nox4 in VSMCs, we used the antibody we raised against Poldip2. As shown, endogenous Poldip2 also co-localizes with Nox4 in focal adhesions, along stress fibers, and in the nucleus of VSMCs (Figure 2.14). For all experiments in which Poldip2 localization was

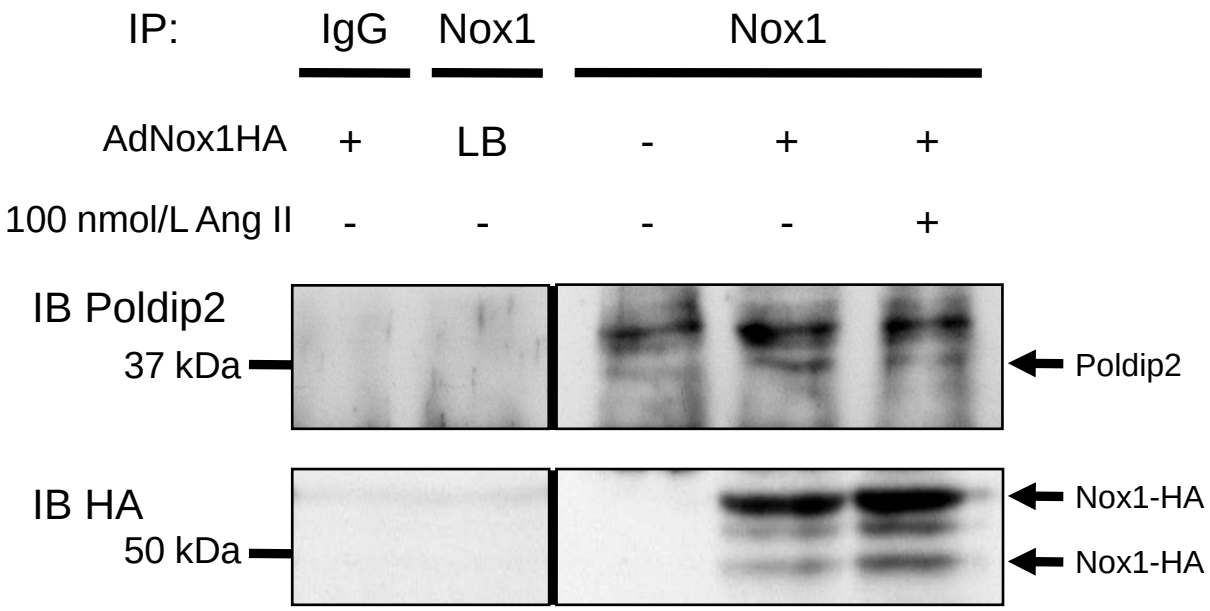


Figure 2.12. Association of Endogenous Poldip2 with HA-tagged Nox1 in Vascular Smooth Muscle Cells. Rat VSMCs transduced with AdGFP (-) or HA-tagged Nox1 (+ , AdNox1HA) adenovirus were untreated or stimulated for 4 hours with 100 nmol/L Angiotensin II (Ang II) prior to being immunoprecipitated (IP) with goat IgGs or a Nox1 antibody and immunoblotted (IB) with a Poldip2 (*upper*) or HA (*lower*) antibody. Lysis buffer (LB) was used as a negative control.

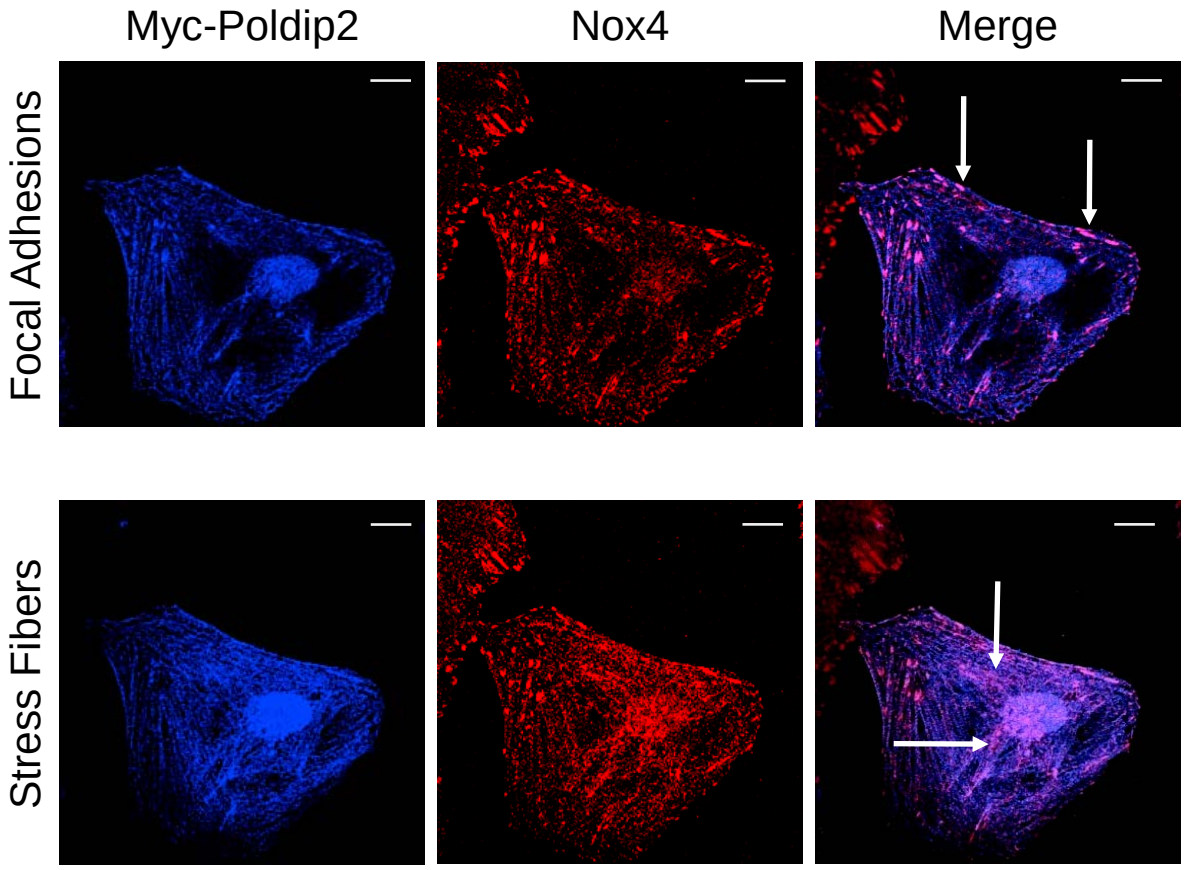


Figure 2.13. Myc-Poldip2 Co-localizes with Nox4 in Vascular Smooth Muscle Cells in Focal Adhesions and Along Stress Fibers. Rat VSMCs were transduced with AdPoldip2 and double labeled with anti-Myc (pseudo-colored blue) and anti-Nox4 (red) antibodies. Arrows indicate areas of co-localization (purple) in the merge at the focal adhesion (*upper panel*) and stress fiber (*lower panel*) planes. Scale bars, 10 μm.

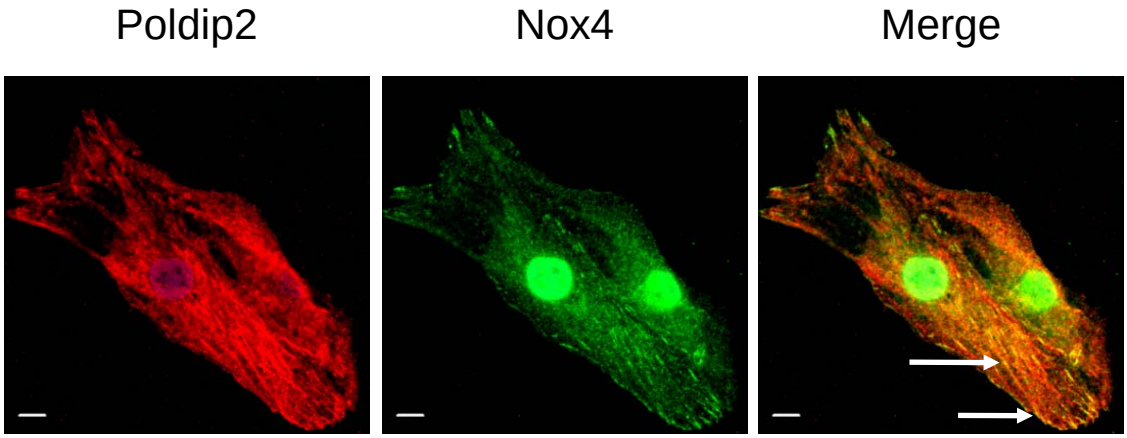


Figure 2.14. Endogenous Poldip2 Co-localizes with Nox4 in Vascular Smooth Muscle Cells in Focal Adhesions and Along Stress Fibers. Rat VSMCs were double labeled with Poldip2 (red) and Nox4 (green) antibodies. Arrows indicate areas of co-localization (yellow) in the merge. Scale bars, 10 µm.

examined, Poldip2 was never detected in caveolae, the compartment in which Nox1 is localized in VSMCs. Altogether, these data suggest that Poldip2 associates with both p22phox and Nox4 in specific subcellular compartments.

2.3.3 Expression and Tissue Distribution of Poldip2

The association between Nox4 and Poldip2 prompted us to examine the expression of Poldip2 in tissues rich in Nox4, such as aorta, lung and kidney (Bedard and Krause, 2007). Analysis of tissue distribution using qRT-PCR, Western blot, and immunohistochemistry indicates that Poldip2 is highly expressed in all three of these Nox4 rich tissues (Figure 2.15 and 2.16). In contrast, Poldip2 is barely detectable in spleen and thymus, which are rich in Nox2 and do not express detectable Nox4.

2.4 Discussion

While previous studies suggest that Nox4 functions as a constitutively active enzyme and demonstrate that none of the currently known cytosolic subunits are required for the activation of Nox4 (Martyn et al., 2006), the existence of novel regulatory proteins for the Nox4 enzyme had not been systematically studied. As noted earlier, the fact that Nox4 regulates such diverse physiological and pathophysiological responses suggests that it modulates a fundamental cellular process. Thus, it becomes critical to understand how this enzyme complex is specifically regulated, what proteins mediate the activation of this enzyme, and how the regulation of Nox4 differs from the regulation of other Noxes, such as Nox1, so that one could potentially pharmacologically target a single Nox subtype without interfering with ROS

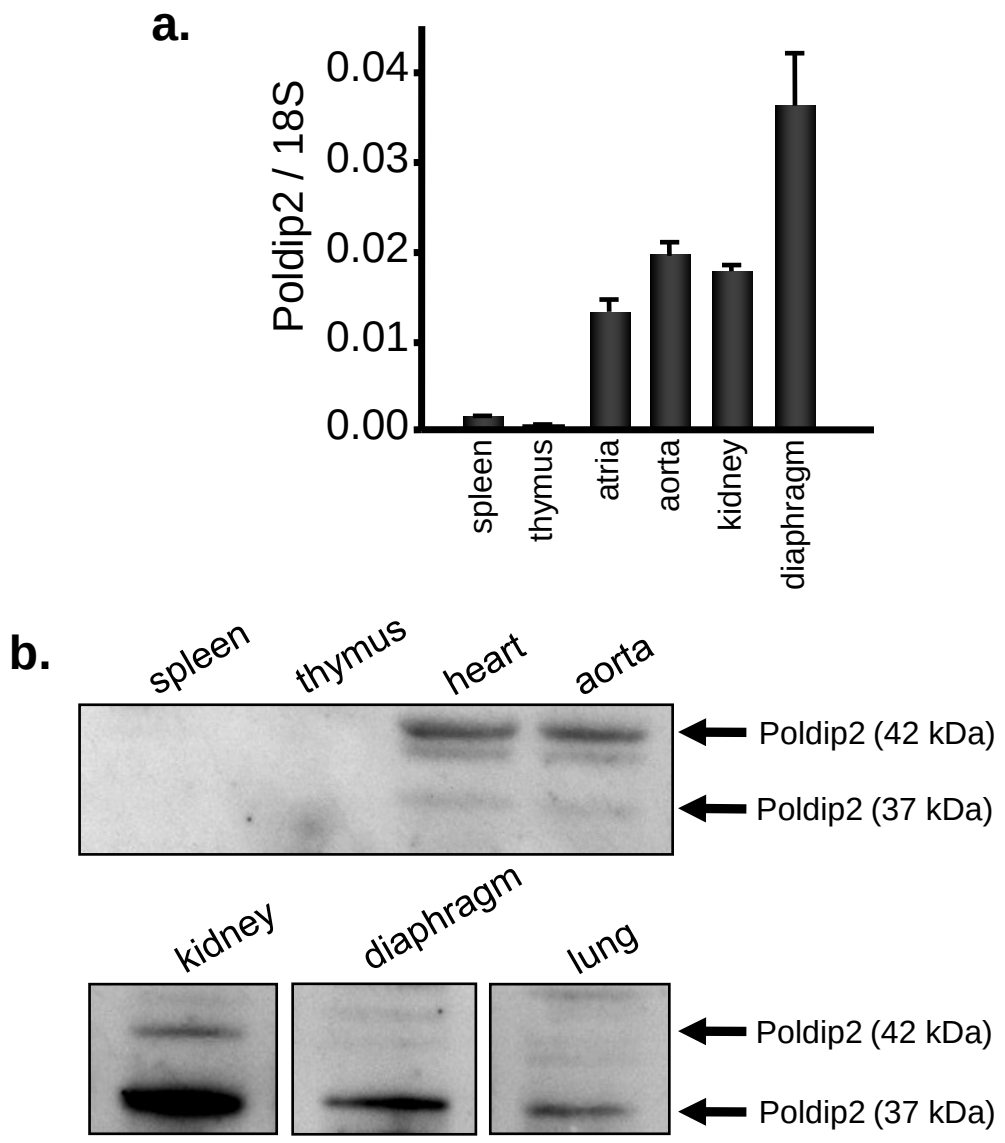


Figure 2.15. a, Poldip2 mRNA Expression in Tissues. Quantitative real-time PCR analysis of poldip2 mRNA expression in tissues from Sprague Dawley rats. mRNA is normalized to 18S. Bars are mean ± S.E.M. of triplicates from a representative experiment, repeated twice. **b, Poldip2 Protein Expression in Tissues.** Western blot of Poldip2 protein expression in rat tissue lysates. The 42 kDa Poldip2, containing the predicted signal peptide, and the 37 kDa Poldip2, after peptide cleavage, are shown.

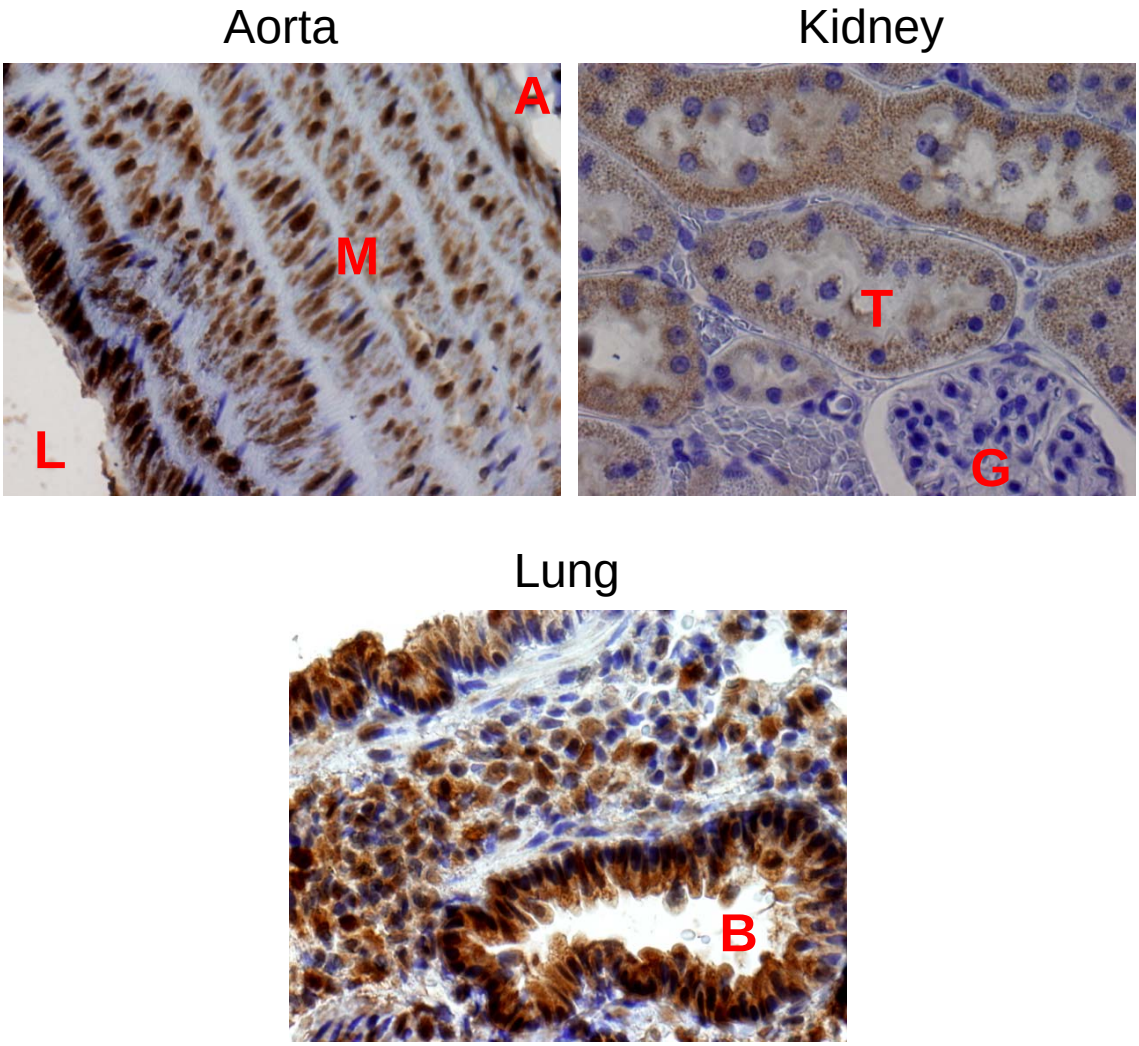


Figure 2.16. Expression of Poldip2 in Tissue Sections.

Immunohistochemical distribution of Poldip2 in rat aorta (L-lumen, M-media, A-adventitia), kidney (G-glomerulus, T-tubule), and lung (B-bronchiole) tissues using an anti-Poldip2 antibody. Poldip2 signal appears brown from use of horse raddish peroxidase (HRP) . Nuclei are counterstained with hematoxylin (blue/purple).

production by other Nox enzymes. In this study, we provided evidence that a new protein, Poldip2, co-immunoprecipitates with and co-localizes with the Nox4/p22phox complex, suggesting that it is a candidate for a novel Nox4 regulatory protein.

We initially carried out our search for novel p22phox binding partners by performing a yeast two-hybrid screen utilizing the cytosolic C-terminal tail of p22phox to identify new p22phox-interacting proteins. From this screen, we identified Poldip2 as a p22phox interacting protein. Further investigation using multiple techniques, including GST-pulldown (Figure 2.4), co-immunoprecipitation (Figures 2.3, 2.5, 2.8 – 2.12), and co-localization (Figures 2.6, 2.7, 2.13, 2.14) confirmed this association and showed a clear functional association between the p22phox/Nox4 complex and Poldip2. We showed that both Myc-tagged Poldip2 and endogenous Poldip2 associate with Nox4 and that this association is dependent on the presence of p22phox. In VSMCs, p22phox/Nox4 and Poldip2 co-localize specifically in focal adhesions, stress fibers, and the nucleus. Additionally, Nox4 and Poldip2 also co-localize in similar cell types and cell structures in tissues from mouse aorta, kidney, and lung.

The cytosolic C-terminal tail of p22phox has classically served as the docking site for other cytosolic regulatory proteins that function to activate Nox enzymatic activity. Association of p22phox with organizer subunits is required for proper activation of several Noxes, including Nox1, Nox2, and Nox3; however, the requirement of the cytosolic C-terminus region of p22phox for proper Nox4 activation was unclear. While a portion of the C-terminal region of p22phox,

specifically amino acids 130-195 of human p22phox, was shown recently to not be required for Nox4 activity (Kawahara et al., 2005; von Lohneysen et al., 2008), our studies indicate that a portion of the C-terminal region of p22phox, specifically amino acids 106-129, is required for Poldip2 association with Nox4. This 24 amino acid domain (aa 106-129) is highly conserved across species and may serve as an additional docking site on p22phox. Consistent with this, functional studies have shown that Nox4 enzymatic activity is p22phox-dependent (Martyn et al., 2006) and that Nox4 both co-immunoprecipitates and co-localizes with p22phox (Hilenski et al., 2004; von Lohneysen et al., 2008). Recent investigations confirmed that Nox4 requires p22phox for maturation, localization, and activity, but that the proline rich region of p22phox was not required for these functions (von Lohneysen et al., 2008). Whether Nox4 requires Poldip2 for maturation, localization, and enzymatic activity will be points of further investigation and discussion in the remaining chapters of this dissertation.

Studies in which Nox4 was heterologously expressed in a reconstituted system suggested that Nox4 functions as a constitutively active enzyme and demonstrate that none of the presently known cytosolic regulatory subunits are required for Nox4 activation (Martyn et al., 2006). Additionally, studies have suggested that the principal mechanism of Nox4 regulation may be at the mRNA level, rather than assembly of an enzymatic complex, like other Noxes (Serrander et al., 2007). The evidence provided in this chapter identifies Poldip2 as the first known cytosolic subunit to associate with Nox4 and proves that

Poldip2 does so in a p22phox-dependent manner. If and how Poldip2 functions to regulate Nox4 enzymatic activity requires further investigation.

In contrast to Nox4, the composition of the active Nox1complex with its cytosolic regulatory proteins is well understood in VSMCs. Activation and ROS generation by Nox1 is known to be dependent on the presence of p22phox (Hanna et al., 2004; Kawahara et al., 2005) and on cytosolic regulatory subunits, specifically p47phox, NoxA1, a p67phox homologue, and Rac in VSMCs (Ambasta et al., 2004; Ambasta et al., 2006; Banfi et al., 2003; Cheng et al., 2006). These subunits in unstimulated cells remain cytosolically localized and do not interact with the cytochrome structure in resting VSMCs. Upon agonist stimulation, p47phox is phosphorylated, which allows p47phox to interact NoxA1 and translocate to the membrane, where these subunits dock with the proline-rich region of p22phox (Kawahara et al., 2005). Additionally, Rac also binds to Nox1, thus forming the functional enzyme capable of producing $O_2^{\bullet}$-. As shown in Figure 2.12, Poldip2 is capable of associating with HA-tagged Nox1, suggesting that Nox4 may not be the only Nox with which Poldip2 forms a complex in VSMCs. However, the decrease in Poldip2 association with HA-tagged Nox1 in Ang II-stimulated VSMCs (Figure 2.12) suggests that Poldip2 does not associate with the active Nox1 complex. If anything, these data seem to suggest that Poldip2 is capable of association with the inactive Nox1 complex in VSMCs and that Poldip2 may potentially be displaced by the binding of p47phox/NoxA1 to p22phox upon agonist stimulation. These findings present a potential mechanism for the opposing stimulation of Nox1 and Nox4 in VSMCs;

therefore, Poldip2 may function to coordinate the inverse regulation of these two Noxes that is required for the physiological responses of VSMCs.

Of interest, we found that in VSMCs the p22phox/Nox4 complex and Poldip2 co-localize specifically in focal adhesions and stress fibers (Figures 2.6, 2.7, 2.13, 2.14), two structures that are primary components of the cytoskeleton. Very little is known about the physiological role of Poldip2, except as a regulator of cell division (Klaile et al., 2008). On the other hand, Nox4 is functionally linked to the regulation of basic cellular processes, such as oxygen sensing and senescence (Geiszt et al., 2000; Shiose et al., 2001), apoptosis (Pedruzzi et al., 2004), and survival (Vaquero et al., 2004) and differentiation (Clempus et al., 2007; Cucoranu et al., 2005; Li et al., 2006). Additionally, Nox4 has recently been implicated in the regulation of fundamental cellular processes such as cell cycle progression (McCrann et al., 2009a; McCrann et al., 2009b; Yamaura et al., 2009) and cytoskeletal modulation (Meng et al., 2008). The fact that Nox4 regulates such diverse physiological and pathophysiological responses suggests that it may potentially regulate a fundamental cellular process. Our data showing the clear association of Nox4 with Poldip2 on cytoskeletal structures may provide a possible mechanism to explain the known functions of both proteins and will be investigated further.

In conclusion, this chapter describes the identification of a novel p22phox binding protein, Poldip2, in VSMCs. This is the first protein to be described to associate with the Nox4/p22phox complex, thus understanding how Poldip2 associates with the Nox4/p22phox enzyme complex and the Nox1/p22phox

complex is the first step in understanding how Poldip2 may function to differentially modulate each of these enzymes in VSMCs. Additional insight into Poldip2's differential association and how that impacts the enzymatic activity of Nox1 and Nox4 in VSMCs will be the primary focus of Chapter 3.

CHAPTER 3

Poldip2 Functions as a Regulator of NADPH Oxidase Enzymatic Activity

3.1 Introduction

While it is well defined that Nox4 enzymatic activity is p22phox-dependent, whether Nox4 requires additional cytosolic subunits for its enzymatic activity has remained controversial. When Nox4 is heterologously expressed in a reconstituted system, the data reported suggest that Nox4 functions as a constitutively active enzyme and show that none of the currently known cytosolic regulatory subunits are required for Nox4 activation in these reconstituted systems (Martyn et al., 2006). However, it is unknown if other potential regulatory proteins for Nox4 exist. We demonstrated clearly that Poldip2 associates with and co-localizes with p22phox, as well as endogenous Nox4, and associates with HA-tagged Nox1 in VSMCs. However, this association with Noxes does not prove that Poldip2 functions to alter the enzymatic activity of these proteins. Therefore, we set out to determine if Poldip2 could positively or negatively modulate the enzymatic activity of Nox4, Nox1, or both in VSMC.

3.2 Methods

3.2.1 Cell Culture

Rat VSMCs, vector control VSMCs and p22AS VSMCs were grown in Dulbecco's Modified Eagle's Media (DMEM; Invitrogen, Carlsbad, CA) as described above in 2.2.1. Cells at passages 6 to 12 were used after 48 hours of quiescence in serum free DMEM for all experiments, unless otherwise noted. nox1$^{y/-}$ VSMCs were isolated from nox1$^{y/-}$ mice, generated by Dr K.H. Krause (Gavazzi et al., 2006; Gavazzi et al., 2007), by enzymatic digestion of nox1$^{y/-}$ mouse aortas, as described previously (Lee et al., 2009). Briefly, the aortas were

removed, cut longitudinally, cleaned of connective tissue, fat and endothelium, and digested with collagenase (Worthington, Lakewood, NJ) and elastase (Worthington, Lakewood, NJ) to remove the adventitia and to dissociate the VSMCs. VSMCs were plated in a culture flask, and grown in DMEM supplemented with 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA). Cells were passaged by trypsinization and mouse aortic smooth muscle cells were used between passages 3 to 10.

3.2.2 Antibodies

p22phox antibodies were kindly provided by Dr. Mark Quinn (Montana State University) or purchased from Santa Cruz, Nox4 antibodies were provided by Dr. David Lambeth (Emory University) or custom made by Sigma (St. Louis, MO). Poldip2 Goat antibody was custom made by GenScript Corporation (Piscataway, NJ) against the peptide sequence NPAGHGSKEVKGKTC. When available, commercial antibodies were used: the CDK4 antibody was purchased from Santa Cruz**.**

3.2.3 siRNA

For transfection with siRNA, VSMCs were trypsinized and plated at 40-50% confluence on collagen-coated substrate. After 4-6 h, cells were washed with serum-free OPTI-MEM (Invitrogen, Mountain View, CA), and incubated with siRNA + Oligofectamine complexes for 48 h. Cells were incubated in fresh serum free OPTI-MEM for an additional 2-4 days. A stealth siRNA against human and rat Poldip2 (siPoldip2, seq.1; annealed siRNA duplexes for Poldip2 seq. 1 were primer 1: 5’GCCCACAUAUAUCUCAGAGAUCUCA3’ and primer 2:

5’UGAGAUCUCUGAGAUAUAUGUGGGC3’) and a stealth control siRNA (siControl) of the corresponding GC content were purchased from Invitrogen (Mountain View, CA). A second siPoldip2 sequence (siPoldip2, seq. 2; annealed siRNA duplexes for Poldip2 seq. 2 were primer 1: 5’ACGUCUAUUGGUGGCGAUACUGUAU3’ and primer 2: 5’AUACAGUAUCGCCACCAAUAGACGU3’) was used in some experiments and gave similar results (Figure 3.12). Cells were transfected with a final Poldip2 siRNA concentration of 15 nmol/L. Nox4 siRNA (siNox4; 25 nmol/L; annealed siRNA duplexes for Nox4 were sense, 5’ACUGAGGUACAGCUGGAUGUU3’ and antisense, 5’CAUCCAGCUGUACCUCAGUUU3’) was used, as described previously, with the Allstars Negative Control (Qiagen, Valencia, CA) (Clempus et al., 2007).

3.2.4 Adenoviruses

The AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA) was used to prepare viruses with either no insert (AdGFP), antisense Nox1 (AdNox1AS), antisense Nox4 (AdNox4AS), or Myc-tagged Poldip2 (AdPoldip2). VSMCs were transduced with recombinant adenoviruses as described in section 2.2.5.

3.2.5 Immunoblotting

VSMCs were lysed in Hunter’s buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 0.1 mmol/L Na-orthovanadate, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, and protease inhibitors) for all experiments as

described in detail in section 2.2.7. Whole cell lysates were utilized for immunoblotting (IB) experiments. For IB analysis, proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with appropriate primary antibodies, as described in 2.2.7. Primary antibodies were used at the following dilutions for immunoblotting: 1) Nox4 at 1:2500, 2) p22phox at 1:2000, and 3) CDK4 at 1:1000. Band intensity was quantified by densitometry using ImageJ 1.38 software.

3.2.6 RNA Isolation and Quantitative RT-PCR

Total RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA), per the manufacturer's recommendations. Superscipt II (Invitrogen, Mountain View, CA) and random primers were used for reverse transcription. Message expression of poldip2 (primer sequences for rat: GTATGAGACGGGACAGCTATTTCTCCA and CTGACATAGTCCAAGCCTGGGATG), nox1, nox4, p22phox, 18S rRNA, were measured by amplification of rat VSMC cDNA using the LightCycler (Roche, Basel, Switzerland) real-time thermocycler and SYBR green dye. Specific rat poldip2, nox1, nox4, or p22phox primers were used to measure mRNA and normalized to 18S rRNA. Copy number was calculated by the instrument software from standard curves of genuine templates.

3.2.7 Immunocytochemistry and Confocal Microscopy

VSMCs were plated on collagen-coated glass coverslips (BD Biosciences, Bedford, MA) were transiently transfected with siPoldip2 for 4 days before labeling. Cells were rinsed, fixed, permeabilized, and quenched as described in

2.2.9. Cells were incubated for 1 h in blocking buffer (3-5% bovine serum albumin in PBS) prior to incubation with a 1:50 dilution of the Poldip2 antibody for 1 h, followed by incubation for 1 h with a secondary antibody conjugated to Rhodamine Red X (The Jackson Laboratory, Bar Harbor, Maine). Cells on coverslips were mounted onto glass slides in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and image acquisition was performed using a confocal microscope as described in 2.2.9. When comparing cells from different treatment groups, all image threshold settings of the confocal microscope remained constant.

3.2.8 Amplex Red Assay

Hydrogen peroxide measurements in intact cells were made by measuring the oxidation of Amplex Red (100 µmol/L, Sigma-Aldrich, St. Louis, MO) in the presence of horseradish peroxidase using the Amplex Red Assay Kit (Molecular Probes, Carslbad, CA), as described previously (Dikalov et al., 2008). Briefly, Amplex Red and horseradish peroxidase type II (0.1 U/mL) were added to the cellular samples. Fluorescence readings were made in triplicate in a 96-well plate at Ex/Em=530/580 nm using 100-µL samples of media. H_2O_2 production was calculated and normalized to cellular protein, measured by the Bradford Assay (Bio-Rad, Hercules, CA).

3.2.9 Lucigenin-Enhanced Chemiluminescence

NADPH oxidase activity in membrane fractions was assessed by measuring the reduction of 5 µmol/L Lucigenin (Sigma-Aldrich, St. Louis, MO) by $O_2^{\bullet}$- in the presence of NADPH (100 µmol/L, Sigma-Aldrich, St. Louis, MO) as

described previously. Briefly, cells were collected in cold HBSS and centrifuged at 1000 x g at 4°C for 10 minutes prior to resuspension in 200 μL of Hypotonic Lysis Buffer (20 mmol/L Phosphate Buffer (100 mmol/L EGTA, pH to 7.4) with 10 μg/mL Aprotinin, 0.5 μg/mL Leupeptin, and 0.5 mmol/L PMSF). Cells were sonicated on ice at a power of 4 watts for 10 seconds, followed by centrifugation at 28,000 x g at 4°C for 15 min to isolate the membrane fraction. Membrane fractions were resuspended in 60 μL of Assay Buffer (53 mmol/L Potassium Phosphate Buffer, sucrose, 100 mmol/L EGTA, pH to 7.4). Measurements were normalized to protein concentration, measured using the Bradford Assay.

3.2.10 Detection of Superoxide using DHE-HPLC

To evaluate intracellular production of $O_2^{\bullet}$-, we measured the conversion of 2-hydroxyethidium from DHE using high-performance liquid chromatography (HPLC). In some samples, polyethylene glycol-conjugated SOD (PEG-SOD; 50 U/mL, Sigma-Aldrich, St. Louis, MO) was added prior to the addition of dihydroethidium (DHE). Results are expressed as signal inhibited by PEG-SOD.

3.2.11 Detection of $O_2^{\bullet}$- and H_2O_2 using Electron Spin Resonance

ESR was used to measure $O_2^{\bullet}$- and H_2O_2 production in membrane fractions, as described previously (Clempus et al., 2007; Dikalov et al., 2008). Briefly, VSMCs were harvested and resuspended in PBS containing protease inhibitors. Cells were sonicated and the membrane pellet was resuspended in electron spin resonance buffer. Electron spin resonance spectroscopy using 1-hydroxy-3-carboxy-pyrrolidine (CPH spin trap) and 200 μmol/L NADPH was used for quantitative measurements of $O_2^{\bullet}$- and H_2O_2 production as described

previously. SOD (50 U/mL) was added directly to the sample to determine the SOD-inhibitable signal when $O_2^{\bullet-}$ was measured.

3.2.12 Fluorescence Activated Cell Sorting (FACS) Analysis

After the desired treatment, cells were trypsinized and washed with cold PBS prior to fixing with 70% ethanol. Fixed cells were washed again with cold PBS, pelleted at 2,000 xg for 10 minutes, and resuspended to a final cell density of approximately 1 x 10^8 cells/mL. Cells were then incubated for 1 hour with primary antibody and, if necessary, stained for 30 minutes with Propidium Iodide (0.1mg/mL; Sigma-Aldrich, St. Louis, MO) in 1X PBS containing 0.6% Triton-X and 2 mg/mL RNase (Sigma-Aldrich, St. Louis, MO). Cells were filtered through wire mesh caps and into fresh Falcon tubes to remove cell clumps and were then kept at 4°C in the dark until FACS analysis was performed. For each sample, 10,000-20,000 cells were counted using a Becton Dickinson FACSort analyzing cytometer. Raw cell count data was collected and analyzed using FlowJo: Flow Cytometry Analysis Software (Tree Star, Inc., Ashland, OR).

3.2.13 Statistical Analysis

Results are expressed as mean ± S.E.M. from at least three independent experiments. Significance of statistical comparisons was assessed using analysis of variance (ANOVA), followed by Bonferroni's Multiple Comparison post-hoc test. A value of $p<0.05$ was considered significant.

3.3 Experimental Results

3.3.1 Poldip2 Overexpression Increases Basal Oxidase Activity in Vascular Smooth Muscle Cells

To determine if Poldip2 regulates oxidase function in VSMCs, we used lucigenin-enhanced chemiluminescense to measure NADPH oxidase activity in membrane fractions of VSMCs transduced with AdPoldip2. The overexpression of Poldip2 alone caused a significant increase in basal oxidase activity in a dose-dependent manner when compared to VSMCs transduced with control adenovirus (AdGFP) (Figure 3.1). This increase in activity was not caused by an increase in nox1, nox4, or p22phox mRNA expression (Figure 3.2, a), nor by an increase in Nox4 or p22phox protein levels (Figure 3.2, b). Because the structure of Poldip2 does not predict intrinsic oxidase activity, we tested whether this increase in NADPH-dependent $O_2^{\bullet}$- production results from activation of either of the VSMC Nox catalytic subunits expressed in VSMCs.

3.3.2 Poldip2 Stimulates ROS Production by Nox4, but Not by Nox1

3.3.2.1 The Increase in ROS Production by Poldip2 Occurs via Nox4 and in a p22phox-Dependent Manner

We have previously shown that basal ROS production in VSMCs is due to Nox4 activity; therefore, we set out to determine if reduction of Nox4 by expression of antisense Nox4 (AdNox4AS) (Figure 3.3) prior to the overexpression of Poldip2 abolishes the Poldip2-mediated increases in Nox activity in the absence of agonist stimulation. We found that the knockdown of Nox4 prevented the increase in oxidase activity caused by the overexpression of Poldip2 in VSMCs (Figure 3.4), suggesting that Nox4 mediates the effects of Poldip2. Additionally, we tested if activation of Nox4 by overexpression of Poldip2 mediates a detectable increase in ROS production within the cell.

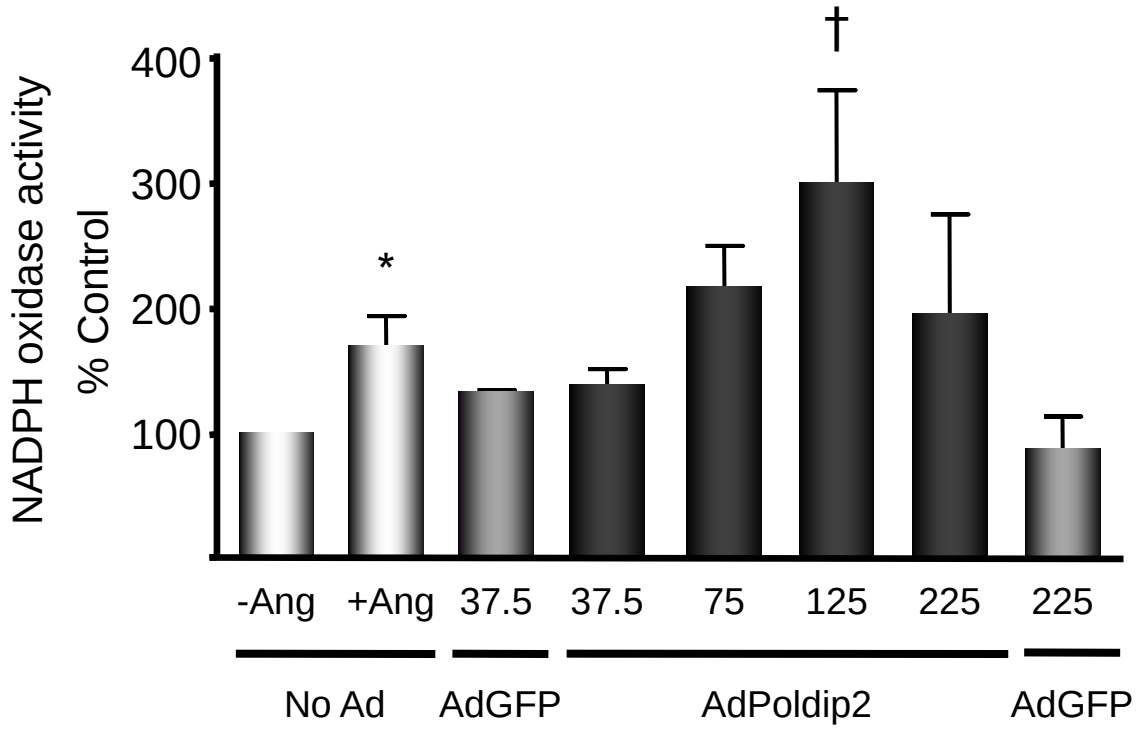


Figure 3.1. Overexpression of Poldip2 Increases Basal Oxidase Activity in a Dose-dependent Manner. Rat VSMCs were transduced with no adenovirus (No Ad), 37.5 or 225 µL/dish of AdGFP, or 37.5 µL/dish to 225 µL/dish of AdPoldip2. Lucigenin enhanced chemiluminescence was used to measure NADPH oxidase activity in membrane fractions. Bars are mean ± S.E.M. of 3-4 independent experiments. †$p<0.05$ vs. AdGFP. Positive control: VSMCs stimulated with 100 nmol/L Ang II for 4 hours. *$p<0.05$ vs. No Ad -Ang.

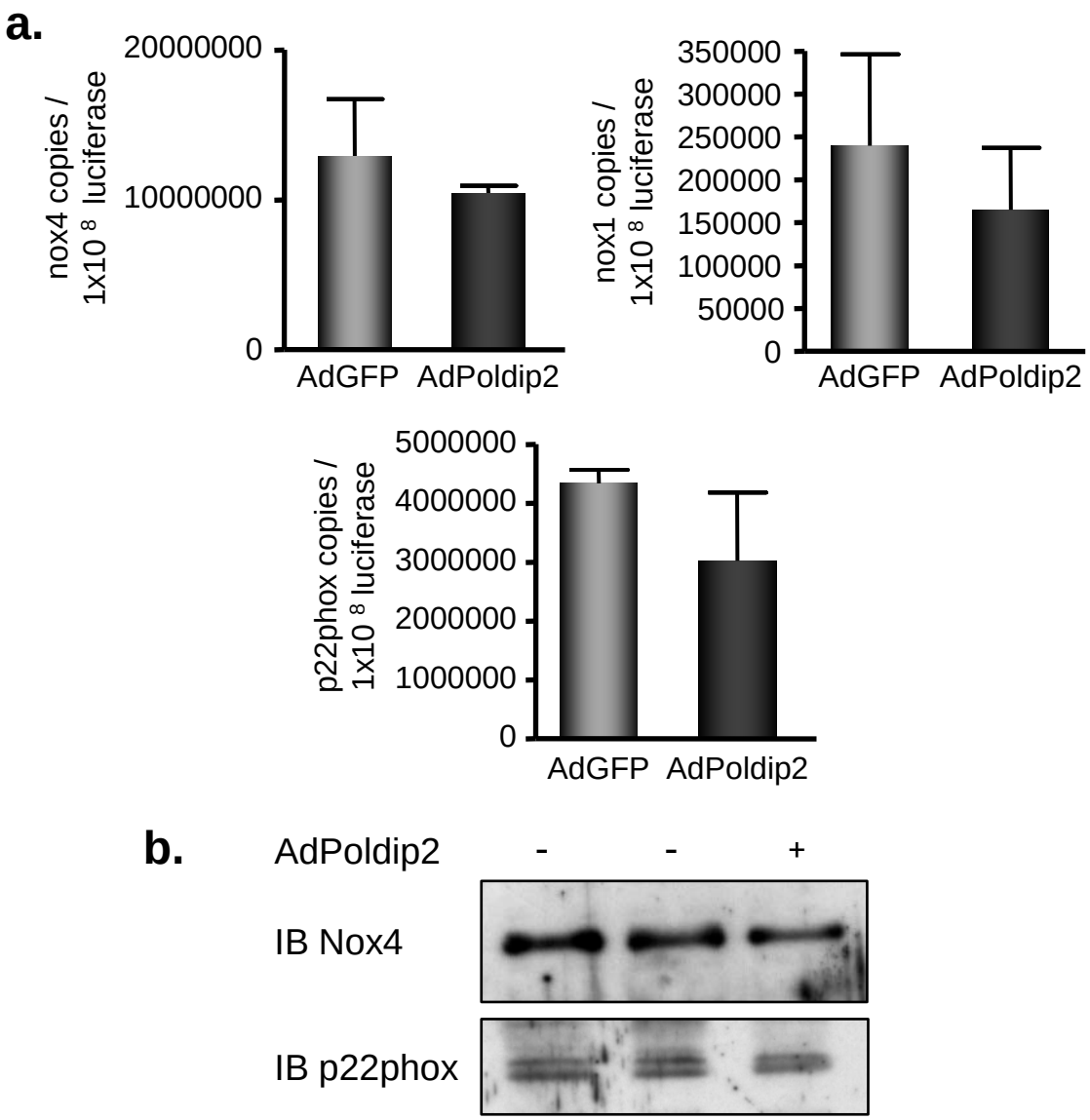


Figure 3.2. The Increase in Basal NADPH Oxidase Activity is Not Due to Increased Oxidase Subunit Expression. a, Quantitative real-time PCR of nox4, nox1, and p22phox mRNA expression in rat VSMCs transduced for 72 hours with control adenovirus (AdGFP) or adenovirus to overexpress Poldip2 (AdPoldip2); mRNA levels are normalized to luciferase. Bars are mean $\pm$ S.E.M of 3 independent experiments. No statistical difference was found. **b,** Western analysis of rat VSMCs transduced with either no adenovirus (- , left), control adenovirus (- , AdGFP, middle), or adenovirus to overexpress Poldip2 (+ , AdPoldip2) for 72 hours prior to immunoblotting (IB) for Nox4 or p22phox.

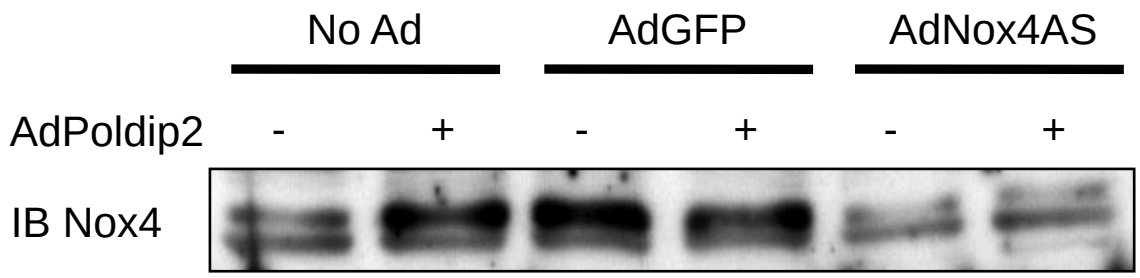


Figure 3.3. Nox4 Antisense Decreases Nox4 Protein Levels. Western analysis of rat VSMCs transduced with either no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to express antisense Nox4 (AdNox4AS) for 48 h prior to transducing cells with either control adenovirus (- , AdGFP) or adenovirus to overexpress Myc-tagged Poldip2 (+, AdPoldip2) for an additional 72 h to verify the knockdown of Nox4 by AdNox4AS.

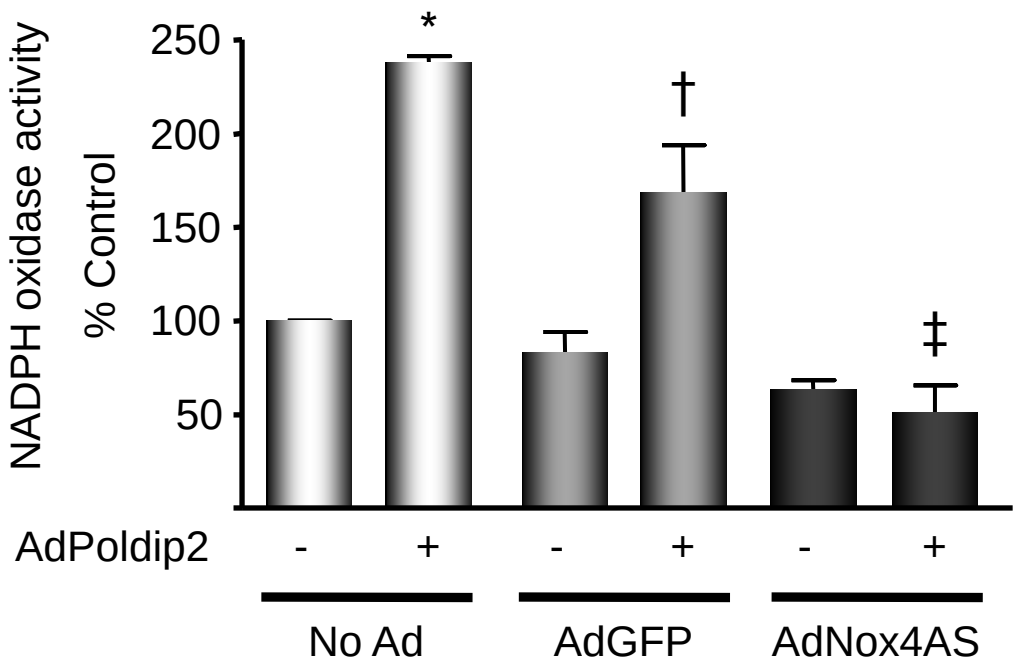


Figure 3.4. Overexpression of Poldip2 Increases Basal Oxidase Activity, which is Blocked by Nox4 Antisense, as Measured by Lucigenin Enhanced Chemiluminescence. Rat VSMCs were transduced with either no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to express antisense Nox4 (AdNox4AS) for 48 h prior to transducing cells with either control adenovirus (- , AdGFP) or adenovirus to overexpress Myc-tagged Poldip2 (+, AdPoldip2) for an additional 72 h before using lucigenin enhanced chemiluminescence to measure NADPH oxidase activity. Bars are mean ± S.E.M. of 4 independent experiments. *$p<0.001$ vs. No Ad – AdPoldip2; †$p<0.01$ vs. AdGFP – AdPoldip2; ‡$p<0.001$ vs. AdGFP + AdPoldip2.

VSMCs transduced with AdNox4AS prior to overexpression of Poldip2 were used for: 1) DHE-HPLC to assess $O_2^{\bullet}$- production and 2) the Amplex Red Assay to assess H_2O_2 production. The overexpression of Poldip2 in VSMCs caused a significant increase in both $O_2^{\bullet}$- (Figure 3.5) and H_2O_2 (Figure 3.6) production and this increase was significantly blunted in VSMCs in which Nox4 expression was knocked down (Figures 3.5 and 3.6). Of interest, Poldip2 was unable to significantly increase H_2O_2 production, as measured by ESR, in VSMCs stably transfected with antisense p22phox and thus lacking p22phox expression (Figure 3.7), suggesting that Poldip2 requires p22phox to increase basal ROS production and Nox4 enzymatic activity. To further verify that the effects on oxidase activity and ROS production mediated by Poldip2 overexpression are through modulation of Nox4 activity, we tested if the effects of Poldip2 overexpression were maintained in VSMCs lacking Nox1 expression, which thereby only express Nox4. Poldip2 overexpression significantly increases ROS production by ~2.5 fold ($p<0.05$) in both Nox1 wild-type ($nox1^{y/+}$; 2.65±0.2 fold increase) and Nox1 knockout ($nox1^{y/-}$; 2.56±0.2 fold increase) cells, as measured by ESR, suggesting that Nox1 is not directly involved in Poldip2-mediated ROS production (Figure 3.10 a, b) and, thus, verifying that the effects of Poldip2 are mediated by Nox4. Taken together, these data strongly indicate that Poldip2 positively regulates Nox4 activity and Nox4-dependent increases in ROS production in VSMCs.

3.3.2.2 Poldip2 Does Not Stimulate Nox1-Dependent ROS Production in Vascular Smooth Muscle Cells

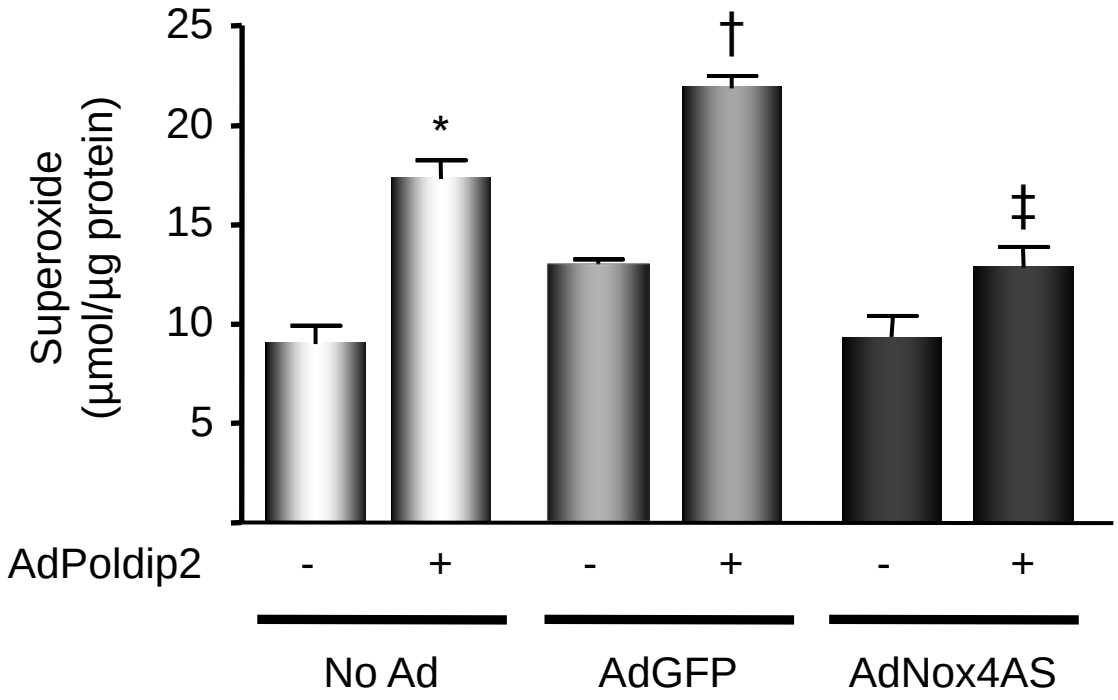


Figure 3.5. Overexpression of Poldip2 Increases Superoxide Production, which is Blocked by Nox4 Antisense, as Measured by DHE-HPLC. Rat VSMCs transduced with either no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to express antisense Nox4 (AdNox4AS) for 48 h prior to transducing cells with either control adenovirus (- , AdGFP) or adenovirus to overexpress Myc-tagged Poldip2 (+ , AdPoldip2) for an additional 72 h prior to using DHE-HPLC to measure $O_2^{\bullet-}$ production. Bars are mean ± S.E.M. of 4 independent experiments. *p<0.001 vs. No Ad – AdPoldip2; †p<0.001 vs. AdGFP – AdPoldip2; ‡p<0.001 vs. AdGFP + AdPoldip2.

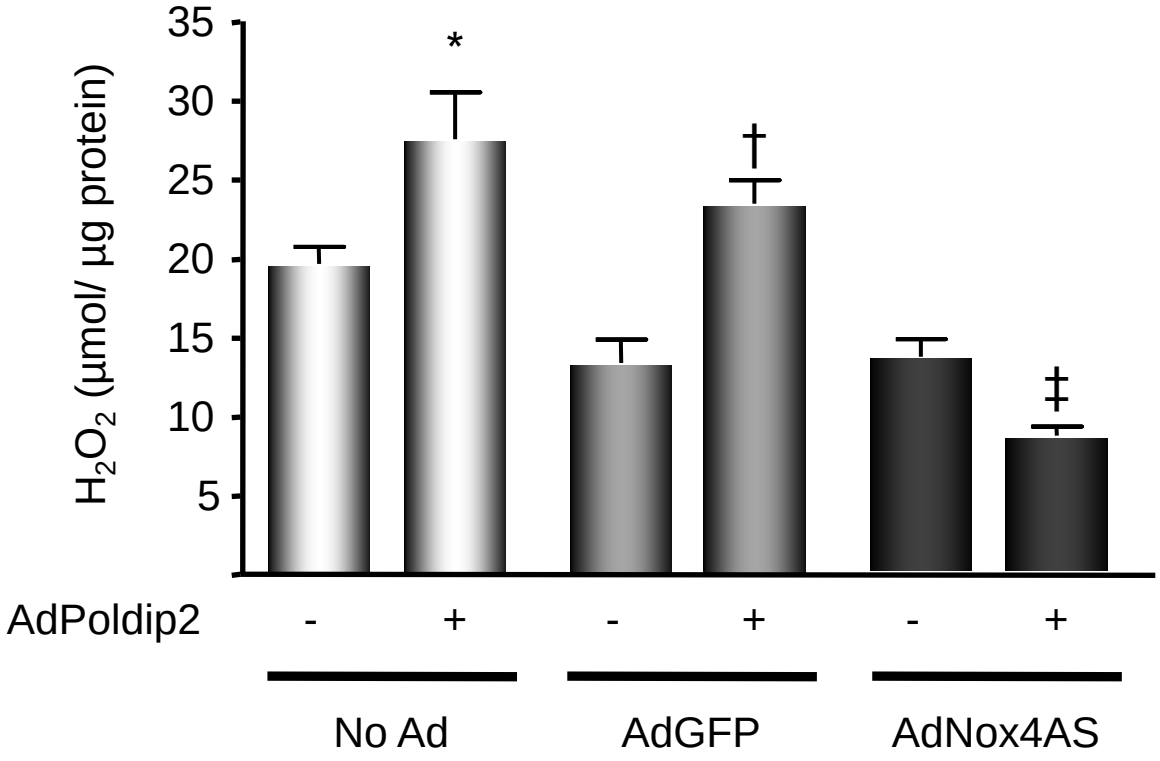


Figure 3.6. Overexpression of Poldip2 Increases Basal Hydrogen Peroxide Production, which is Blocked by Nox4 Antisense, as Measured by Amplex Red Assay. Rat VSMCs transduced with either no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to express antisense Nox4 (AdNox4AS) for 48 h prior to transducing cells with either control adenovirus (- , AdGFP) or adenovirus to overexpress Myc-tagged Poldip2 (+ , AdPoldip2) for an additional 72 h prior to using the Amplex Red Assay to measure H_2O_2 production. Bars are mean ± S.E.M. of 4 independent experiments. *$p<0.05$ vs. No Ad – AdPoldip2; †$p<0.01$ vs. AdGFP – AdPoldip2; ‡ $p<0.001$ vs. AdGFP + AdPoldip2.

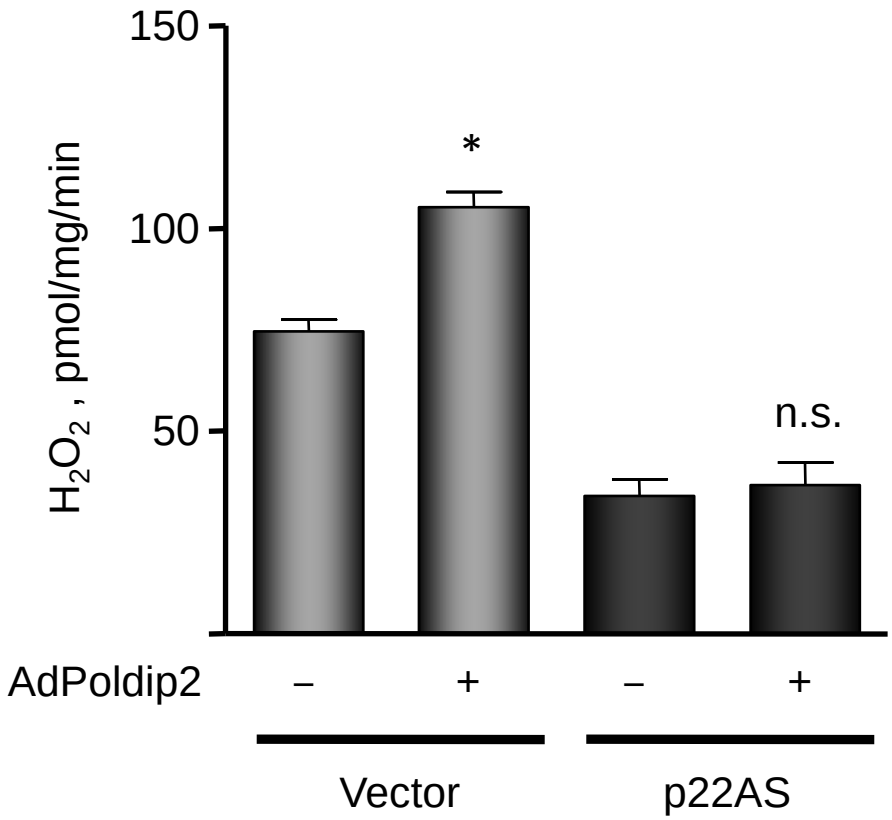


Figure 3.7. Nox4-Dependent ROS Production Mediated by Poldip2 Overexpression Requires the Presence of p22phox, as Demonstrated in p22phox Antisense Cells . Rat VSMCs stably transfected with empty vector (Vector) or antisense p22phox (p22AS) were transduced for 72 h with AdPoldip2 (+) or AdGFP (-) prior to using ESR to detect H_2O_2 levels. Bars are mean $\pm$ S.E.M. of 3 independent experiments. *$p<0.01$ vs. Vector – AdPoldip2, not significant (n.s.) vs. p22AS – AdPoldip2.

As shown in Figure 2.12, Poldip2 is capable of association with HA-tagged Nox1 and this association decreases in response to Ang II stimulation, suggesting that Poldip2 associates with the inactive Nox1 complex. Based on this observation, we set out to determine if reduction of Nox1 by expression of antisense Nox1 (AdNox1AS) prior to the overexpression of Poldip2 abolishes the Poldip2-mediated increases in Nox activity. In contrast to the results obtained with antisense Nox4, when Nox1 is depleted using antisense Nox1, the increase in H_2O_2 production caused by Poldip2 overexpression is potentiated, rather than inhibited (Figure 3.8). Interestingly, increases in ROS production by Nox1 oxidase activity in VSMCs stimulated by 4 hours of 100 nmol/L Ang II is blocked in cells overexpressing Poldip2 (Figure 3.9). These data suggest that Poldip2 is not a positive regulator of Nox1, but may potentially serve as a negative regulator of Nox1 activity in VSMCs.

3.3.3 siPoldip2 Significantly Decreases Poldip2 Levels, Decreases Basal NADPH Oxidase Activity, and Changes Cell Phenotype

3.3.3.1 Characterization of siPoldip2

The experiments performed in which Poldip2 was overexpressed, clearly established the ability of Poldip2 to enhance Nox4 enzymatic activity and Nox4-dependent ROS production in VSMCs, but do not indicate whether Poldip2 is required for Nox4 activation. We have previously shown that basal ROS production in VSMCs is due to Nox4 activity; therefore, we sought to determine the effects of Poldip2 knockdown on basal $O_2^{\bullet}$- and H_2O_2 production in VSMCs transfected with siRNA to deplete Poldip2 (siPoldip2). To do this, we first

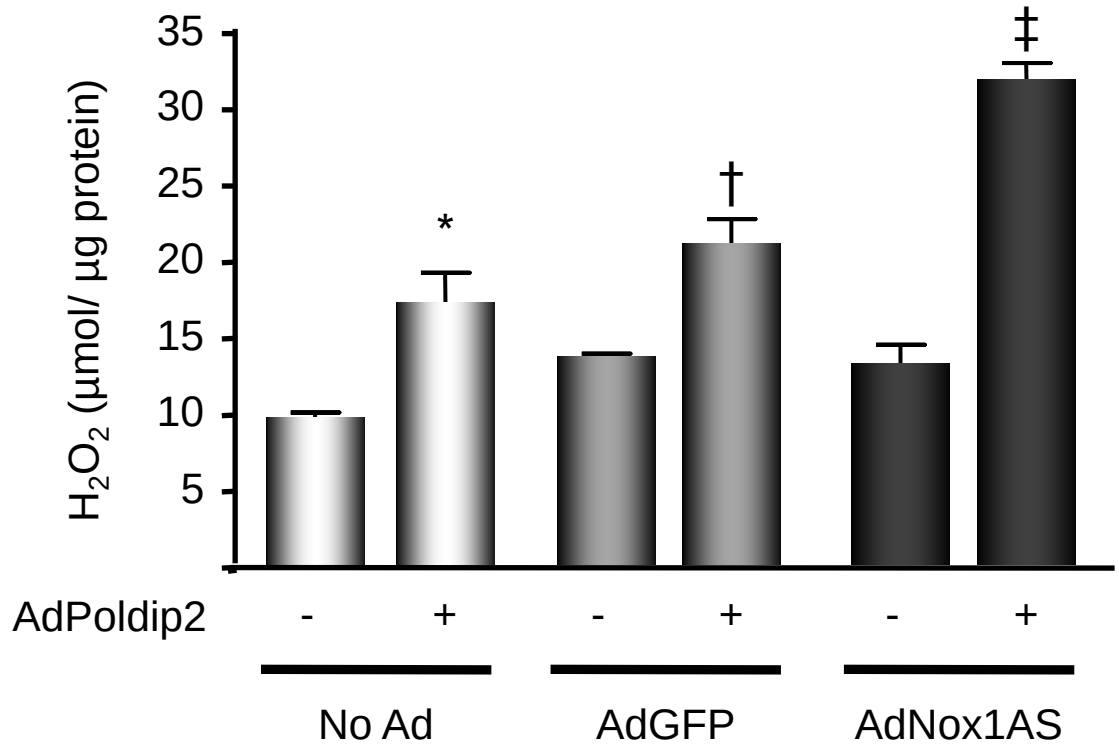


Figure 3.8. Overexpression of Poldip2 Increases Basal Hydrogen Peroxide Production, which is Not Blocked by Nox1 Antisense, as Measured by Amplex Red Assay. Rat VSMCs were transduced with either no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to express antisense Nox1 (AdNox1AS) for 48 h prior to transducing cells with either control adenovirus (- , AdGFP) or adenovirus to overexpress Myc-tagged Poldip2 (+ , AdPoldip2) for an additional 72 h prior to using the Amplex Red Assay to measure H_2O_2 production. Bars are mean ± S.E.M. of 4 independent experiments. *$p<0.05$ vs. No Ad – AdPoldip2; †$p<0.05$ vs. AdGFP – AdPoldip2; ‡$p<0.001$ vs. AdGFP + AdPoldip2.

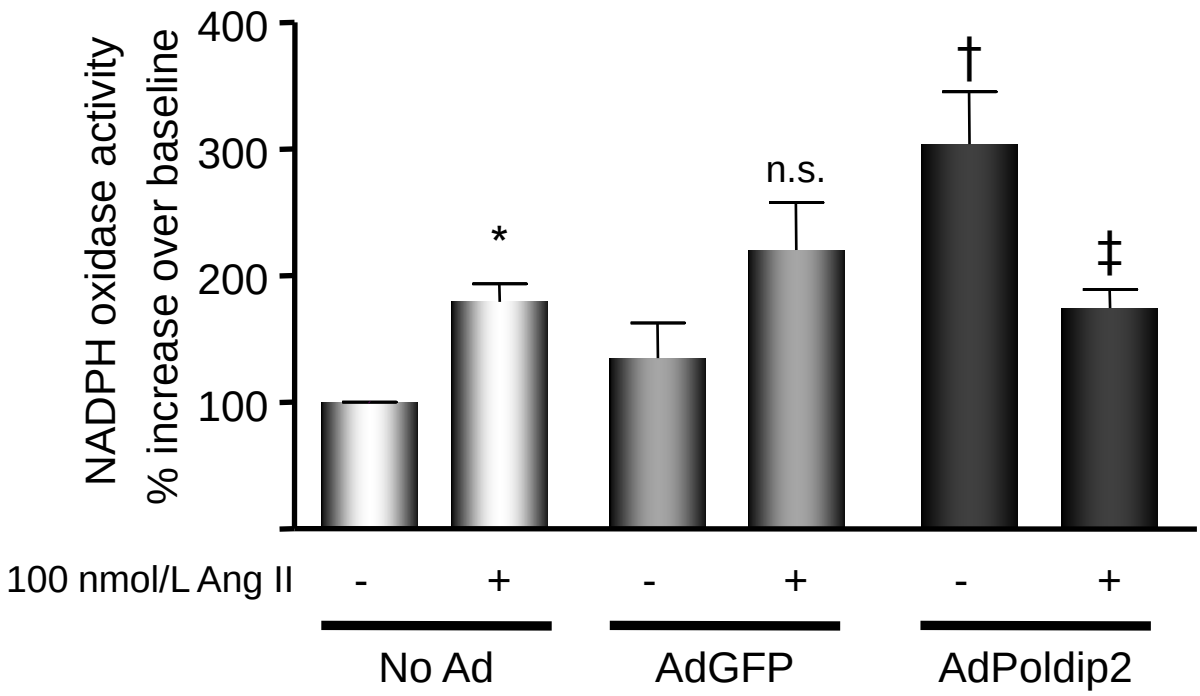


Figure 3.9. Overexpression of Poldip2 Does Not Increase ROS Production by Nox1 in Response to Ang II Stimulation of VSMCs. Rat VSMCs were transduced with no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 h prior to no stimulation or stimulation for 4 hours with 100 nmol/L of angiotensin II (Ang II). Lucigenin enhanced chemiluminescence was then used to measure NADPH oxidase activity. Bars are mean ± S.E.M. of 4-6 independent experiments. *$p<0.01$ vs. No Ad – Ang II; not significant (n.s.) vs. AdGFP – Ang II; †$p<0.01$ vs. AdGFP – Ang II; ‡ $p< 0.05$ vs. AdPoldip2 – Ang II.

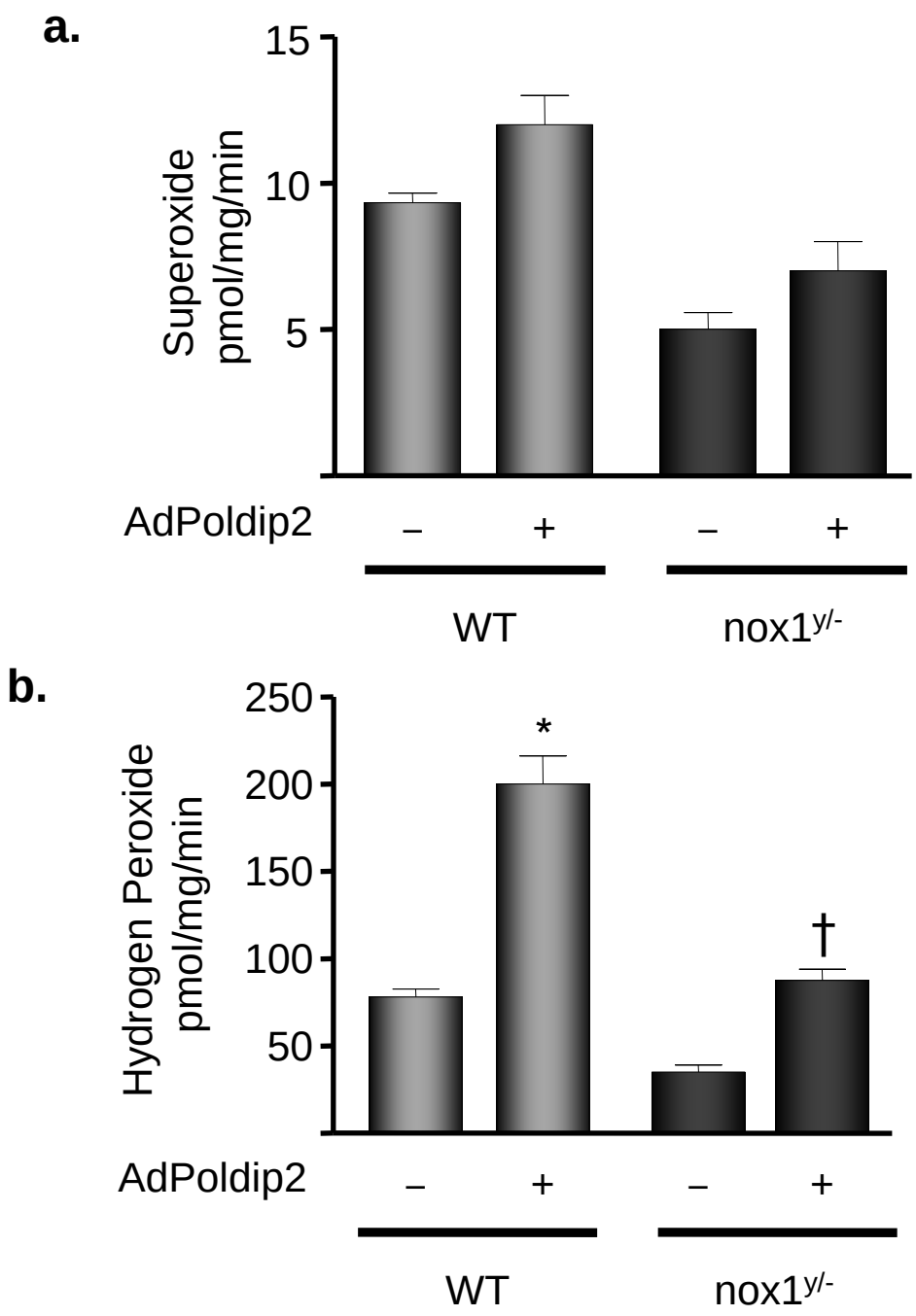


Figure 3.10. Overexpression of Poldip2 Increases Nox4-Mediated ROS Production in nox1$^{y/-}$ VSMCs, as Measured by ESR. Mouse nox1 wild-type (WT) or mouse nox1 knockout (nox1$^{y/-}$) VSMCs were transduced for 72 h with AdGFP (-) or AdPoldip2 (+) prior to using ESR to detect **a,** $O_2^{\bullet}$- and **b,** H_2O_2 levels. Bars are mean ± S.E.M. of 3 independent experiments. No statistical difference was detected in $O_2^{\bullet}$- measurements. * $p<0.001$ vs. WT – AdPoldip2; † $p<0.05$ vs. nox1$^{y/-}$ – AdPoldip2.

generated siRNAs against endogenous Poldip2. As shown, siPoldip2 significantly decreases Poldip2 mRNA (Figure 3.11) and protein levels, as measured by western blot analysis (Figure 3.12) and immunocytochemistry (Figure 3.13). Of note, we used two different siRNA sequences to ensure specificity (Figure 3.12), both of which promote significant knockdown of Poldip2.

3.3.3.2 Knockdown of Poldip2 Decreases Basal NADPH Oxidase Activity in Vascular Smooth Muscle Cells and Alters Cell Phenotype

VSMCs in which endogenous Poldip2 was knocked down using siRNA showed significant decreases in basal $O_2^{\bullet}$- and H_2O_2 production (Figure 3.14) compared to control siRNA (siControl). These data suggest that Poldip2 is required for basal Nox4 activity and ROS production in VSMCs. Interestingly, during the initial process of testing and characterizing siPoldip2, we observed distinct changes in VSMC morphology after siPoldip2 treatment. Cells became elongated, spindly, and seemed to have fewer points of contact with the dish, reminiscent of the phenotype observed in siNox4 treated cells, as shown in Figure 3.15. Importantly, the changes in VSMC phenotype mediated by siPoldip2 are not attributable to an increase in apoptosis, as measured by the lack of increase in anti-phosphatidylserine, an early marker of cellular apoptosis, compared to untreated cells and cells treated with siControl (Figure 3.16).

3.4 Discussion

The functional studies performed in this chapter suggest that Nox4 does, in fact, require a novel cytosolic subunit for proper enzymatic activity and ROS production and suggest that Nox4 may not function only as a constitutively active

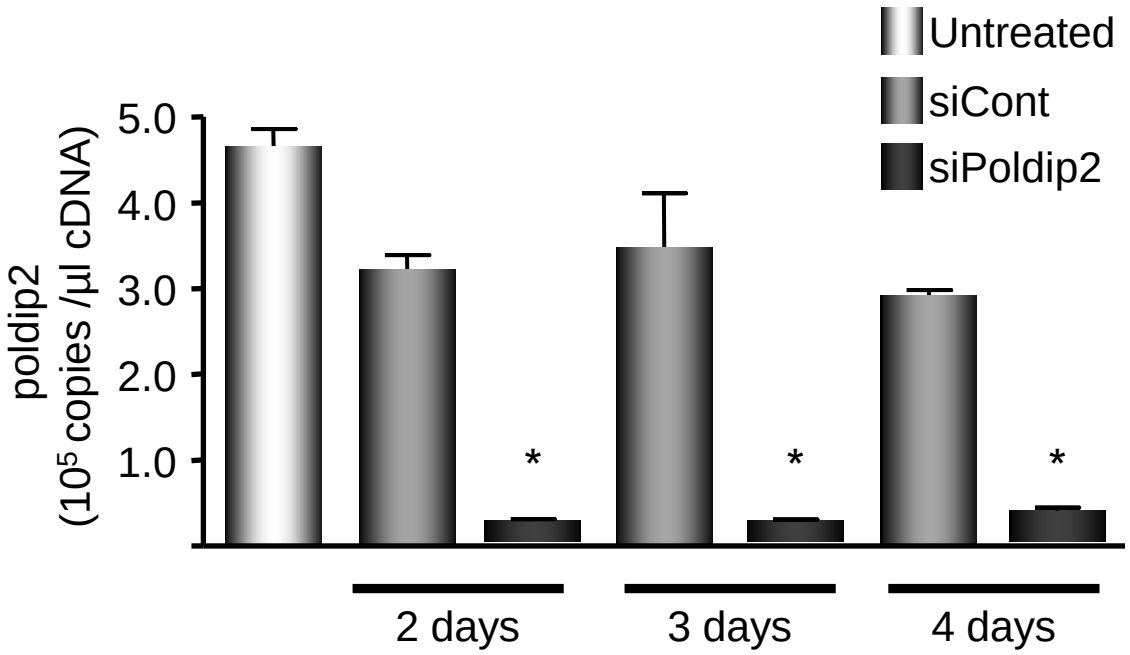


Figure 3.11. siPoldip2 Decreases Endogenous Poldip2 mRNA Expression. Quantitative real-time PCR of poldip2 mRNA expression in rat VSMCs transfected with control siRNA (siCont) or siRNA against Poldip2 (siPoldip2) for the indicated times. Bars are mean ± S.E.M. of 3 independent experiments. *p<0.001 vs. siCont.

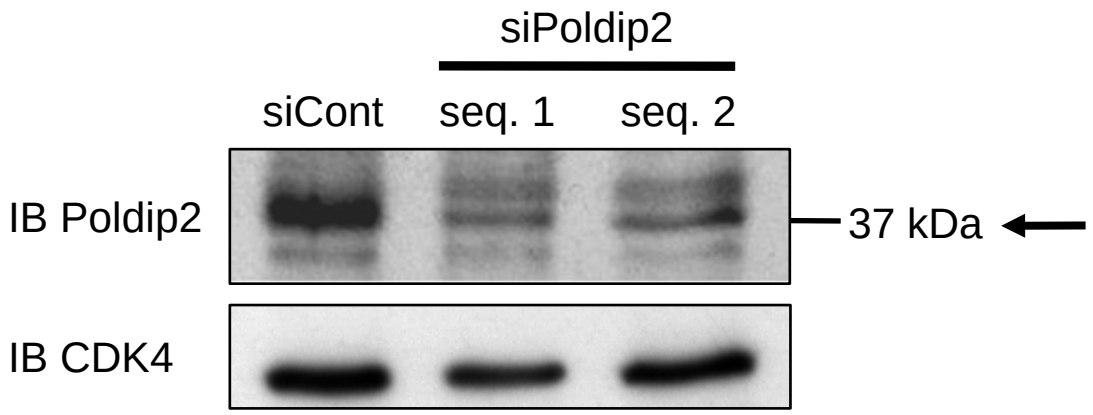


Figure 3.12. siPoldip2 Decreases Endogenous Poldip2 Protein Expression, as Shown by Western Blot Analysis. Western analysis of Poldip2 protein expression in extracts from rat VSMCs transiently transfected for 4 days with 15 nmol/L of either control siRNA (siCont), siRNA against Poldip2 (siPoldip2) sequence 1 (seq.1), or siPoldip2 sequence 2 (seq. 2). CDK4 levels (lower blot) were included as a loading control. siPoldip2 sequence 1 was used for all siPoldip2 experiments shown herein, unless otherwise indicated.

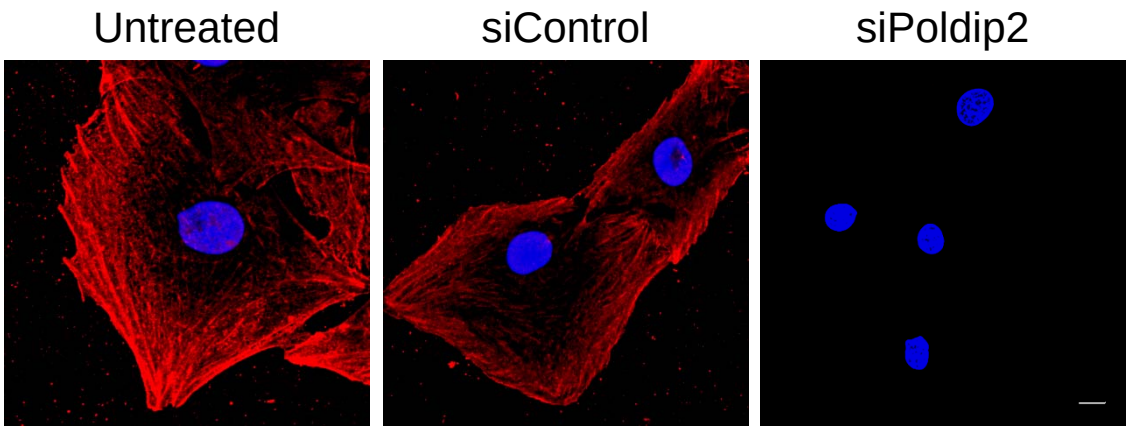


Figure 3.13. siPoldip2 Decreases Endogenous Poldip2 Protein Expression, as Shown by Immunocytochemistry. Rat VSMCs were either untreated or transiently transfected for 4 days with 15 nmol/L of either siControl or siPoldip2 before single labeling with an anti-Poldip2 antibody (red). Immunocytochemistry images were acquired using confocal microscopy. Images acquired at the focal adhesion plane are depicted. Scale bars, 10 µm.

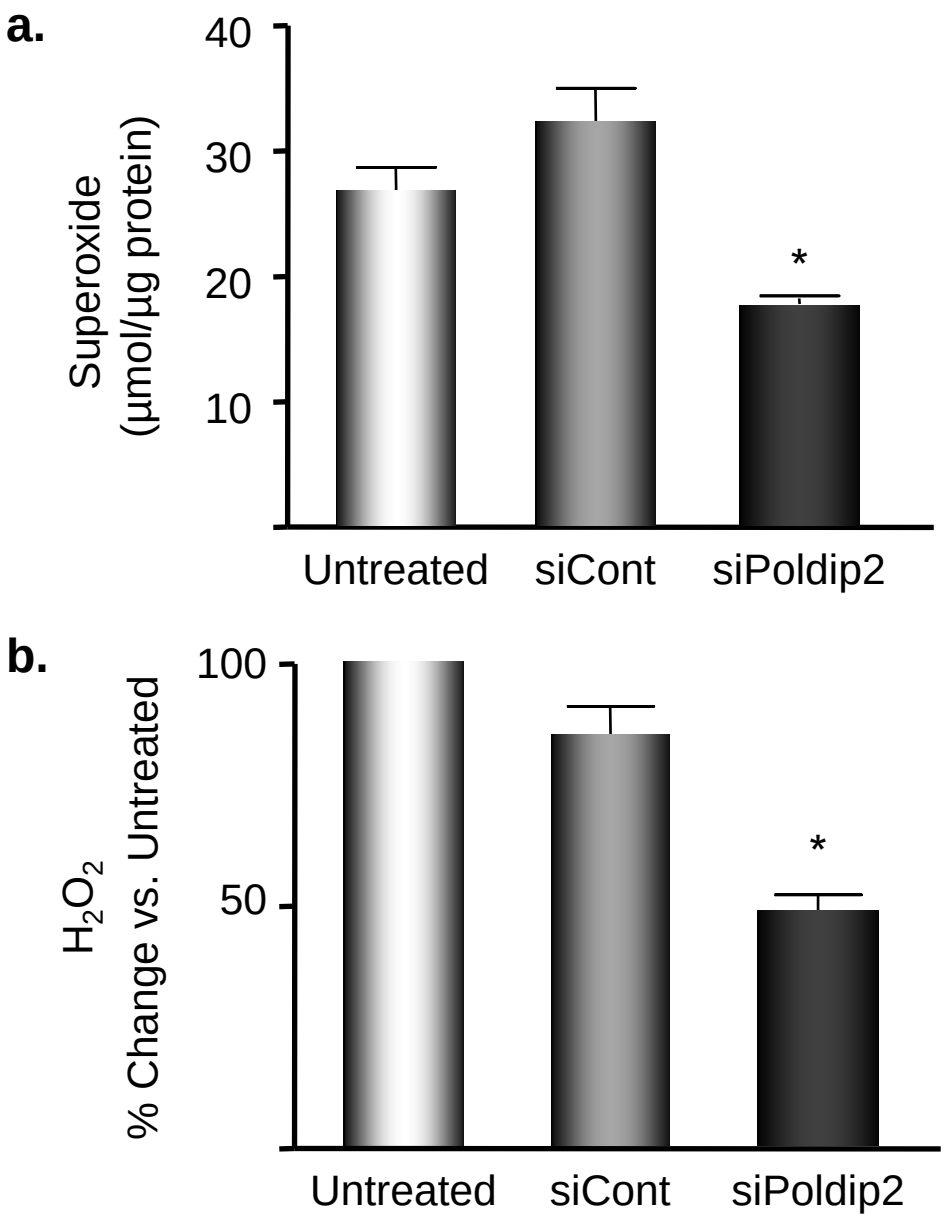


Figure 3.14. siPoldip2 Decreases Basal Superoxide Production, as Measured by DHE-HPLC, and Basal Hydrogen Peroxide Production, as Measured by Amplex Red Assay. **a,** DHE-HPLC was used to assess intracellular $O_2^{\bullet}$- production in rat VSMCs that were either untreated or transiently transfected for 4 days with 15 nmol/L of either siControl or siPoldip2. Bars are mean ± S.E.M. of 4 independent experiments. *p<0.01 vs. siCont. **b,** Amplex Red Assay was used to assess H_2O_2 production in rat VSMCs that were either untreated or transiently transfected for 4 days with 15 nmol/L of either siControl or siPoldip2 VSMCs. Bars are means ± S.E.M. of 4 independent experiments. *p<0.001 vs. siCont.

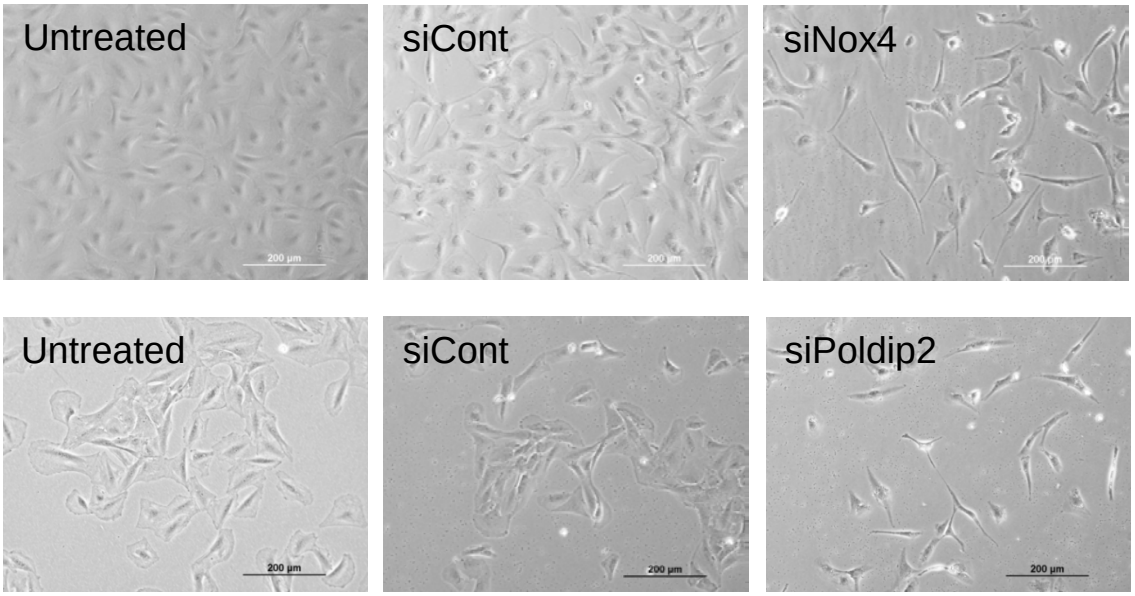


Figure 3.15. siPoldip2 Changes VSMC Phenotype, Similarly to siNox4.

Rat VSMCs were either untreated or transiently transfected for 5 days with 25 nmol/L of siControl (siCont) or siNox4 (*upper*) for maximal Nox4 downregulation, or for 4 days with 15 nmol/L of siControl (siCont) or siPoldip2 (*lower*) for maximal Poldip2 downregulation and visualized by phase contrast microscopy. Scale bars, 200 µm.

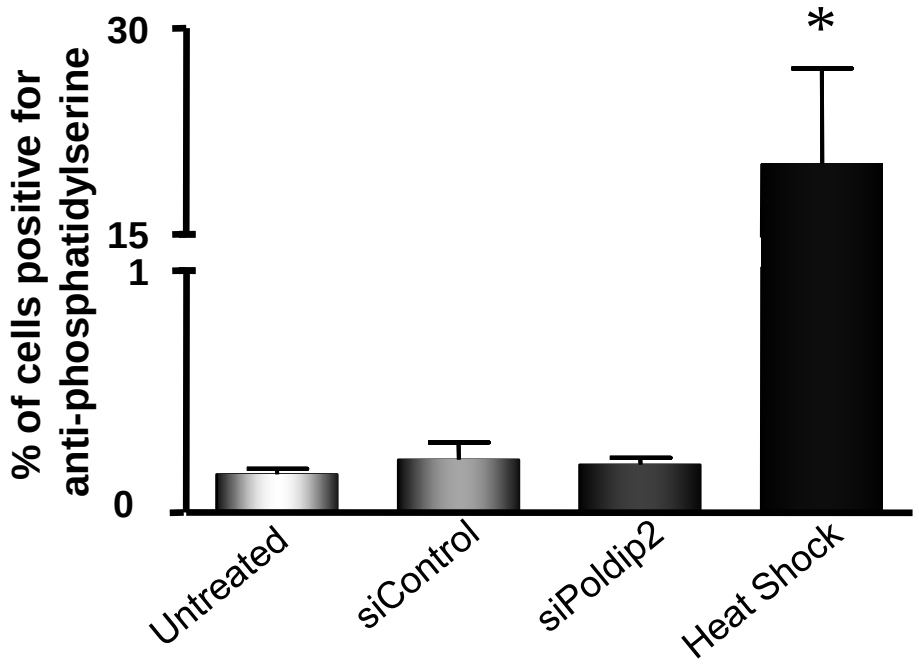


Figure 3.16. Changes in Cell Phenotype Mediated by siPoldip2 are Not Due to an Increase in Apoptosis. Rat VSMCs that were either untreated or transiently transfected for 4 days with 15 nmol/L of either siControl or siPoldip2 were stained with propidium iodide and anti-Phosphatidylserine, a marker of early apoptosis, prior to assessment of cell surface expression of Phosphatidylserine by fluorescence activated cell sorting (FACS) analysis. Untreated rat VSMCs heat shocked at 50˚C for 1 h prior to Phosphatidylserine staining were included as a positive control. Bars are mean ± S.E.M. of 3 independent experiments. No statistical difference was found. *$p<0.05$ vs. all other treatments.

enzyme, as previously reported. We provide evidence that Poldip2 functions to positively modulate Nox4 enzymatic activity. Poldip2 overexpression significantly increases both basal oxidase activity (Figure 3.1) and ROS production (Figures 3.5 and 3.6), and the ability of Nox4 antisense to block Poldip2-induced increases in NADPH oxidase activity and ROS production (Figures 3.4, 3.5, and 3.6) indicates that Poldip2/Nox4-mediated ROS generation is responsible for these changes. In contrast to what was reported by von Lohneysen, et al. (von Lohneysen et al., 2008), our studies indicate that a portion of the C-terminal region of p22phox, specifically aa 106-129, is required for Poldip2 association with Nox4. Our data support that this 24 amino acid domain (aa 106-129) serves as the binding site on p22phox for Poldip2. Evidence from functional studies performed in p22phox antisense VSMCs, in which Poldip2's effects on Nox4-dependent ROS production were significantly impaired, strongly suggests that Poldip2 modulates Nox4 activity in a p22phox-dependent manner (Figure 3.7). Furthermore, overexpression of Poldip2 in nox1$^{y/-}$ VSMCs, which only express Nox4, has the same effect on ROS production as it does in wild-type VSMCs (Figure 3.10), suggesting that Poldip2 exerts its effects on ROS and NADPH oxidase activity specifically through Nox4/p22phox.

While overexpression studies clearly indicate that Poldip2 enhances Nox4-dependent ROS production and activity, it was unclear if Poldip2 is required for the basal activity and ROS production by Nox4 in VSMCs. To test this hypothesis, siRNA against Poldip2 was generated and characterized to verify Poldip2 knockdown (Figures 3.11, 3.12, and 3.13). VSMCs lacking Poldip2

expression showed a significant decrease in basal ROS production (Figure 3.14), suggesting that Poldip2 is required for Nox4 basal oxidase activity. Interestingly, VSMCs lacking Nox4 or its newly found regulatory protein, Poldip2, undergo distinct phenotypic changes in which the cells become elongated, spindly, and seem to have fewer points of contact with the dish (Figure 3.15). This phenotypic change will be explored further in Chapter 4. Altogether, these data support the conclusion that Poldip2 is a novel positive modulator of Nox4 enzymatic activity.

It should be noted that when we increased Poldip2 protein levels by overexpression or decreased Poldip2 mRNA and protein levels by siRNA, we were able to detect measurable changes in Nox4-dependent $O_2^{\bullet}$- and H_2O_2 by multiple methods including the Lucigenin Assay, using DHE-HPLC, ESR, and the Amplex Red Assay. Recently, investigators working in reconstituted systems have suggested that H_2O_2, and not $O_2^{\bullet}$-, is the principal product generated by the Nox4 enzyme (Dikalov et al., 2008; Martyn et al., 2006). Some of the experiments performed by Martyn, et al. (Martyn et al., 2006) measured $O_2^{\bullet}$- production in intact cells, which would limit any detectable $O_2^{\bullet}$- to that generated extracellularly, since $O_2^{\bullet}$- is a charged molecule and does not freely traverse cell membranes. Many cell types, including VSMCs, are believed to express Nox enzymes in intracellular membrane structures, such as signaling endosomes (Miller et al., 2007); therefore, the $O_2^{\bullet}$- generated by these enzymes would primarily be intracellular, in contrast to phagocytic cells, which do generate extracellular $O_2^{\bullet}$-. Alternatively, it is proposed that the $O_2^{\bullet}$- produced by Nox4 may be so rapidly converted to H_2O_2 that the release of $O_2^{\bullet}$- from this enzyme is

almost undetectable (Dikalov et al., 2008; Serrander et al., 2007). While the primary form of ROS generated by Nox4 remains controversial, our data support the idea that the Nox4/Poldip2 complex generates $O_2^{\bullet}$-, which is then rapidly converted to H_2O_2.

As mentioned previously, recent studies suggested that the principal mechanism of Nox4 regulation may be at the mRNA level, rather than by assembly of an enzymatic complex, like other Noxes (Serrander et al., 2007). While it remains to be determined exactly how Poldip2 functions to regulate Nox4 oxidase activity, as there are multiple possible levels of Nox4 regulation, we did confirm that altering Poldip2 mRNA and protein levels does not alter Nox4 mRNA or protein levels (Figure 3.2 and Figure 3.3). Potentially, Poldip2 could modulate Nox4 include through post-translational modification, regulation of proper protein maturation, coordination of appropriate subcellular localization, or modulation of enzymatic activity. This latter possibility is supported by the experiments described herein (Figures 3.1, 3.4 – 3.6, 3.10, and 3.14), but may not be the only, or even principal, mechanism of regulation. Data presented in Chapter 6 support a role for Poldip2 in determining the subcellular localization of Nox4, but the other possibilities remain to be investigated.

While our functional studies indicate that Poldip2 does not positively influence Nox1 activity, they do suggest that Poldip2 may serve to negatively modulate Nox1 enzymatic activity in VSMCs (Figure 3.8 and Figure 3.9), as shown by the ability of Poldip2 overexpression to significantly blunt the increase in Nox1 activity normally mediated by 4h of 100 nmol/L Ang II treatment, and by

the increase in ROS production in Poldip2-transfected cells treated with nox1 antisense. However, how Poldip2 functions to modulate Nox1 activity and influences Nox1-specific signaling requires further investigation. The potentiation of ROS production caused by Poldip2 overexpression after Nox1 depletion with antisense Nox1 (Figure 3.8) may occur because the acute absence of Nox1 may increase the amount of p22phox available to stabilize Nox4, or may release a pool of Poldip2 that is then free to interact with Nox4. Alternatively, the presence of Poldip2 in a complex with Nox1 may inhibit binding of its regulatory, activating subunits. While Poldip2 overexpression increases ROS in $nox1^{y/-}$ cells (Figure 3.10), the effect of Poldip2 overexpression in Nox1 knockout cells is not the same potentiation shown antisense Nox1 treated cells (Figure 3.8), which may be attributable to compensatory adjustments in the cells from these $nox1^{y/-}$ animals that may not occur when Nox1 is acutely depleted.

The ability of Poldip2 to both positively regulate Nox4-dependent ROS production and to negatively regulate Nox1-dependent ROS production raises the possibility that this single protein may serve to functionally coordinate the activation of a single Nox pool in VSMCs in response to specific agonist stimulation. The involvement of Poldip2 in both processes may help explain why the two Noxes expressed in VSMCs, Nox1 and Nox4, are differentially regulated and mediate different biological responses.

CHAPTER 4

Poldip2 Regulates Proper Nox4 and p22phox Localization, Focal Adhesion Integrity and Stress Fiber Formation

4.1. Introduction

As noted in Figure 3.14, VSMCs in which Nox4 or its newly found regulatory protein, Poldip2, were knocked down by siRNA showed distinct phenotypic changes in which the cells become elongated, spindly, and seem to have fewer points of contact with the dish. The localization of Poldip2, Nox4 and p22phox in focal adhesions and the profound change in cell phenotype in siPoldip2- and siNox4 treated cells raises the possibility that the Poldip2/Nox4/p22phox complex may function to modulate or maintain proper cytoskeletal integrity. Focal adhesions are specialized sites of cell attachment to the extracellular matrix where transmembrane integrins link the matrix to the actin cytoskeleton. These sites of cell attachment contain a number of proteins including, but not limited to, FAK, vinculin, paxillin, p130Cas, and a number of PTPs (Zaidel-Bar et al., 2003). The precise mechanisms regulating focal adhesion maintenance are unclear, but appear to require the activation of RhoA (Nobes and Hall, 1995a). While recent work shows that Rho-GTPases contain a conserved redox-sensitive motif (Heo and Campbell, 2005) and raises the possibility of direct regulation of Rho-GTPases by ROS, the NADPH oxidase responsible for this regulation remains to be defined. Additionally, a number of PTPs localize to focal adhesions and maintain their stability. Of these, the LMW-PTP and PTP-PEST are regulated by ROS (Angers-Loustau et al., 1999; Chiarugi et al., 2000a; Chiarugi et al., 2000b), the latter of which is strongly linked with focal adhesion turnover and the modulation of a number of signaling proteins upstream of RhoA, such as FAK, and therefore may serve as a potential

target for ROS mediated regulation of VSMC migration. While both ROS and Noxes are linked to cytoskeletal modulation, how Poldip2/Nox4 functions to regulate cytoskeletal integrity in VSMCs is completely unknown, and is the focus of the studies conducted in this chapter.

4.2. Methods

4.2.1. Cell Culture

Rat VSMCs were grown in Dulbecco's Modified Eagle's Media (DMEM; Invitrogen, Carlsbad, CA) as described above in 2.2.1. nox1$^{y/-}$ VSMCs were grown in DMEM as described in 3.2.1. Cells at passages 6 to 12 were used after 48 hours of quiescence in serum free DMEM for all experiments, unless otherwise noted.

4.2.2 Antibodies

The p22phox and Nox4 antibodies used for immunocytochemistry were from Dr. Mark Quinn (Montana State University) and Dr. David Lambeth (Emory University), respectively. The Poldip2 goat antibody was custom made by GenScript Corporation (Piscataway, NJ) against the peptide sequence NPAGHGSKEVKGKTC. When available, commercial antibodies were used: Nox1 and RhoA antibodies were purchased from Santa Cruz (Santa Cruz, CA), the biotin antibody was purchased from Vector Labs (Burhingame, CA), the vinculin and β-Tubulin antibodies were from Sigma (St. Louis, MO), the paxillin and p190RhoGAP antibodies were from BD Biosciences (Bedford, MA), the HA-tag antibody was obtained from Abcam (Cambridge, MA), and the phospho-FAK,

total FAK, PTP-PEST, and Myc-tag antibodies were all purchased from Cell Signaling (Danvers, MA).

4.2.3 siRNA

For transfection with siRNA, VSMCs were trypsinized and plated at 40-50% confluence on collagen-coated substrate. After 4-6 h, cells were washed with serum-free OPTI-MEM (Invitrogen, Mountain View, CA), and incubated with siRNA + Oligofectamine complexes as described in detail in section 3.2.3. Cells that were both transfected with siRNA and transduced with adenovirus were treated as follows: 1) cells were trypsinized and plated at 40-50% confluence on collagen-coated substrate for 4 hours, 2) cells were then transduced with recombinant adenovirus for 2 h at 37°C in serum-free DMEM, and 3) cells were rinsed with OPTI-MEM prior to transfecting with siRNA using the same technique described in detail in section 3.2.3 prior to experimental use.

4.2.4 Adenoviruses

The AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA) was used to prepare viruses with either no insert (AdGFP) or Myc-tagged Poldip2 (AdPoldip2). The LacZ control (AdLacZ), HA-tagged constitutively active RhoA (AdRhoAGV), and HA-tagged dominant negative RhoA adenoviruses (AdRhoTN) were kind gifts of Dr. Aviv Hassid (University of Tennessee). VSMCs were transduced with recombinant adenoviruses for 2 h at 37°C in serum-free DMEM, followed by incubation for 48 h-4 days in serum-free DMEM without virus.

4.2.5 **Immunoblotting**

VSMCs were lysed in standard lysis buffer (50 mmol/L HEPES, 50 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L Na-pyrophosphate, 50 mmol/L NaF, 1 mmol/L Na-orthovanadate, 1% Triton X 100, and protease inhibitors) or in Hunter's buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 0.1 mmol/L Na-orthovanadate, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, and protease inhibitors) for all experiments. Whole cell lysates were utilized for immunoblotting (IB) experiments. For IB analysis, proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with appropriate primary antibodies, as described in 2.2.7. Primary antibodies were used at the following dilutions for immunoblotting: 1) paxillin at 1:2000, 2) vinculin at 1:2000, 3) β-Tubulin at 1:10,000, 4) FAK at 1:1000, 5) RhoA at 1:500, 6) phospho-FAK Tyr397 at 1:1000, 7) phospho-FAK Tyr576/577 at 1:1000, 8) Biotin at 1:1000, 9) Myc at 1:1000, and 10) PTP-PEST at 1:1000. Band intensity was quantified by densitometry using ImageJ 1.38 software.

4.2.6 Immunocytochemistry and Confocal Microscopy

VSMCs were plated on collagen-coated glass coverslips (BD Biosciences, Bedford, MA) were 1) transiently transfected with siPoldip2 for 4 days before labeling, 2) transiently transfected with siNox4 for 5 days before labeling, 3) transduced with recombinant adenovirus for 72h to overexpress Poldip2, 4) transduced with either control adenovirus (AdLacZ) or HA-tagged constitutively active Rho (AdRhoGV) prior to transiently transfecting with siControl or siPoldip2, or 5) transduced with AdGFP or AdPoldip2 prior to transduction with either

AdLacZ or an HA-tagged dominant negative Rho (AdRhoTN). Cells were rinsed, fixed, permeabilized, and quenched as described in 2.2.9. Cells were incubated for 1 h in blocking buffer (3-5% bovine serum albumin in PBS) prior to incubating with primary antibody for 1 hour at room temperature. Cells were then incubated for 1 hour at room temperature with fluorophore-conjugated secondary antibodies purchased from The Jackson Laboratory (Bar Harbor, Maine). Nuclei were labeled with DAPI for 10 minutes at room temperature. Cells on coverslips were mounted onto glass slides in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and image acquisition was performed using a confocal microscope as described in 2.2.9. The primary antibodies used for immunocytochemistry experiments include: 1) Nox4 at 1:50, 3) p22phox Rb5554 at 1:50, 4) vinculin at 1:50, 5) paxillin at 1:50, 6) HA-tag at 1:500, 7) Phalloidin at 1:100, and 8) DAPI (nuclei) at 1:1000. When comparing cells from different treatment groups, all image threshold settings of the confocal microscope remained constant.

4.2.6. Rho Activity Assay

Rho activity was measured by two independent methods. The first method used to assess RhoA activation was a GST-pulldown assay, which utilizes a GST fusion protein containing the Rho binding domain (RBD) of the Rho effector protein, Rhotekin (GST-Rhotekin RBD, MilliPore, Billerica, MA). The Rhotekin RBD binds specifically to the active GTP-bound, and not GDP-bound, form of RhoA. Rho activity was assessed by measuring the amount of RhoA that immunoprecipitates from cell lysates using beads bound to the GST-Rhotekin

RBD. The amount of active RhoA was quantified by western blot analysis and normalized to total RhoA. The second method used to measure RhoA activation was a G-LISA method in which the amount of active GTP-bound Rho from cell lysates that binds to the Rho-GTP-binding protein substrate is detected with a RhoA specific antibody and luminometry (Cytoskeleton, Denver, CO), very similar to an ELISA assay.

4.2.7. Oxidation of Proteins

The oxidation of proteins was assessed using a N-(biotinoyl)-N-(iodoacetyl)ethylenediamine (BIAM) labeling assay, as described previously (Kim et al., 2000). Briefly, VSMCs were transduced with AdGFP or AdPoldip2 for 3 days prior to being lysed in acidic (pH 6.5) MES-NaOH buffer containing 20 μM BIAM to label reduced cysteines. Reduced PTP-PEST that was labeled with BIAM was detected by Western blot with anti-PTP-PEST antibody, where the loss of signal is indicative of in vivo oxidation.

4.2.9 Migration Assay

Migration was measured using Boyden Chamber assays as described previously. Briefly, 5 x 10^4 cells from each treatment group were seeded in a Transwell plate with 6.5 mm inserts with 8-μm pores that were coated with 0.5 mg/mL collagen I (BD Biosciences, Bedford, MA). Cells were subsequently allowed to migrate through the Transwell membrane. Cells that did not migrate were removed from the upper surface of the membrane, and cells that migrated were fixed and stained with DAPI (diluted at 1:1000, 1 μg/mL, Sigma-Aldrich, St. Louis, MO). The number of migrated cells was quantified using a Zeiss Axioskop

microscope where 5 images from 5 random fields were quantified from each of 3 independent experiments. The number of migrated cells was then quantified using ImageJ 1.38 software.

4.2.10 Statistical Analysis

Results are expressed as mean ± S.E.M. from at least three independent experiments. Significance of statistical comparisons was assessed using analysis of variance (ANOVA), followed by Bonferroni's Multiple Comparison post-hoc test. A value of $p<0.05$ was considered significant.

4.3. Experimental Results

4.3.1. Modulation of Poldip2 or Nox4 Regulates Focal Adhesion and Stress Fiber Formation

Because siPoldip2 and siNox4 cells became elongated, spindly, and seemed to have fewer points of contact with the dish, as shown in Figure 3.14, we set out to further elucidate the effects of Poldip2/Nox4 on the cytoskeleton. The profound cytoskeletal phenotype observed in cells treated with siPoldip2 or siNox4 suggests that this enzyme complex may regulate focal adhesion integrity and/or stress fiber formation. To determine if this is the case, we first treated VSMCs with siPoldip2 and stained for stress fibers, as detected by phalloidin staining, and the two resident focal adhesion proteins, vinculin and paxillin. siPoldip2 treatment resulted in wavy, disorganized stress fibers, as well as a complete loss of focal adhesion-like structures (Figure 4.1). This loss of vinculin and paxillin staining in siPoldip2 treated VSMCs cannot be completely attributed to a downregulation of these proteins, as there was only a 50% decrease in

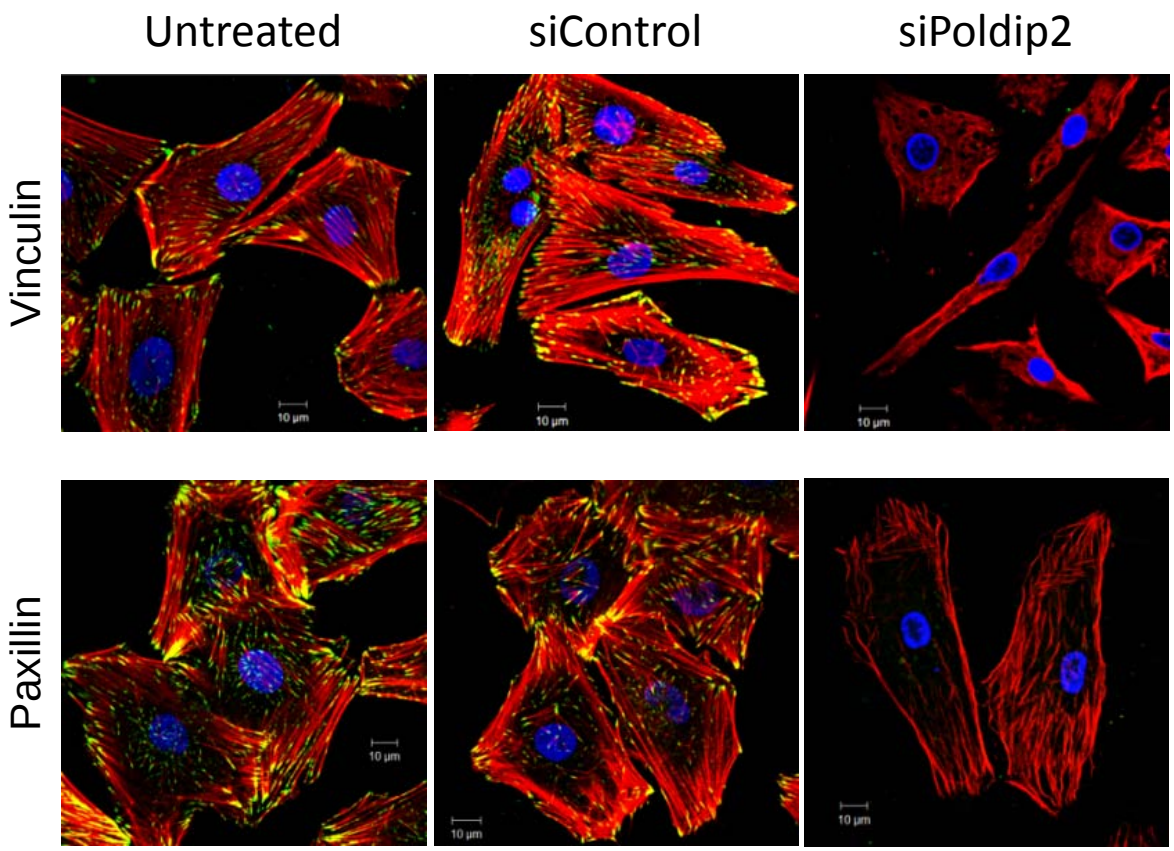


Figure 4.1. siPoldip2 Decreases the Focal Adhesion Markers Vinculin and Paxillin and Stress Fiber Formation. Rat VSMCs were either untreated or transiently transfected with 15 nmol/L of siControl or siPoldip2. ***Vinculin, upper.*** Cells were double labeled with vinculin (green) and phalloidin (red). ***Paxillin, lower.*** Cells were double labeled with paxillin (green) and phalloidin (red). Yellow appears where stress fibers (red) insert into focal adhesions (green). Nuclei are labeled with DAPI (blue). Confocal images acquired at the focal adhesion plane are depicted. Scale bars, 10 µm.

paxillin protein levels and no significant decrease in vinculin protein levels (Figure 4.2). This discrepancy may potentially be explained by a possible change the structure of vinculin when not localized in focal adhesions, such that the epitope region against which the vinculin antibody was raised is no longer exposed. VSMCs treated with siNox4, similarly, resulted in stress fiber disorganization and a loss of focal adhesions (Figure 4.3). In contrast, the overexpression of Poldip2 resulted in an increase in both focal adhesions (Figure 4.4) and stress fiber formation (Figure 4.5). As shown, siPoldip2 treatment caused a loss of paxillin and vinculin localization to focal adhesions. Because Poldip2, Nox4, and p22phox all localize in focal adhesions, the apparent loss of these structures in siPoldip2-treated cells led us to investigate if Nox4 and p22phox are mislocalized in VSMCs when Poldip2 is depleted. We stained for these two proteins, as well as for stress fibers, in siPoldip2 treated cells. Both Nox4 and p22phox protein levels seem slightly reduced in siPoldip2 treated VSMCs; however, the most striking effect is the loss of Nox4 and p22phox localization to focal adhesions (Figure 4.6). Thus, Poldip2 seems to be a critical regulator of proper Nox4/p22phox localization to focal adhesion structures in VSMCs and appears to regulate the integrity of these important cytoskeletal structures.

4.3.2. Poldip2 Regulates Focal Adhesion and Stress Fiber Formation by Activating RhoA via Nox4

Manipulation of Poldip2 has profound effects on the cytoskeleton, particularly on stress fibers and focal adhesions. Because both focal adhesion turnover and stress fiber formation are mediated through RhoA activation

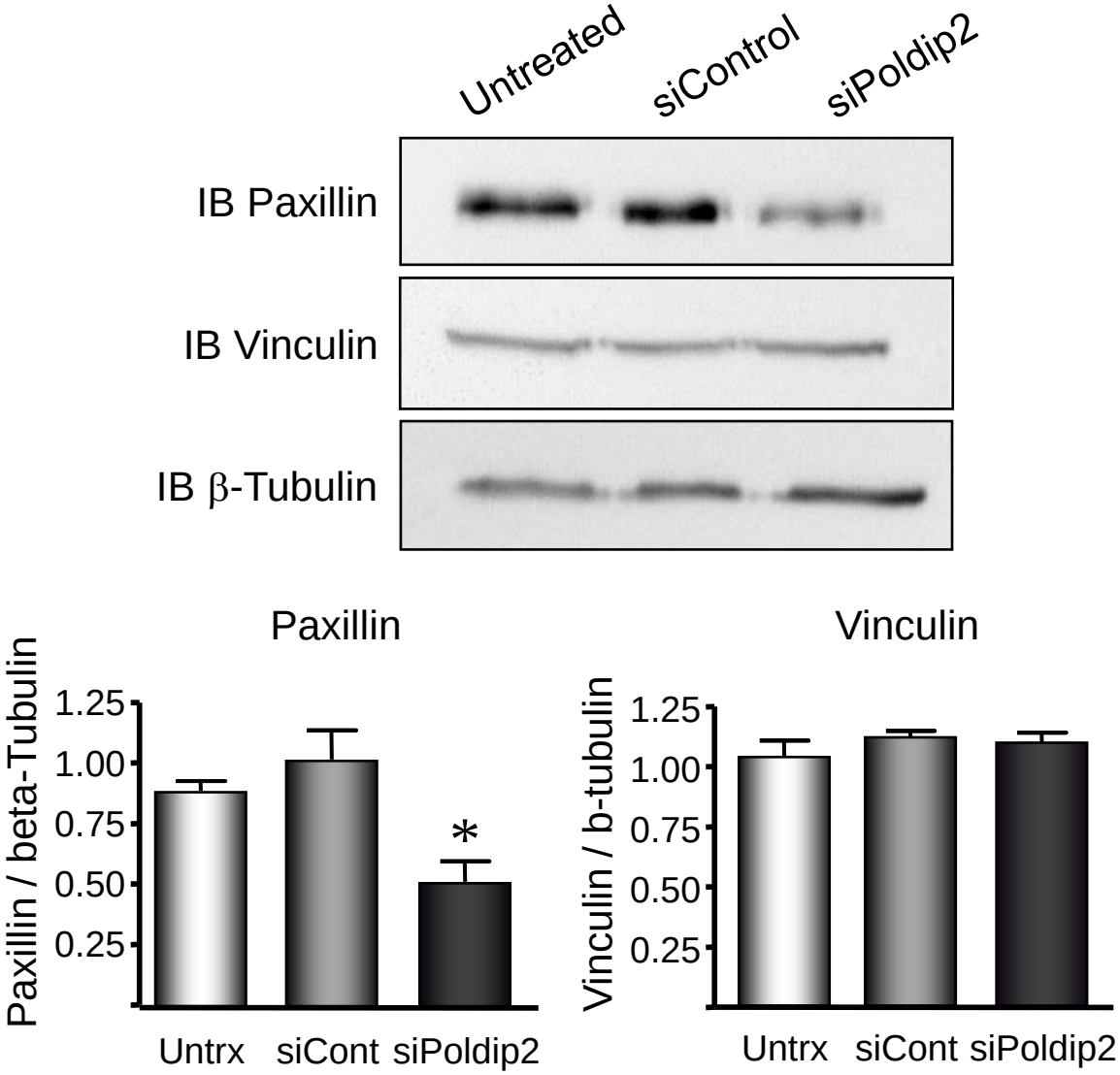


Figure 4.2. siPoldip2 Alters Paxillin, but Not Vinculin, Protein Expression Levels. Western analysis of paxillin and vinculin protein expression in protein extracts from rat VSMCs that were either untreated (Untrx) or transiently transfected with 15 nmol/L of siControl (siCont) or siPoldip2. β-Tubulin levels (lower blot) were included as a loading control. Bars are mean ± S.E.M. of 3 independent experiments. *$p<0.05$ vs. siCont. No statistical difference in vinculin was found.

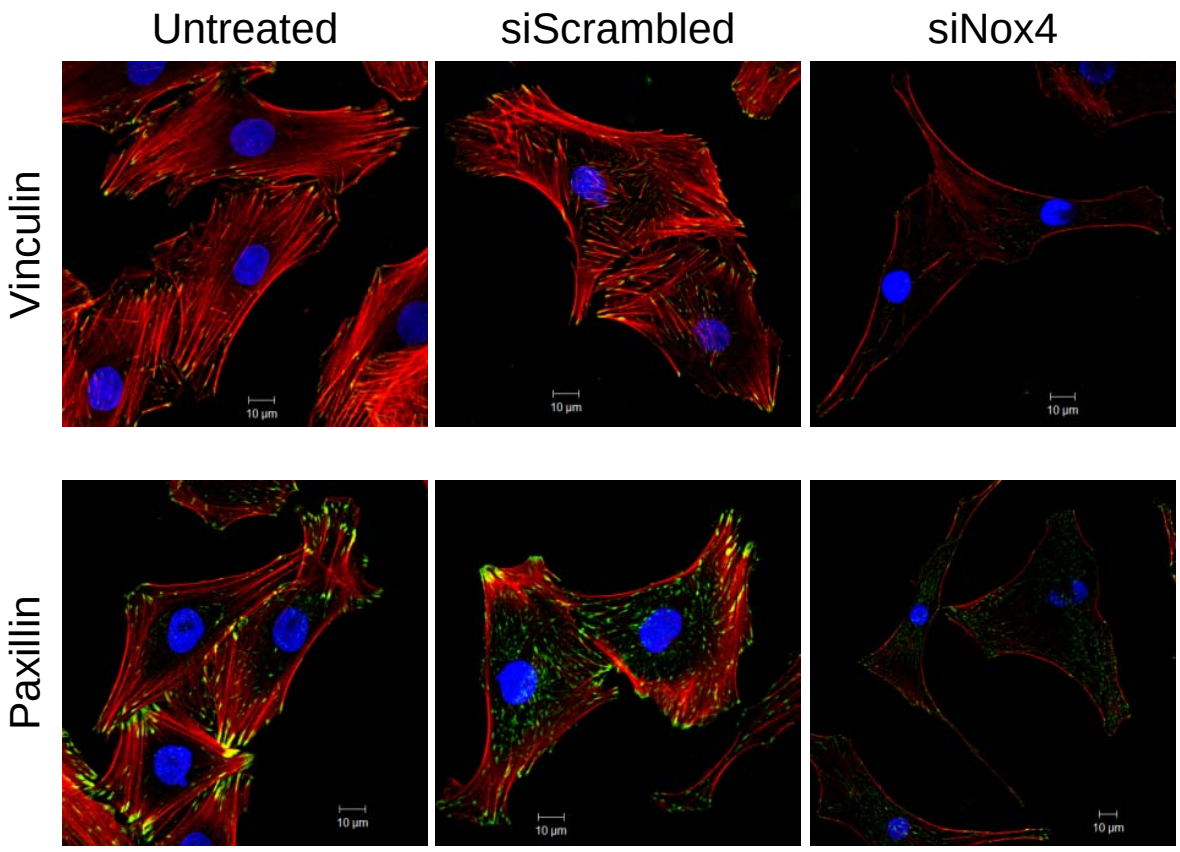


Figure 4.3. siNox4 Decreases the Focal Adhesion Markers Vinculin and Paxillin and Stress Fiber Formation. Rat VSMCs were either untreated or transiently transfected with 25 nmol/L of siScrambled or siNox4. ***Vinculin, upper.*** Cells were double labeled with vinculin (green) and phalloidin (red). ***Paxillin, lower.*** Cells were double labeled with paxillin (green) and phalloidin (red). Yellow appears where stress fibers (red) insert into focal adhesions (green). Nuclei are labeled with DAPI (blue). Confocal images acquired at the focal adhesion plane are depicted. Scale bars, 10 µm.

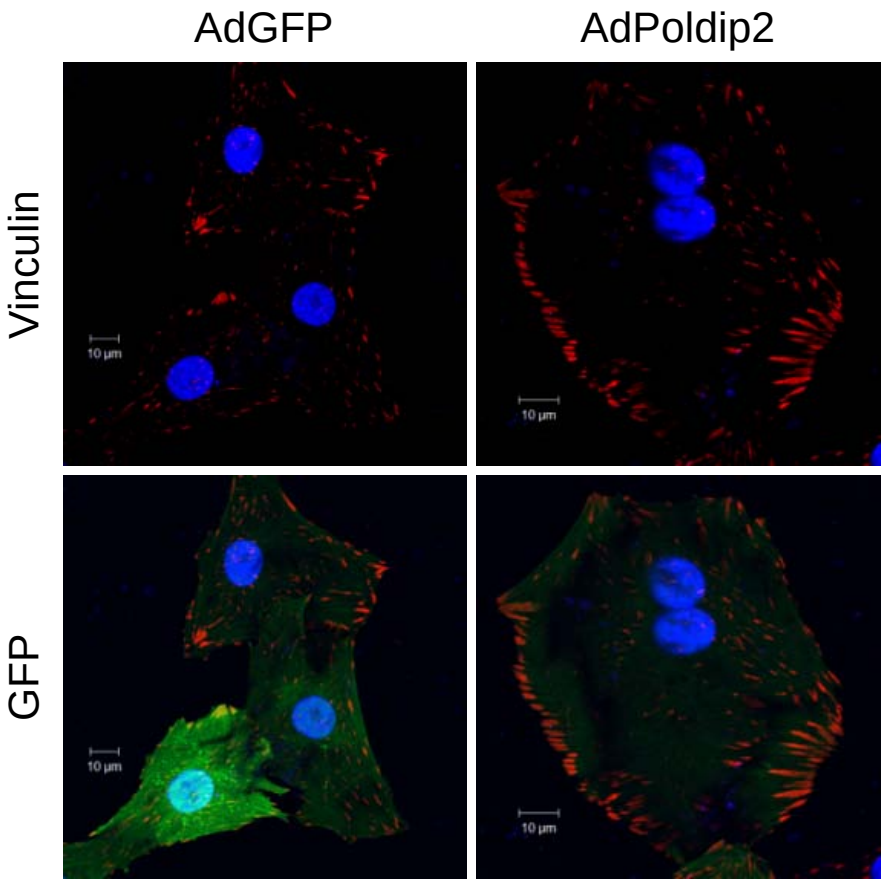


Figure 4.4. Overexpression of Poldip2 Increases Focal Adhesion Formation. Rat VSMCs were transduced with control adenovirus (AdGFP) or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 hours before labeling with the focal adhesion marker vinculin (red). Cells transduced with adenovirus are detected as green in the GFP panels (*lower*). Nuclei are labeled with DAPI (blue). Confocal images acquired at the focal adhesion plane are depicted. Scale bars, 10 µm.

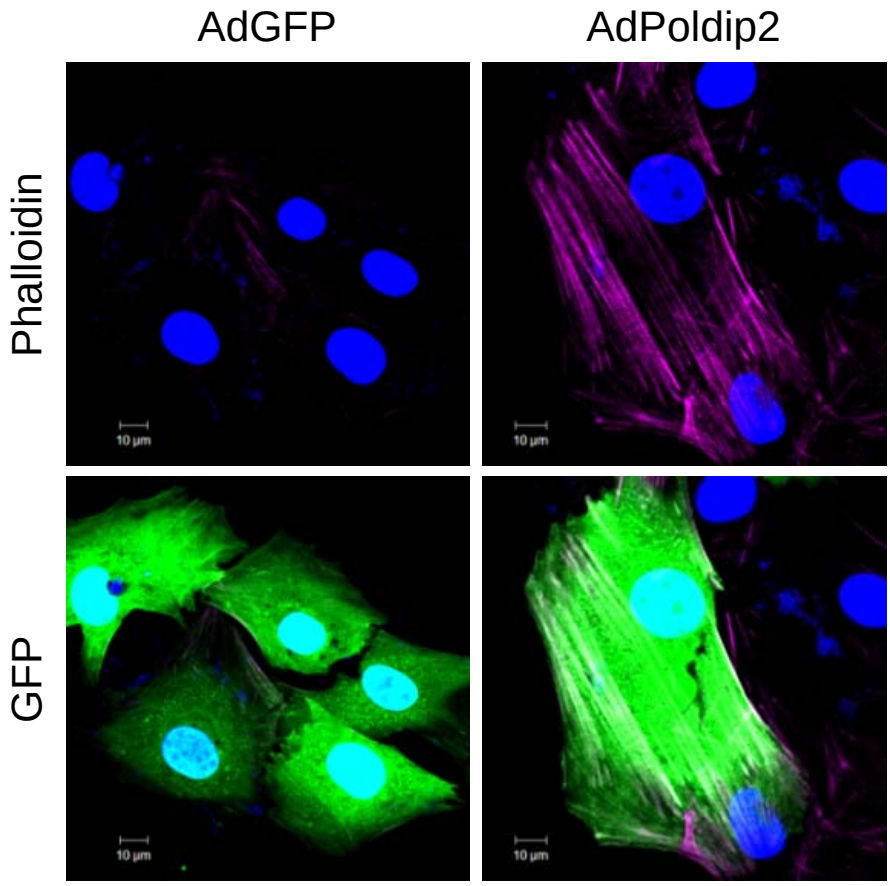


Figure 4.5. Poldip2 Overexpression Increases Stress Fiber Formation. Rat VSMCs were transduced with control adenovirus (AdGFP) or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 hours before labeling with the stress fiber marker phalloidin (purple). Cells transduced with adenovirus are detected as green in the GFP panels (*lower*). Nuclei are labeled with DAPI (blue). Confocal images acquired at the focal adhesion plane are depicted. Scale bars, 10 µm.

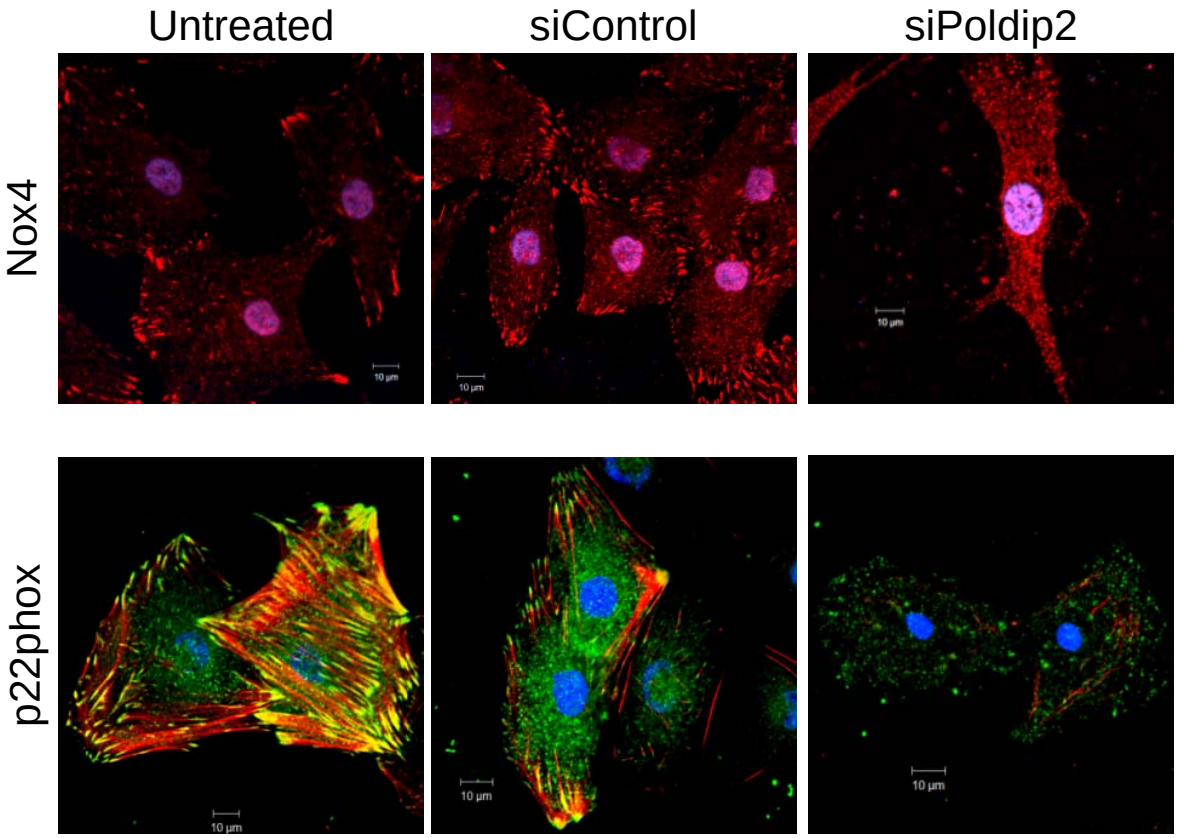


Figure 4.6. The Absence of Poldip2 Prevents Proper Localization of Nox4 and p22phox to Focal Adhesions. Rat VSMCs were either untreated or transiently transfected with 15 nmol/L of siControl or siPoldip2. ***Nox4, upper.*** Cells were single labeled with anti-Nox4 (red) antibody. ***p22phox, lower.*** Cells were double labeled with anti-p22phox (green) and phalloidin (red) to stain stress fibers. Yellow appears where stress fibers (red) insert into focal adhesions (green). Nuclei are labeled with DAPI (blue). Confocal images acquired at the focal adhesion plane are depicted. Scale bars, 10 µm.

(Chrzanowska-Wodnicka and Burridge, 1996), we hypothesized that Poldip2/Nox4 may exert their effects by activating RhoA. Previous work has shown that RhoA is activated by ROS, suggesting that RhoA is a potential target of this complex (Montezano et al., 2008). To assess if modulation of Poldip2 affects RhoA activation, we first performed a GST-pulldown utilizing a GST fusion protein containing the Rho binding domain of the Rho effector protein, Rhotekin (GST-Rhotekin RBD), which binds specifically to the GTP-bound, and not GDP-bound, RhoA. We found that the overexpression of Poldip2 in VSMCs results in a substantial increase in RhoA activation (Figure 4.7). These effects on RhoA activation could be mediated through Nox4, in which case the effects would be ROS mediated, or could be due to a function of Poldip2 independent of its role as a Nox4 regulatory protein. To determine if the effects of Poldip2 on RhoA activation are mediated through ROS, we overexpressed Poldip2 in VSMCs in the absence or presence of the potent antioxidant NAC and found that the pronounced increase in RhoA activity mediated by Poldip2 was significantly decreased in the presence of NAC (Figure 4.8).

To determine what signaling pathways are activated by Poldip2-mediated activation of Nox4 and RhoA, we assessed some of the kinases and PTPs known to be upstream of RhoA. FAK, a nonreceptor tyrosine kinase upstream of RhoA, is activated by integrin receptors and phosphorylates paxillin and p130Cas, thereby regulating their translocation to focal adhesions and enhancing focal adhesion formation. To determine if the significant increase in RhoA activity caused by Poldip2 overexpression is mediated by an increase in FAK

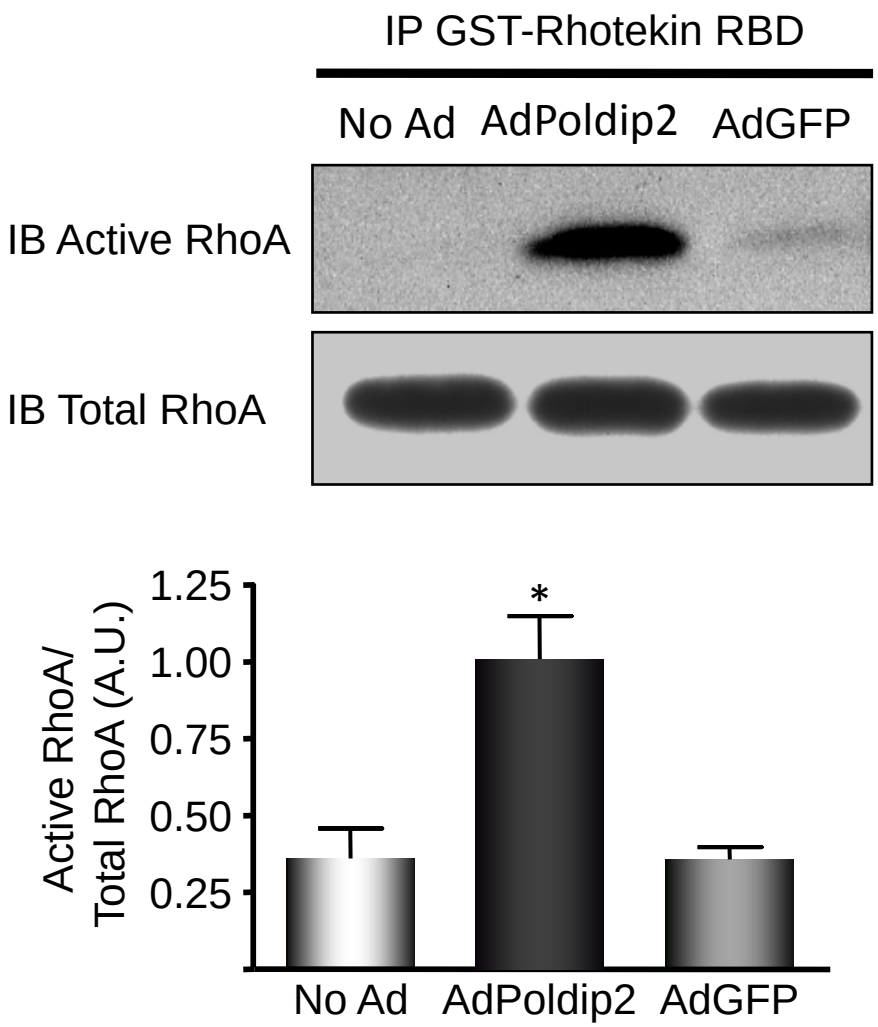


Figure 4.7. Poldip2 Overexpression Increases Active RhoA. Rat VSMCs were transduced with no adenovirus (No Ad), adenovirus to overexpress Myc-Poldip2 (AdPoldip2), or with control adenovirus (AdGFP) for 72 hours prior to being used to measure active, GTP-bound RhoA. Cells were immunoprecipitated (IP) with beads bound to GST-Rhotekin RBD before immunoblotting (IB) for GTP-bound, active RhoA (*upper*) and total RhoA (*lower*). The western blots shown are from a representative experiment. Bars are mean ± S.E.M. from 3 independent experiments. *$p<0.05$ vs. AdGFP.

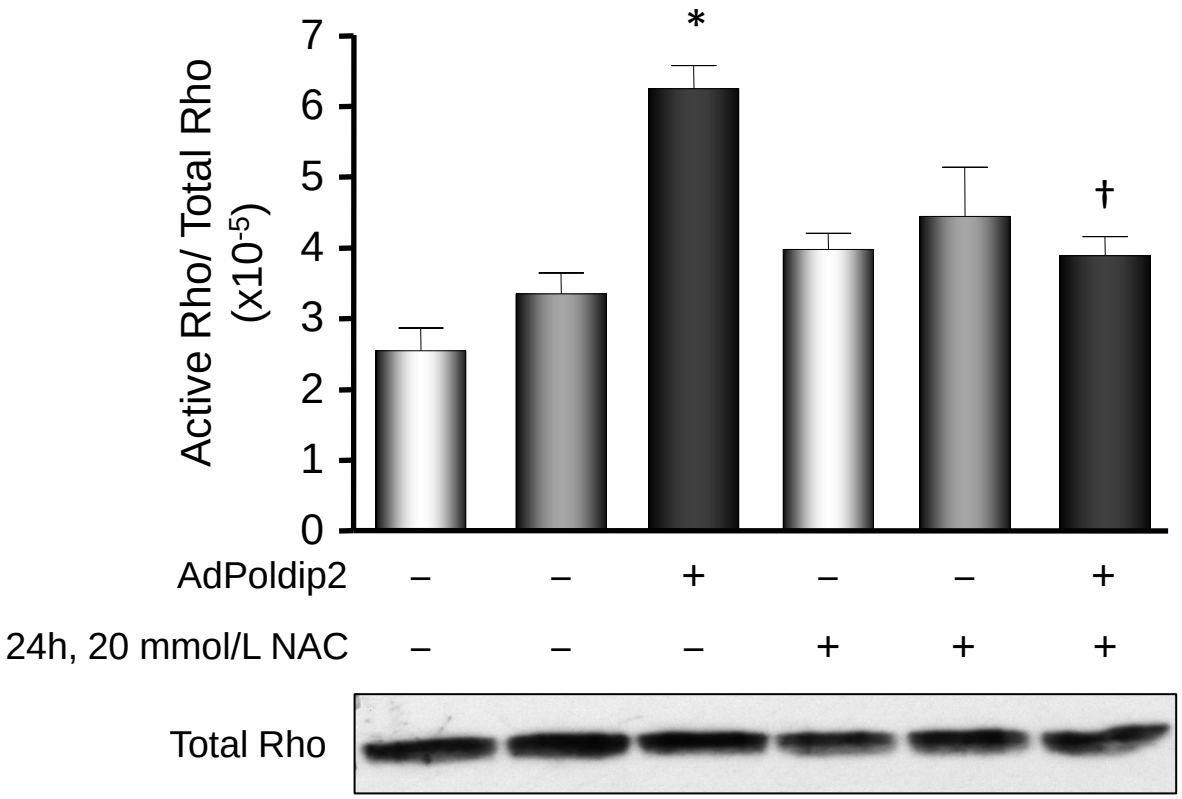


Figure 4.8. The Increase in Active RhoA Mediated by Poldip2 Overexpression is Blunted by N-acetyl Cysteine. Rat VSMCs were transduced with no adenovirus (- , white bars), control adenovirus (- , medium grey bars), or adenovirus to overexpress Myc-Poldip2 (+ , dark grey bars) for 48 hours prior to no treatment (-) or treatment with 20 mmol/L N-acetyl cysteine (+ , NAC) for 24 h. Active, GTP-bound RhoA was quantified using a G-LISA method in which the amount of active GTP-bound Rho, which binds to the Rho-GTP-binding substrate, is detected with a RhoA specific antibody and a luminometer. The Total RhoA western blot shown is from a representative experiment. Bars are mean ± S.E.M. from 3 independent experiments. *p<0.01 vs. AdGFP – NAC; †p<0.05 vs. AdPoldip2 – NAC.

phosphorylation, we assessed this by western blot analysis. We found no increases in FAK phosphorylation or in total FAK expression (Figure 4.9 a) to account for the increase in RhoA activity, nor was the increase mediated by a decrease in the expression of the GTPase activating protein (GAP), p190RhoGAP (Figure 4.9 b), a Rho-GTPase that catalyzes the exchange of GTP to GDP and, thereby, inactivates Rho. We next set out to assess if there was any significant increase in the oxidation of Cys residues in the protein tyrosine phosphatase PTP-PEST, a phosphatase previously shown to be ROS sensitive and known to localize in focal adhesions, regulate focal adhesion turnover, and to be upstream of RhoA signaling. To measure the oxidation of PTP-PEST, we utilized a method using a BIAM labeling assay as previously described by Kim et al. (Kim et al., 2000). Briefly, VSMCs were transduced with AdGFP or AdPoldip2 for 3 days prior to being lysed in acidic (pH 6.5) MES-NaOH buffer containing 20 μM BIAM to label reduced cysteines. Biotinoylated proteins were pulled down using Streptavadin. Reduced PTP-PEST that was labeled with BIAM was detected by Western blot with an anti-PTP-PEST antibody, where the loss of signal is indicative of in vivo oxidation. As shown in Figure 4.10, the amount of PTP-PEST in its reduced form was diminished in VSMCs overexpressing Poldip2, thus allowing us to conclude that PTP-PEST oxidation was significantly increased, since total PTP-PEST protein levels were unchanged in transduced cells.

To further establish that the effects on stress fibers and focal adhesions mediated by Poldip2 were Nox4-dependent, we tested if the changes in these

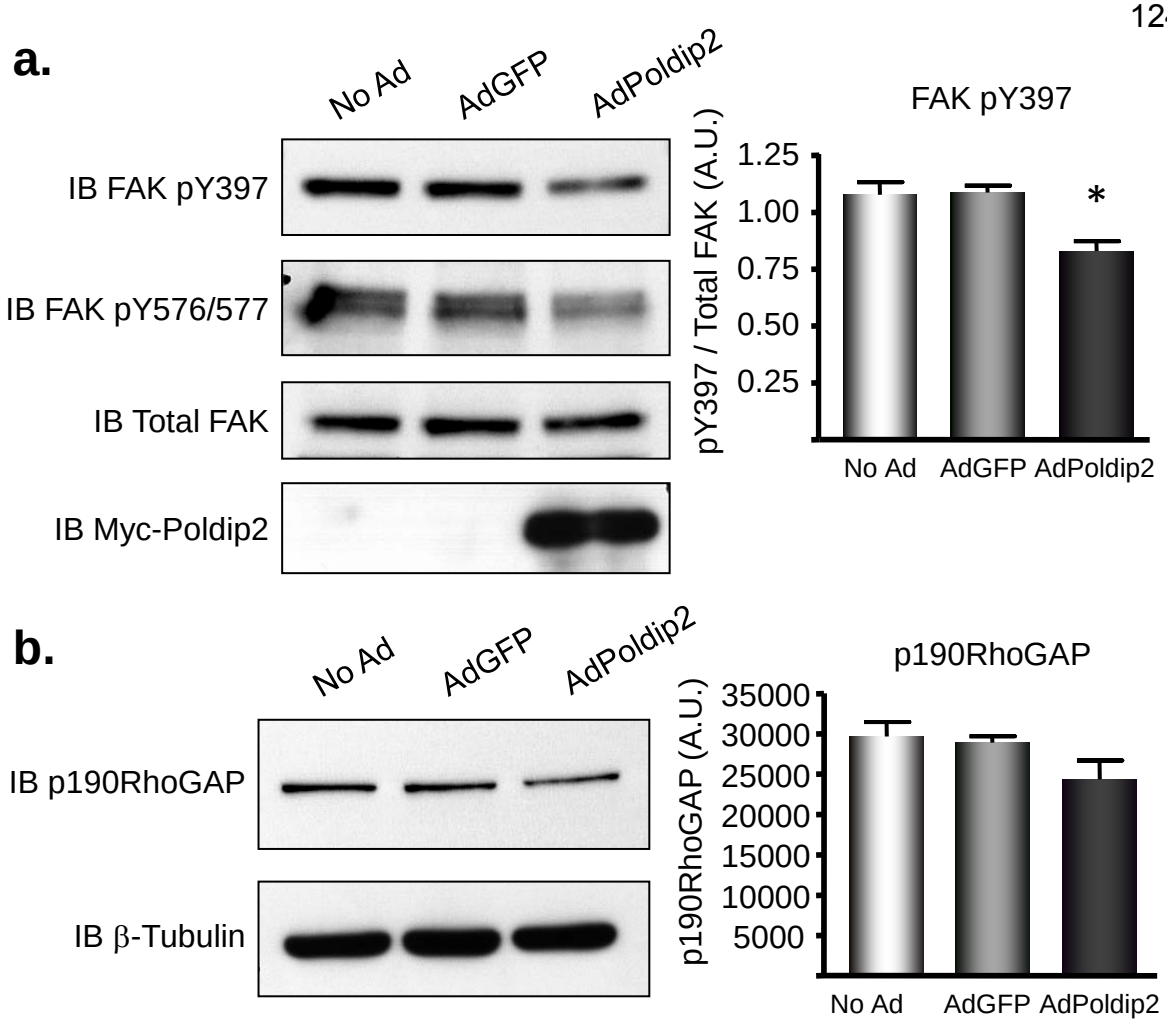


Figure 4.9. Poldip2 Mediated Increases in Active RhoA are Not Due to Increases in Phospho-FAK, Total FAK, or p190RhoGAP Expression. a, Rat VSMCs were transduced with no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 hours prior to immunoblotting (IB) for phospho-FAK Y397 and Y576/577 and total FAK. Lysates were immunoblotted for Myc to show overexpression of Myc-Poldip2. * $p<0.05$ vs. AdGFP. **b,** The same lysates were immunoblotted for total p190RhoGAP. β-Tubulin levels were included as a loading control. The western blots shown are from a representative experiment. Bars are mean ± S.E.M. from 3 independent experiments. No statistical difference in p190RhoGAP was found.

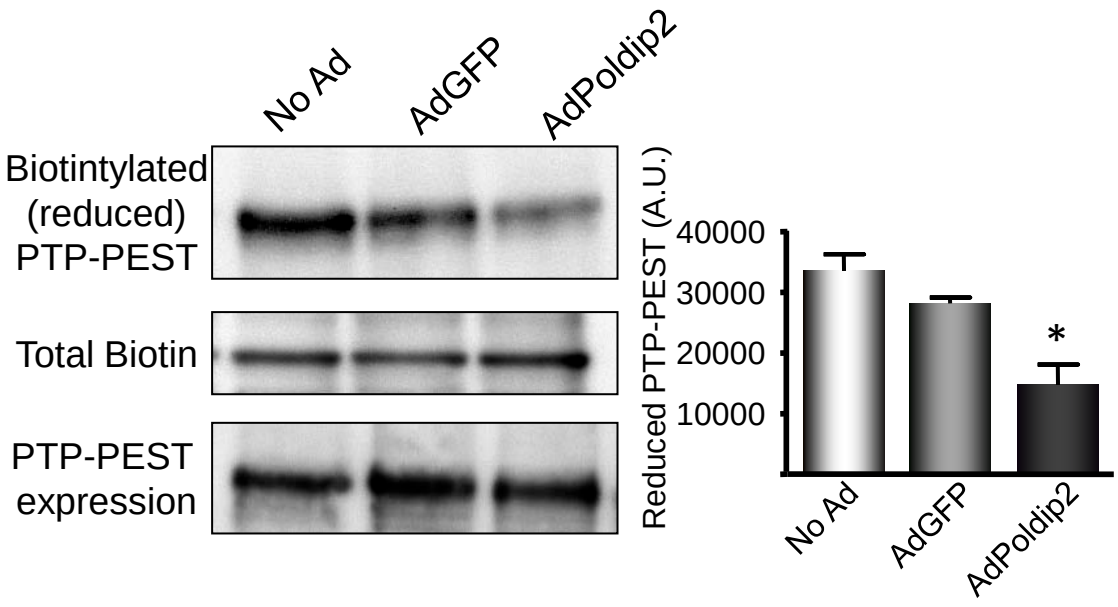


Figure 4.10. Poldip2 Overexpression Increases PTP-PEST Oxidation.

Rat VSMCs were transduced with no adenovirus (No Ad), control adenovirus (AdGFP), or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 hours. The oxidation of proteins was assessed using a N-(biotinoyl)-N-(iodoacetyl) ethylenediamine (BIAM) labeling assay by lysing cells in acidic (pH 6.5) MES-NaOH buffer containing 20 μM BIAM to label reduced cysteines. Biotinylated proteins were pulled down using Streptavadin. Reduced PTP-PEST that was labeled with BIAM was detected by Western with a PTP-PEST antibody, where the loss of signal is indicative of in vivo oxidation. Lysates were immunoblotted with Total Biotin, to show the same amout of BIAM was used in each sample, and with anti-PTP-PEST to show total PTP-PEST protein expression. The western blots shown are from a representative experiment. Bars are mean ± S.E.M. from 3 independent experiments. *$p<0.05$ vs. AdGFP.

endpoints by manipulation of Poldip2 levels are maintained in VSMCs lacking Nox1 expression (nox1$^{y/-}$), which therefore only express Nox4. Indeed, siPoldip2 decreased stress fiber organization and focal adhesions (Figure 4.11), while Poldip2 overexpression increased stress fibers and focal adhesions in nox1$^{y/-}$ VSMCs (Figure 4.12), supporting the conclusion that the phenotypic changes caused by modulation of Poldip2 are mediated by Nox4.

To further evaluate the role of RhoA in mediating the effects of Poldip2/Nox4, we transduced siPoldip2-treated VSMCs with constitutively active RhoA (AdRhoGV) to determine if we could rescue the loss of focal adhesions and stress fibers. Indeed, as shown in Figure 4.13, RhoGV countered the phenotypic loss of stress fibers and focal adhesions caused by Poldip2 depletion. Additionally, dominant negative RhoA (AdRhoTN) blocked the phenotypic increase in stress fiber and focal adhesion formation in VSMCs overexpressing Poldip2 (Figure 4.14). Taken together, these data strongly indicate that Poldip2 functionally regulates focal adhesion and stress fiber formation through a RhoA-dependent pathway, although the specific pathway and all its targets have not been definitively identified.

4.3.3. Modulation of Poldip2 or Nox4 Inhibits Vascular Smooth Muscle Cell Migration

VSMCs participate in the development of vascular lesions through their ability to proliferate and migrate (Doran et al., 2008). Migration, in particular, is dependent upon dynamic focal adhesion turnover and cytoskeletal reorganization, where focal adhesion turnover requires both the proper

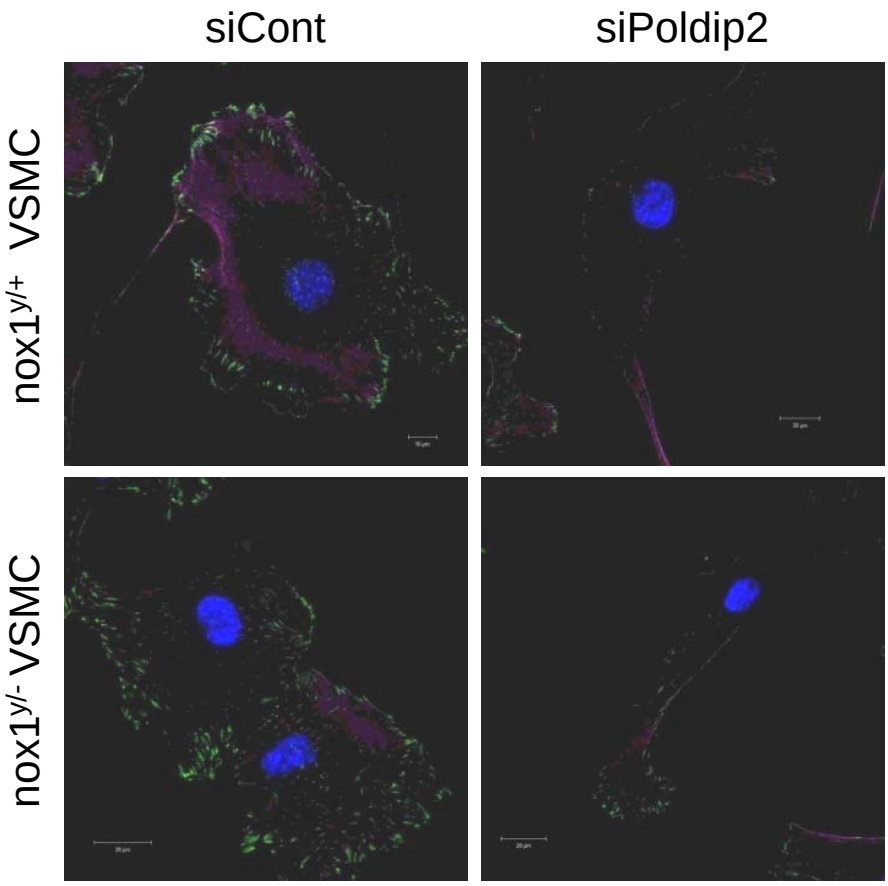


Figure 4.11. The Phenotypic Changes in Focal Adhesions and Stress Fiber Formation Associated with siPoldip2 are Mediated by Nox4, as Demonstrated in nox1$^{y/-}$ VSMCs. Confocal images of mouse nox1 wild-type (nox1$^{y/+}$) or mouse nox1 knockout (nox1$^{y/-}$) VSMCs that were transiently transfected with 15 nmol/L of either control siRNA (siCont) or siPoldip2 before labeling with vinculin (green) and phalloidin (purple). Nuclei are labeled with DAPI (blue). Confocal images acquired at the focal adhesion plane are depicted. Scale bars, 10 µm.

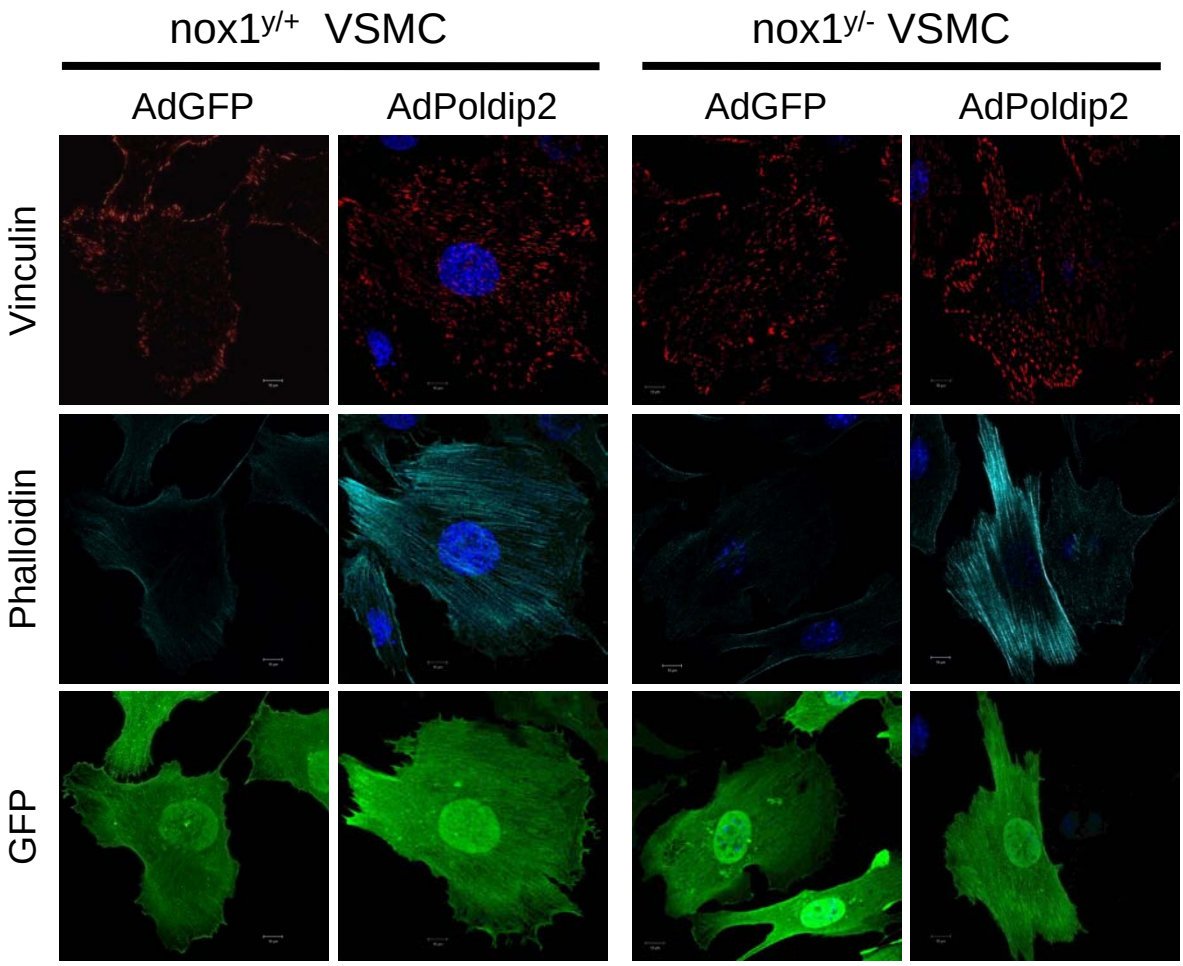


Figure 4.12. The Phenotypic Changes in Focal Adhesions and Stress Fiber Formation Associated with Poldip2 Overexpression are Mediated by Nox4, as Demonstrated in nox1$^{y/-}$ VSMCs. Confocal images of mouse nox1 wild-type (nox1$^{y/+}$) or mouse nox1 knockout (nox1$^{y/-}$) VSMCs transduced with control adenovirus (AdGFP) or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 hours before labeling with vinculin (red) or with phalloidin (pseudocolored cyan). Cells transduced with adenovirus are detected as green in the GFP panels (*lower*). Nuclei are labeled with DAPI (blue). Confocal images acquired at the focal adhesion (*upper*) and stress fiber (*middle*) planes are depicted. Scale bars, 10 μm.

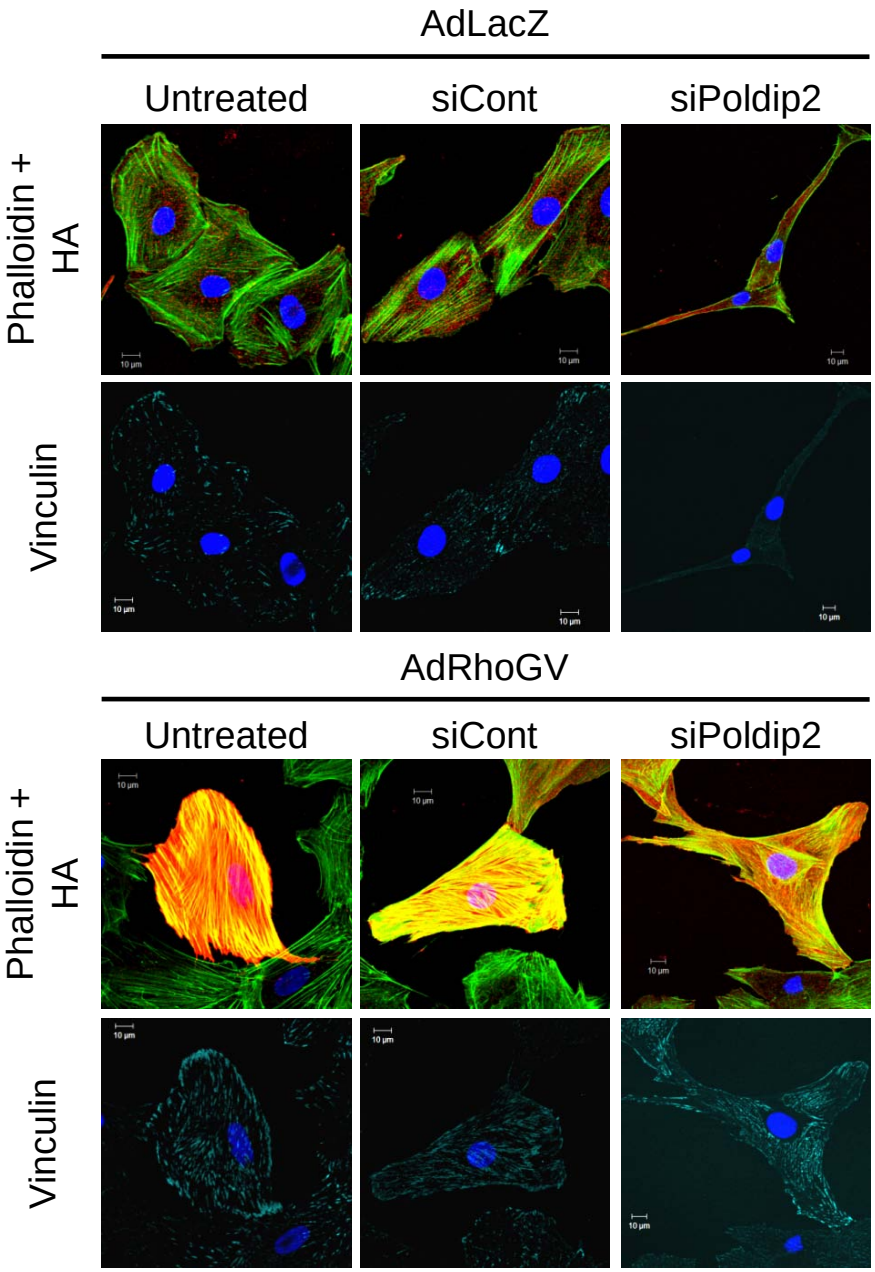


Figure 4.13. CA-RhoA Rescues the Loss of Stress Fibers and Focal Adhesions Caused by siPoldip2 . Rat VSMCs were transduced with either control adenovirus (AdLacZ) or HA-tagged constitutively active Rho (AdRhoGV) prior to leaving untreated or transiently transfecting with 15 nmol/L of either siCont or siPoldip2. VSMCs were triple labeled with anti-HA antibody (HA; red) to detect cells transduced with AdRhoGV, Phalloidin (green) to detect stress fibers, and vinculin (pseudo-colored cyan) to detect focal adhesions. Confocal images acquired at the stress fiber and focal adhesion planes are depicted. Scale bars, 10 μm.

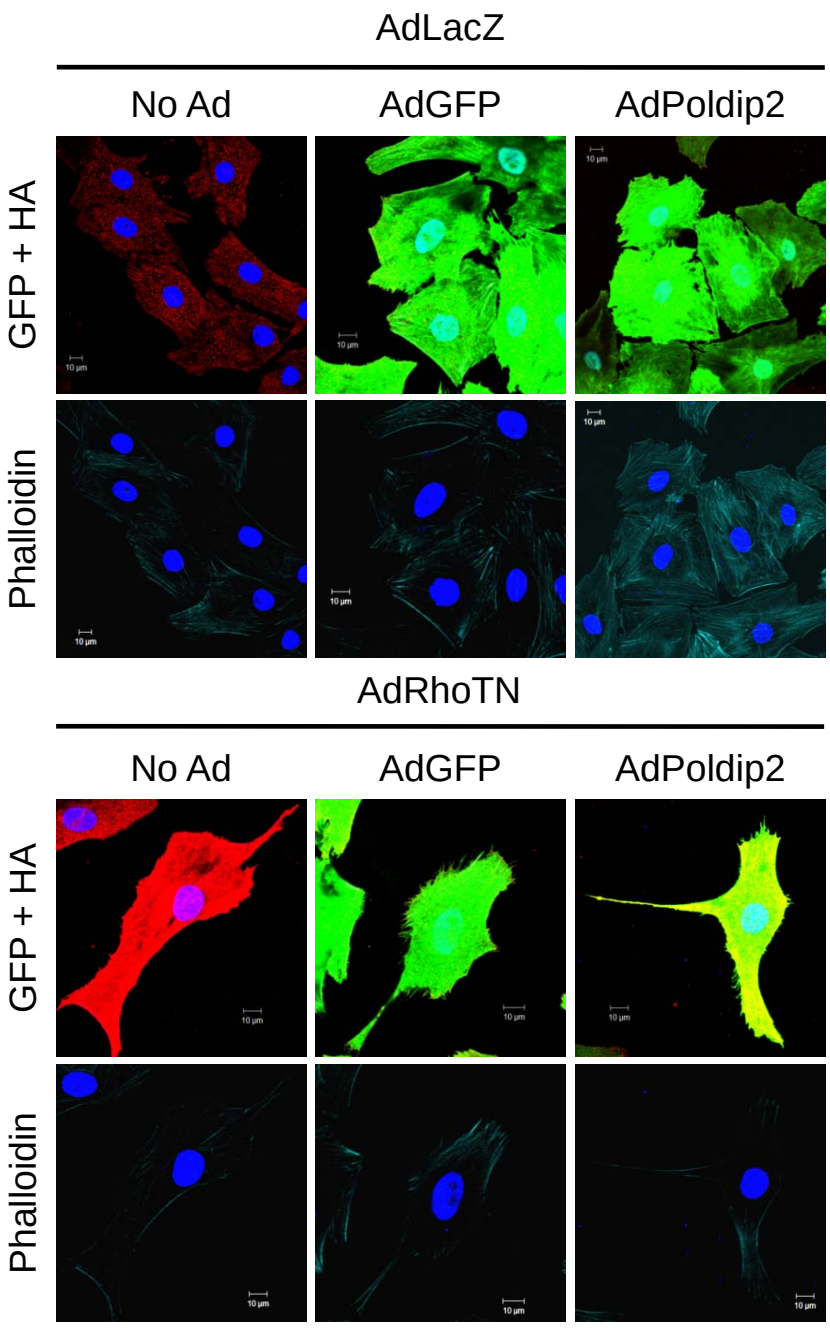


Figure 4.14. DN-RhoA Blocks the Increase in Stress Fibers and Focal Adhesions Caused by Poldip2 Overexpression. VSMCs non-transduced (No Ad) or transduced with AdGFP or AdPoldip2 prior to transduction with either AdLacZ or an HA-tagged dominant negative Rho (AdRhoTN). Cells transduced with AdGFP or AdPoldip2 appear green from GFP. Cells were double labeled with anti-HA antibody (HA; red) to detect cells transduced with AdRhoTN, and Phalloidin (pseudo-colored cyan) to detect stress fibers. Nuclei are labeled with DAPI (blue). Scale bars,10 µm.

dissolution of existing adhesions and the formation of focal contacts and their maturation into focal adhesions. To determine whether siPoldip2 affects the formation of focal adhesions, we performed a cell spreading assay. Cells were treated with siPoldip2 for 4 days, trypsinized and replated. Cell attachment was measured after 30 min. As shown in Figure 4.15, siPoldip2 had a minimal effect on the process of cell attachment, suggesting that Poldip2 may be more important in regulating focal adhesion maturation than focal adhesion formation. Because either upregulation or downregulation of Poldip2/Nox4 negatively impacts focal adhesion turnover, we hypothesized that either overexpression or knockdown of these proteins would impair cell migration. To assess cell migration, we used a Boyden Chamber Assay. As shown in Figure 4.18, overexpression of Poldip2, which strengthens focal adhesions, blocks migration in response to PDGF (10 ng/mL, 4 hours), perhaps because focal adhesions cannot release from the trailing edge of the cell to allow forward movement. Knockdown of Poldip2 using siRNA, which we have shown induces a loss of focal adhesions, also inhibits migration (Figure 4.16), in this case presumably because new focal adhesions cannot form or mature at the leading edge of the cell. Knockdown of Nox4 using an antisense construct resulted in similar decreases in VSMC migration (Figure 4.17). Altogether, the data from these studies provide a direct link between Poldip2/Nox4 and the regulation of the cytoskeleton in VSMCs, ultimately providing a link between Poldip2/Nox4 and VSMC migration.

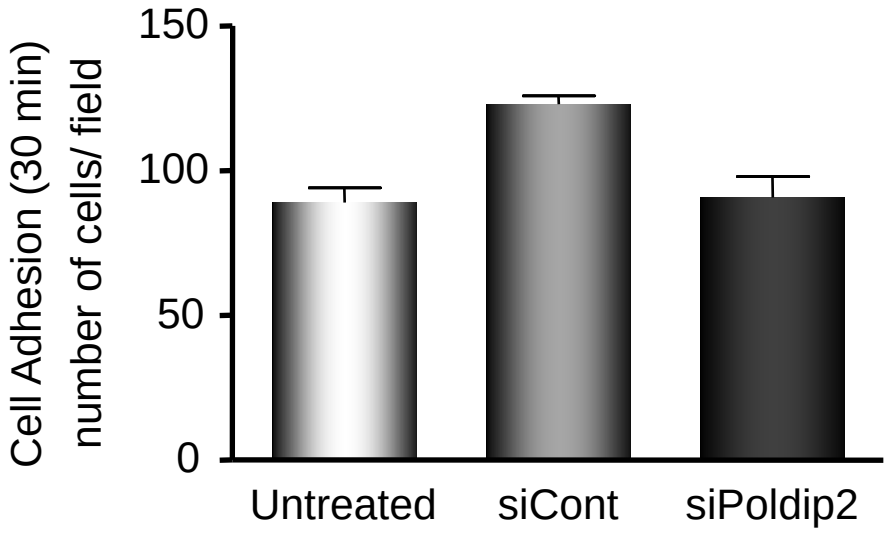


Figure 4.15. siPoldip2 Does Not Interfere with Cell Adherence and Spreading. Rat VSMCs were either untreated or transiently transfected with 15 nmol/L of siControl (siCont) or siPoldip2 for 4 days prior to trypsinizing and replating. Cell attachment was measured after 30 minutes. Cell attachment was quantified by phase contrast microscopy where images were taken of 5 random fields of view per treatment group and counted. No statistical difference was found.

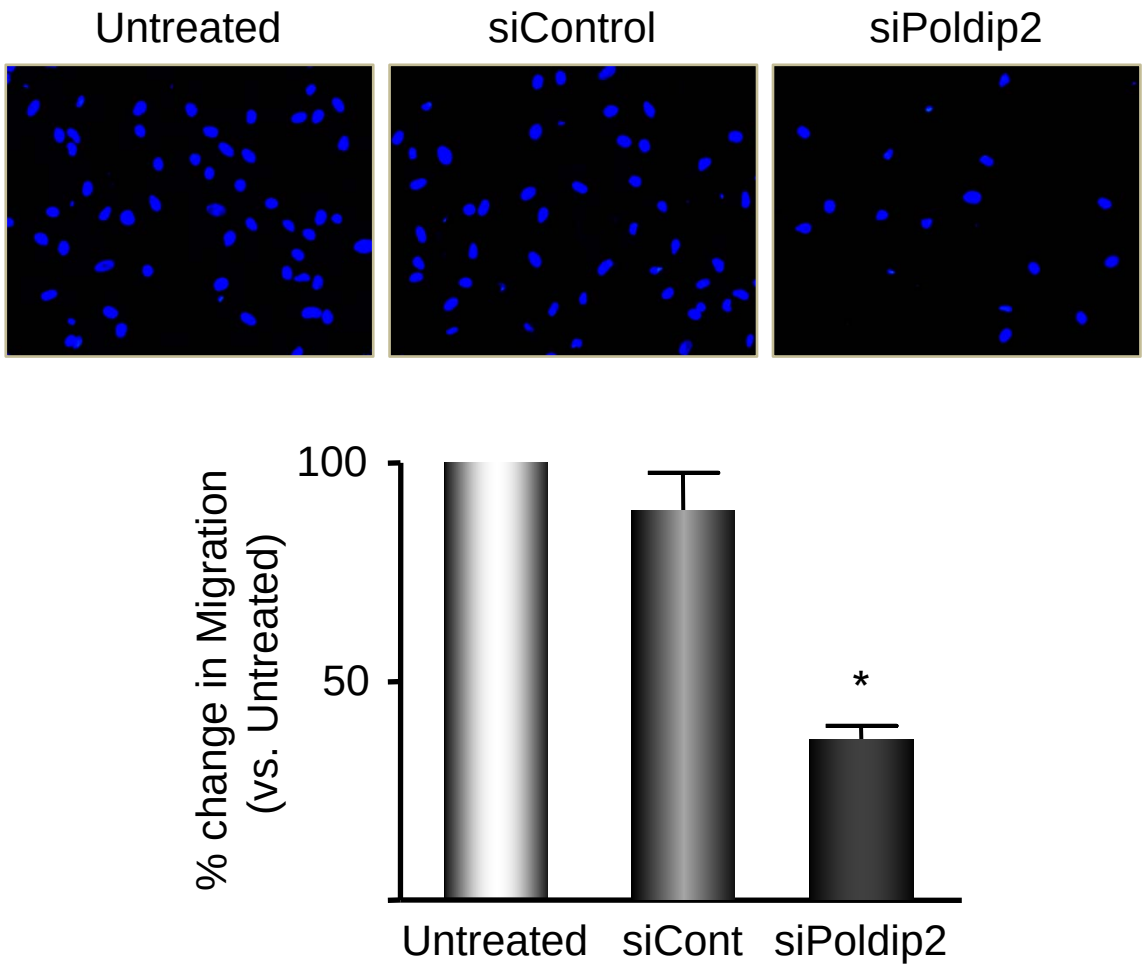


Figure 4.16. siPoldip2 Decreases Vascular Smooth Muscle Cell Migration. Rat VSMCs were transiently transfected with no siRNA (Untreated), control siRNA (siCont), or siRNA against Poldip2 (siPoldip2) prior to using the Boyden Chamber Assay to measure cell migration in response to 10 ng/mL platelet-derived growth factor (PDGF) for 4 h. Bars are means ± S.E.M. of 3 independent experiments. *p<0.01 vs. siCont.

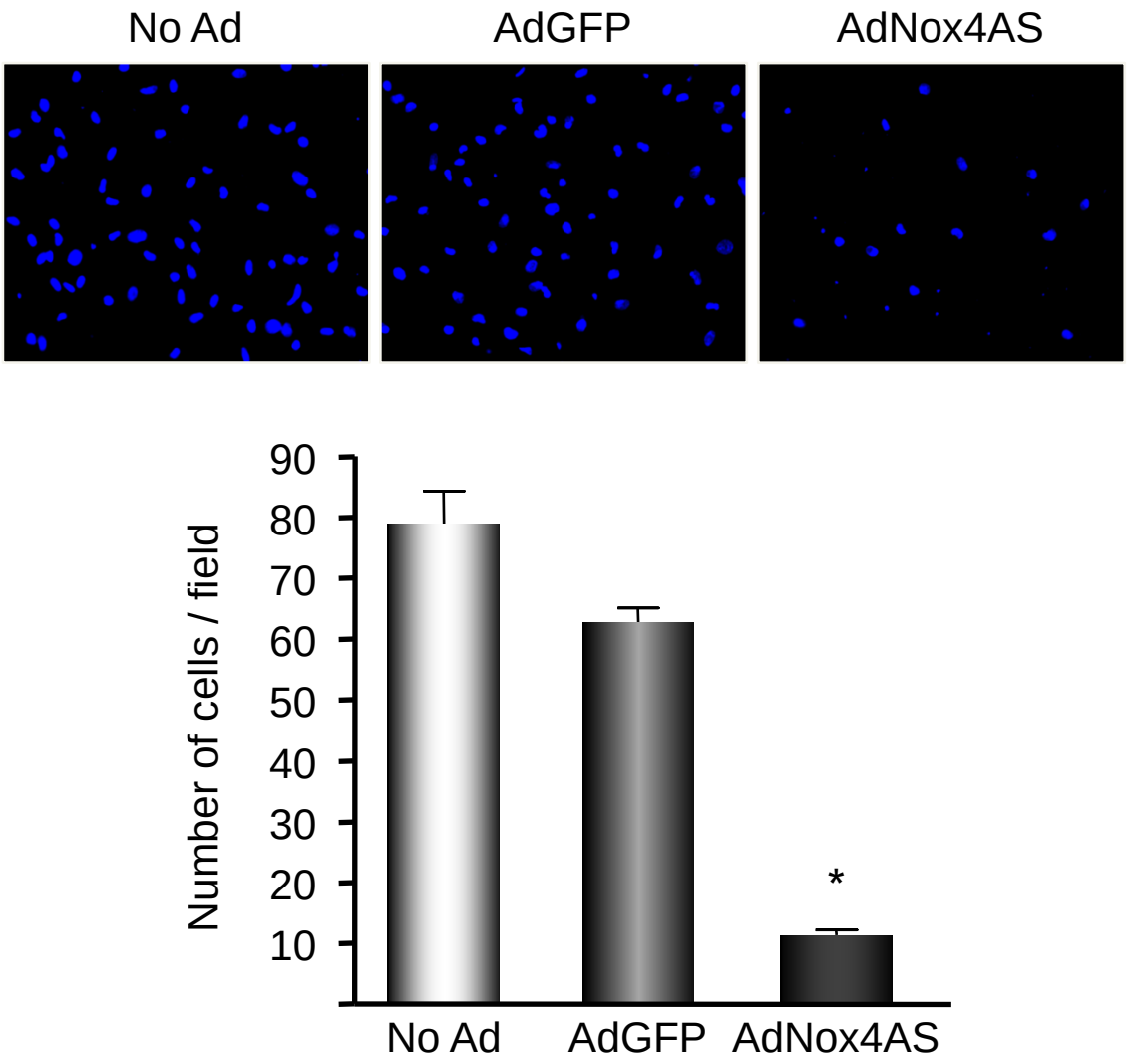


Figure 4.17. Nox4 Antisense Decreases VSMC Migration. Rat VSMCs were transduced with no adenovirus (No Ad), control adenovirus (AdGFP) or adenovirus expressing antisense Nox4 (AdNox4AS) prior to using the Boyden Chamber Assay to measure cell migration in response to 10 ng/mL platelet-derived growth factor (PDGF) for 4 h. Bars are means ± S.E.M. of 3 independent experiments. *p<0.01 vs. AdGFP.

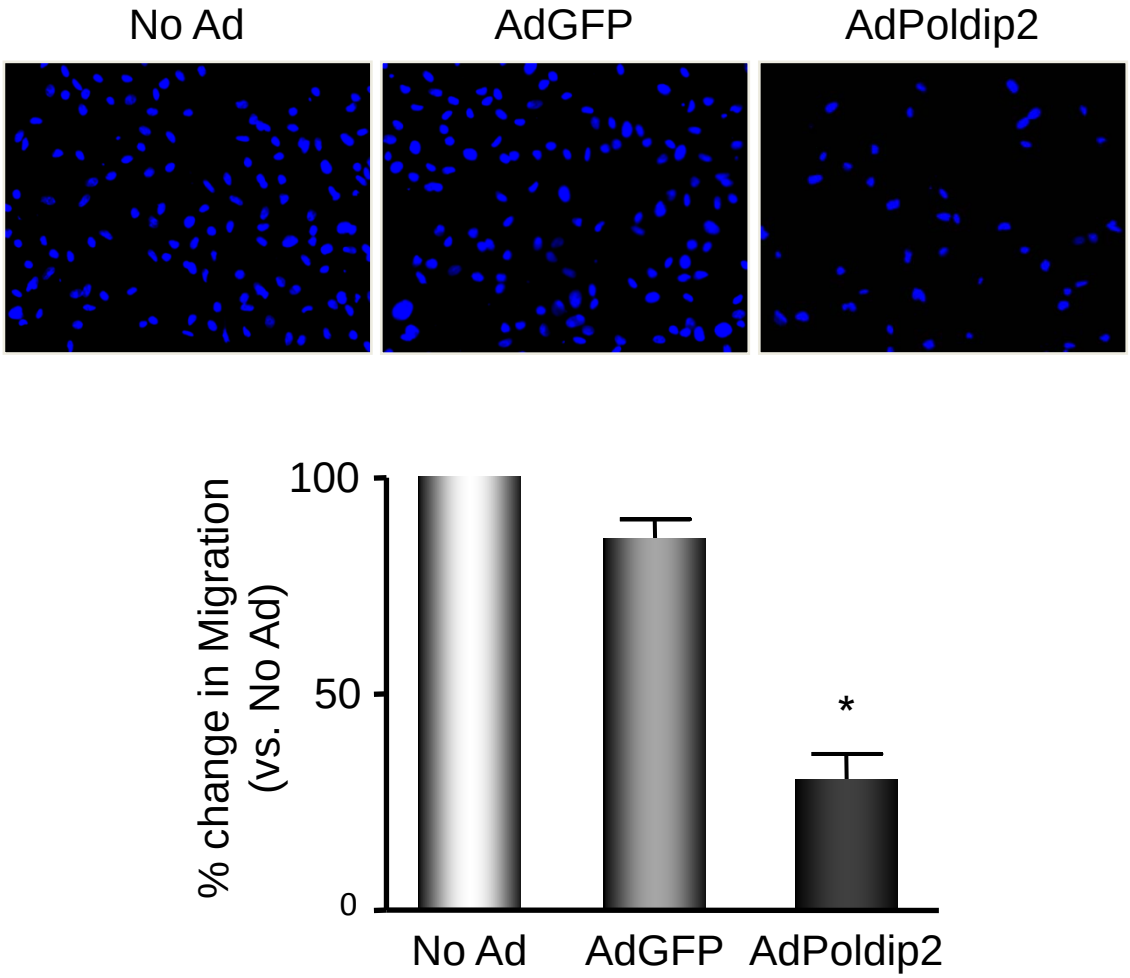


Figure 4.18. Poldip2 Overexpression Decreases Vascular Smooth Muscle Cell Migration. Rat VSMCs were transduced with no adenovirus (No Ad), control adenovirus (AdGFP) or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) prior to using the Boyden Chamber Assay to measure cell migration in response to 10 ng/mL platelet-derived growth factor (PDGF) for 4 h. Bars are means $\pm$ S.E.M. of 3 independent experiments. *$p<0.01$ vs. AdGFP.

4.4. Discussion

As noted earlier, the fact that Nox4 regulates such diverse physiological and pathophysiological responses suggests that it modulates a fundamental cellular process that may influence multiple functions such as proliferation, apoptosis, senescence, and migration. The association between Poldip2 and Nox4 predicts that Poldip2 should mediate these functions as well. Our data clearly implicate the cytoskeleton as a target of Poldip2. As shown in Figures 3.14 and 4.1, knockdown of Poldip2 using siRNA promotes VSMCs to become elongated and spindly, with few detectable focal adhesions and wavy stress fibers. In contrast, overexpression of Poldip2 increases the thickness of stress fibers and causes focal adhesions to elongate and mature (Figures 4.4 and 4.5). It is likely that these effects are mediated through Nox4, since knockdown of Nox4 yields a similar phenotype (Figures 3.14 and 4.3) and previous studies have linked ROS directly to the regulation of the actin cytoskeleton by showing that actin polymerization is blocked by the flavin oxidase inhibitor diphenylene iodonium (DPI) (Moldovan et al., 2000). Interestingly, oxidative modifications of focal adhesion proteins were shown to be induced by the overexpression of a NADPH oxidase component, p47phox (Wu, 2005), which suggests the possibility that ROS produced in focal adhesions may regulate the integrity of these structures by specifically modifying resident proteins. The functional data provided in this chapter strongly implicate Poldip2/Nox4 as the Nox responsible for the modulation of focal adhesions in VSMCs, especially given their localization to focal adhesion structures (Figures 2.6, 2.7, 2.12, and 2.13), the

loss of these vital structures upon knockdown of either of these proteins (Figures 3.14, 4.1, and 4.3), and the strengthening of focal adhesion structures upon overexpression of Poldip2 (Figure 4.4).

A major target of the Nox4/Poldip2 complex appears to be RhoA, which is known to be critical in the process of focal adhesion maturation and turnover, as well as stress fiber formation. Poldip2 overexpression significantly increases RhoA activation (Figure 4.7), and this increase is blocked by the potent antioxidant NAC (Figure 4.8). The phenotypic changes initiated by the overexpression of Poldip2 are blocked by dominant negative RhoA (Figure 4.14). Moreover, expression of constitutively active RhoA reverses the loss of stress fibers and focal adhesions induced by knockdown of Poldip2 (Figure 4.13). Additionally, our data suggest that Poldip2/Nox4 mediates these effects through generation of ROS that function to oxidize a target found upstream of RhoA activation and known to function in maintenance of focal adhesions, PTP-PEST (Figure 4.10). The ability of PTP-PEST, known to be inactivated by ROS (Heneberg and Draber, 2005), to regulate multiple cytoskeleton-associated functions most likely derives both from its phosphatase activity towards focal adhesion substrates and from its proline-rich regions that may allow it to serve as a scaffold for assembly of oxidant-sensitive focal adhesion signaling complexes, thereby explaining the extreme loss of focal adhesion complexes in siPoldip2 (Figure 4.1) or siNox4 (Figure 4.3) treated VSMCs. RhoA is one of the most important factors regulating focal adhesion turnover. Basal Rho activity is required to maintain cell substrate adhesion (Nobes and Hall, 1999), the

maturation of focal adhesions from focal complexes is mediated by Rho-dependent actin-myosin contraction of stress fibers (Chrzanowska-Wodnicka and Burridge, 1996), and focal adhesion dissolution requires inhibition of Rho (Iwanicki et al., 2008). Rho also regulates microtubule stabilization (Bartolini et al., 2008), suggesting an additional mechanism to explain the siPoldip2 phenotype.

The pronounced effects of Poldip2/Nox4 on a number of cytoskeletal changes suggests that other cytoskeleton-mediated events, such as cell division and proliferation, as well as protein trafficking, may also be influenced by the Poldip2/Nox4 axis, as will be evaluated further in Chapters 5 and 6 of this body of work. Moreover, these new findings explain why both Nox1 and Nox4 are required for PDGF-induced VSMC migration. Our results establish that either upregulation or downregulation of Poldip2/Nox4 negatively impacts focal adhesion turnover, which is required for proper cell migration; therefore, one would expect that the alteration of Poldip2 expression or Nox4 expression would impair VSMC migration, which was indeed found to be the case (Figures 4.16, 4.17, and 4.18). These new data on Nox4/Poldip2 and focal adhesion turnover allow us to conclude that Nox1 is clearly involved in signaling events at the cell membrane, regulating cofilin-mediated actin remodeling (Lee et al., 2009; San Martin et al., 2008), while Nox4/Poldip2 likely regulates focal adhesion turnover in the trailing edge of the cell, both of which are required events for forward cell progression in VSMC migration.

In this regard, it is of interest to examine what little is presently known about the functions of Poldip2. Poldip2 (also known as PDIP38 and Mitogenin I) was originally identified as a PCNA- and DNA polymerase δ-interacting protein, implicating a possible function in the regulation of gene expression, DNA duplication, or DNA repair (Liu et al., 2003). In the present context, this is of particular import because we also see Nox4 in the nucleus, but the role of Nox4 in these processes is unknown. While Poldip2 is reportedly associated with the mitochondria, these studies were performed in HeLa cells transfected with GFP-tagged Poldip2, which may confer different subcellular localizations compared to endogenous Poldip2 (Arakaki et al., 2006; Xie et al., 2005). In fact, a more recent study reported localization of Poldip2 in the nucleus, cytoplasm and plasma membrane in epithelial and endothelial cells (Klaile et al., 2007). Interestingly, these authors found an association between Poldip2 and carcinoembryonic antigen-related cell adhesion molecule-1 (CD66a) at the plasma membrane, which induced shuttling of Poldip2 to the nucleus, a process that requires cytoskeletal integrity. Additional reports link Poldip2 to mitotic spindle organization and chromosome segregation, which both require proper microtubule and cytoskeletal dynamics (Klaile et al., 2008). Taken in conjunction with the observations reported here, these data substantiate a role for Poldip2 as a novel modulator of cytoskeletal coordination, a function important in VSMC migration and endothelial barrier function (Bogatcheva and Verin, 2008).

Given our recent findings, we propose that Poldip2 functions to regulate Nox4 enzymatic activity and, thereby, to modulate the cytoskeleton via RhoA and

that this principal mechanism may underlie many of the previously described functions of Poldip2. While Poldip2 likely has additional binding partners, its association with Nox4 seems to be a major determinant of cell phenotype. Conversely, previous knowledge of cell processes affected by Poldip2 suggests potential new functions for Nox4 in the regulation of DNA repair. The identification of Poldip2 as a Nox4 regulatory protein provides an important new mechanism for regulation of basal ROS production. While previous studies have focused on transcriptional control of Nox4 as the principal mechanism of regulation (Serrander et al., 2007), our data implicate Poldip2 as an additional critical regulator not only of Nox4 activity, but also of its subcellular localization, which is investigated further in Chapter 6. It is thus conceivable that the interaction of these two proteins to coordinate ROS generation and cytoskeletal organization represents a novel mechanism that explains their shared and individual functions.

CHAPTER 5

Poldip2 Controls Vascular Smooth Muscle Cell Ploidy through Modulation of Cytoskeletal Integrity

5.1. Introduction

The pronounced effects of Poldip2/Nox4 on cytoskeletal integrity suggest that cytoskeleton-mediated events other than migration, such as cell division and proliferation, may also be influenced by the Poldip2/Nox4 axis. The cell cycle is one of the essential biological processes of life. The process of cell-cycle progression is highly ordered and ensures high fidelity replication of a cell's genome and subsequent division into two identical daughter cells. A growing body of evidence links ROS produced by Noxes to cell cycle regulation in VSMCs. Previous studies in VSMCs indicate that H_2O_2 both promotes growth and induces apoptosis, depending on its concentration, and several cell cycle regulatory proteins that control translation initiation and gene expression are ROS sensitive (Deshpande et al., 2002). ROS generation was shown to occur during cell cycle progression at both the G_1/S and G_2/M checkpoints (McCrann et al., 2009b; Menon et al., 2003). More specifically, Nox1-dependent ROS production modulates cyclin D1 and thereby regulates G_1/S transition (Ranjan et al., 2006), whereas Nox4-dependent ROS production alters the protein tyrosine phosphatase cdc25, and thereby modulates Cyclin B activation to regulate G_2/M transition and cell polyploidy (Yamaura et al., 2009). Because the cell cycle is such a critical and fundamental cellular function, gaining further insight into how the Nox4/Poldip2 axis functions to regulate cell cycle control will be investigated in this chapter.

5.2. Materials and Methods

5.2.1. Cell Culture

Rat VSMCs were grown in Dulbecco's Modified Eagle's Media (DMEM; Invitrogen, Carlsbad, CA) as described in 2.2.1. Cells at passages 6 to 12 were used for the immunocytochemistry and western analysis experiments and cells at passage 2 were used for FACS analysis experiments.

5.2.2 Antibodies

The following commercial antibodies were used: the cdc2, phospho-cdc2 Tyr15, and Myc-tag antibodies were all purchased from Cell Signaling (Danvers, MA).

5.2.3 Immunoblotting

VSMCs were lysed in Hunter's buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 0.1 mmol/L Na-orthovanadate, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, and protease inhibitors). Whole cell lysates were utilized for immunoblotting (IB) experiments. For IB analysis, proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with appropriate primary antibodies, as described in 2.2.7. Primary antibodies were used at the following dilutions for immunoblotting: 1) cdc2 at 1:1000, 2) phospho-cdc2 at 1:1000, and 3) Myc at 1:1000. Band intensity was quantified by densitometry using ImageJ 1.38 software.

5.2.4 Immunocytochemistry and Confocal Microscopy

VSMCs were plated on collagen-coated glass coverslips (BD Biosciences, Bedford, MA) and grown to 50-60% confluence before transducing for 72 h with recombinant adenovirus to express control (AdGFP) or Myc-tagged Poldip2

(AdPoldip2). Cells were rinsed, fixed, permeabilized, and quenched as described in 2.2.9. Cells were incubated for 1 h in blocking buffer (3-5% bovine serum albumin in PBS) prior to incubating with fluorophore-conjugated Phalloidin (The Jackson Laboratory, Bar Harbor, Maine) diluted at 1:100 for 1 hour at room temperature. Nuclei were labeled with DAPI for 10 minutes at room temperature. Cells on coverslips were mounted onto glass slides in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and image acquisition was performed using a confocal microscope as described in 2.2.9. When comparing cells from different treatment groups, all image threshold settings of the confocal microscope remained constant.

5.2.5 Adenoviruses

The AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA) was used to prepare viruses with either no insert (AdGFP) or Myc-tagged Poldip2 (AdPoldip2). VSMCs were transduced with recombinant adenoviruses for 2 h at 37°C in serum-free DMEM as described in section 2.2.5

5.2.6. FACS Analysis

After the desired treatment, cells were trypsinized and washed with cold PBS prior to fixing with 70% ethanol. Fixed cells were stained with Propidium Iodide (0.1 mg/mL; Sigma-Aldrich, St. Louis, MO), filtered, and FACS analysis was performed as described in detail in section 3.2.12. Raw cell count data was collected and analyzed using FlowJo: Flow Cytometry Analysis Software (Tree Star, Inc., Ashland, OR).

5.2.7 Statistical Analysis

Results are expressed as mean ± S.E.M. from at least three independent experiments. Significance of statistical comparisons was assessed using analysis of variance (ANOVA), followed by Bonferroni's Multiple Comparison post-hoc test. A value of $p<0.05$ was considered significant.

5.3. Experimental Results

5.3.1. Overexpression of Poldip2 Increases the Frequency of Polyploidy in Vascular Smooth Muscle Cells

As shown in Chapter 4, modulation of Poldip2/Nox4 results in pronounced cytoskeletal changes. Therefore, we set out to determine if other processes dependent on cytoskeleton-mediated events, such as cell division and proliferation, may also be influenced by the Poldip2/Nox4 axis. One of our preliminary observations while working with VSMCs overexpressing Poldip2 was an increase in the frequency of bi-nucleated cells (Figure 4.4 and 5.1), suggesting a misregulation of cell division. To further quantify this observation, we performed fluorescence-activated cell sorting (FACS) analysis on cells that were not transduced (No Ad) or that were transduced for 72 hours with control adenovirus (AdGFP) or adenovirus to overexpress Poldip2 (AdPoldip2), and stained with propidium iodide (PI), which intercalates and labels DNA. Cellular DNA content was quantified as diploid (2N) or polyploidy (4N and greater). As shown in Figure 5.2, Poldip2 overexpression increases the mean number of tetraploid VSMCs; however, this was trend was not statistically significant.

5.3.2. Poldip2 Regulates Cell Cycle Progression

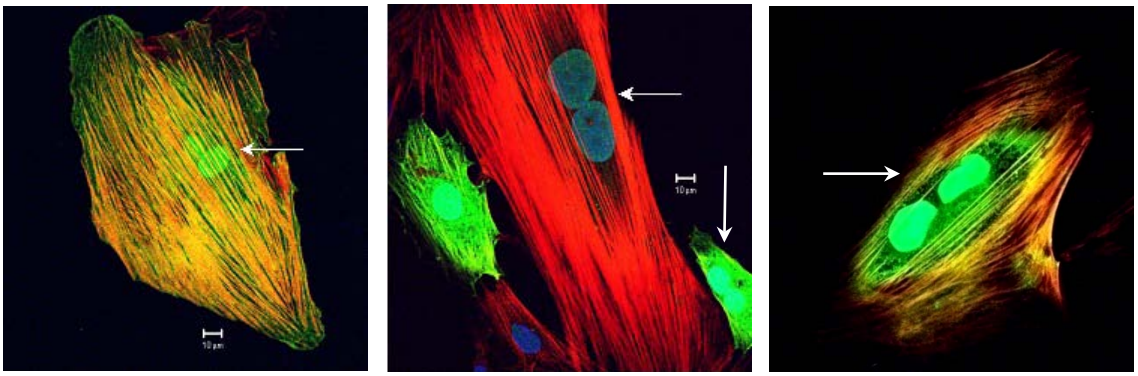


Figure 5.1. Overexpression of Poldip2 Increases the Frequency of Polyploid, Bi-nucleated VSMCs. Rat VSMCs transduced with adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 hours were single labeled with Phalloidin (red) to detect stress fibers. Cells transduced with AdPoldip2 appear green from GFP. Nuclei are labeled with DAPI (blue). Arrows indicate polyploid, bi-nucleated cells. Scale bars,10 µm.

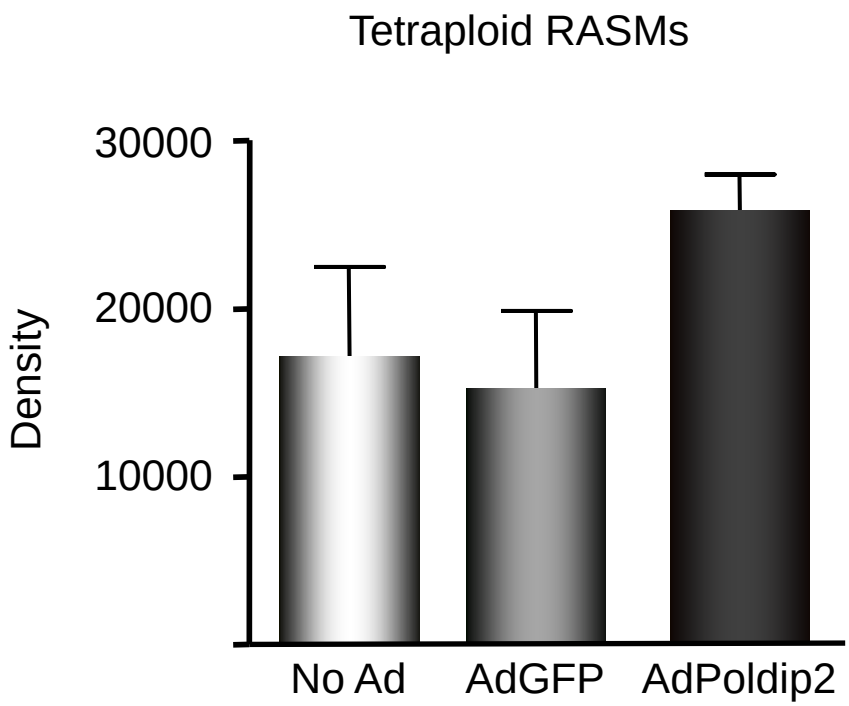


Figure 5.2. Effect of Poldip2 Overexpression on VSMC Polyploidy, as Measured by FACS Analysis. Passage 2 rat VSMCs were transduced with no adenovirus (No Ad), control adenovirus (AdGFP) or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 hours prior to labeling with propidium iodide. Cellular DNA content was quantified as diploid (2N) or polyploid (4N and greater) by FACS analysis. Bars are mean $\pm$ S.E.M. of 3 independent experiments. No statistical difference was found.

Polyploidy, however, is a common indication that the cell cycle is misregulated within the G2/M phase; therefore, we hypothesized that increased ROS production by Nox4, via Poldip2, regulates cell cycle progression through this phase. To investigate this concept, we performed FACS analysis on VSMCs that were not transduced (No Ad) or that were transduced for 72 hours with control adenovirus (AdGFP) or adenovirus to overexpress Poldip2 (AdPoldip2), and stained with propidium iodide (PI) to quantify cellular DNA content according to their position in the cell cycle. Poldip2 overexpression in VSMCs significantly increases the percentage of cells in G_2/M phase (Figure 5.3). These data, together with the trend toward an increase in polyploidy in Poldip2 overexpressing cells, suggest that ROS production by Nox4/Poldip2 may regulate the G_2/M phase of the cell cycle.

Transition through the G_2/M checkpoint requires two primary alterations in the protein cell division cycle 2 (cdc2), which then allow cyclin B to be activated and the cell to progress through mitosis. Cdc2 requires: 1) phosphorylation on Thr161 by cyclin activated kinase (CAK) and 2) dephosphorylation of Tyr15 by the protein tyrosine phosphatase (PTP) cdc25. Because of the known sensitivity of phosphatases, including cdc25, to ROS (Rudolph, 2005a; Rudolph, 2005b; Sarsour et al., 2009), we hypothesized that Poldip2 blocks G_2/M transition in VSMCs by blocking dephosphorylation of cdc2 on Tyr15 by cdc25. To investigate this idea, we measured the phosphorylation of cdc2 on Tyr15 in cells transduced with AdGFP or AdPoldip2 for 72 hours. As shown in Figure 5.4, basal cdc2 phosphorylation on Tyr15 is substantially higher in VSMCs

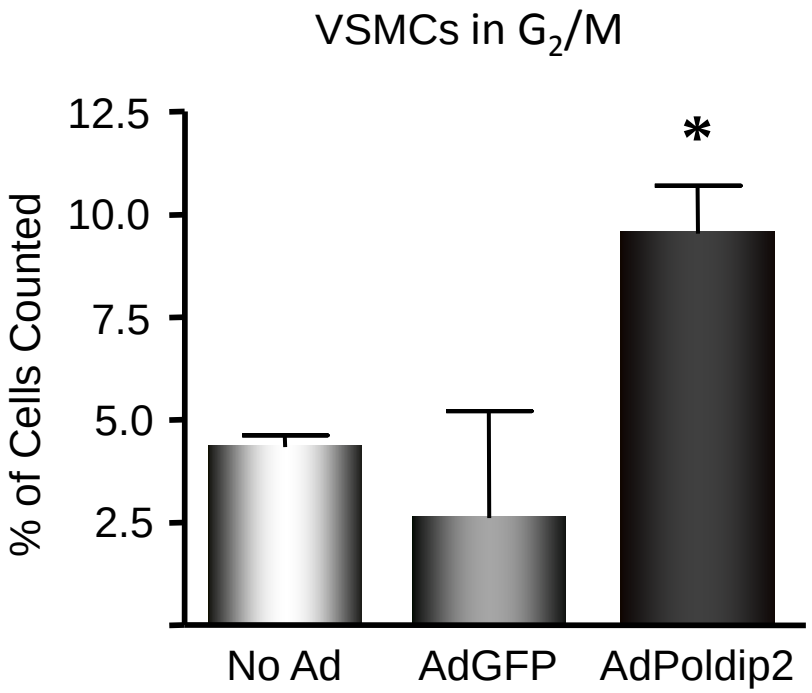


Figure 5.3. Overexpression of Poldip2 Increases the Number of Cells in G_2/M of the Cell Cycle, as Measured by FACS Analysis. Passage 2 rat VSMCs were transduced with no adenovirus (No Ad), control adenovirus (AdGFP) or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 hours prior to labeling with propidium iodide. Cellular DNA content was used to quantify cellular position in the cell cycle by FACS analysis. Bars are mean ± S.E.M. of 3 independent experiments. *$p<0.05$ vs. AdGFP.

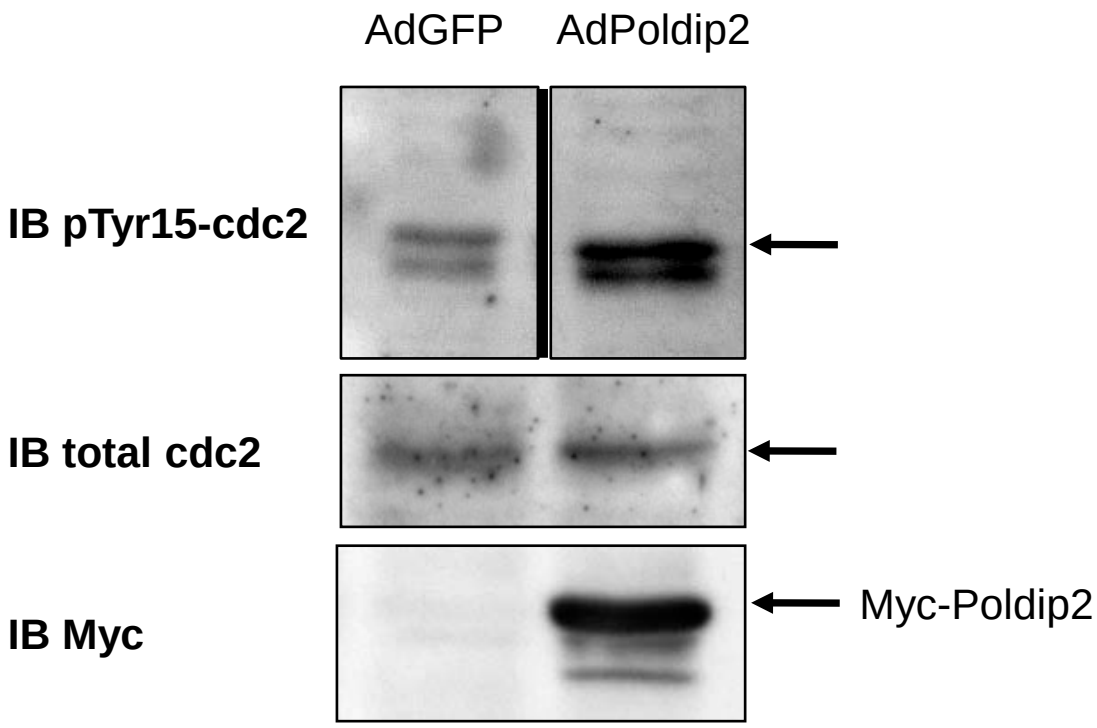


Figure 5.4. Overexpression of Poldip2 Increases Basal cdc2 Phosphorylation. Rat VSMCs were transduced with control adenovirus (AdGFP) or adenovirus to overexpress Myc-Poldip2 (AdPoldip2) for 72 hours prior to western analysis to assess phosphorylation of cdc2 on Tyr15 and total cdc2 protein levels. Lysates were also immunoblotted for Myc to show overexpression of Myc-Poldip2.

overexpressing Poldip2; however, total cdc2 protein levels are unaffected. Whether this increase in phosphorylation is due to the increase in ROS caused by Poldip2 overexpression and thus oxidation and inactivation of cdc25, the phosphatase required to dephosphorylate cdc2 at this residue, remains to be investigated.

5.4. Discussion

The initial observation that the overexpression of Poldip2 in VSMCs appears to increase the frequency of tetraploid cells (Figures 5.1, 5.2) led us to further investigate if Poldip2 modulates progression through the G_2/M phase of the cell cycle. FACS analysis on VSMCs overexpressing Poldip2 showed that cells accumulate in the G_2/M phase of the cell cycle (Figure 5.3). It is known that several regulators of the cell cycle are ROS sensitive and that ROS can specifically modify signaling molecules such as PTKs and PTPs. Therefore, we hypothesized that the increased Nox4-dependent ROS production caused by the overexpression of Poldip2, as shown in Chapter 2, blocks G_2/M transition in VSMCs by blocking dephosphorylation of cdc2 on Tyr15 by cdc25, which is required for cyclin B activation. As shown in Figure 5.4, basal phosphorylation on Tyr15 of cdc2 is higher in cells overexpressing Poldip2, suggesting that the phosphatase required to dephosphorylate cdc2, cdc25, may be inactivated, potentially by an increase in oxidation of cdc25s Cys residues. This, however, remains to be further investigated.

Several publications released within the last year have linked Nox4 to polyploidy and regulation of the transition through the G_2/M checkpoint of the cell

cycle. Polyploidy, a common phenotypic consequence of misregulation of mitosis, was shown to increase in VSMCs in vivo and this increase in frequency of polyploidy correlated with an increase in Nox4 protein expression (McCrann et al., 2009b). Interestingly, ROS generation was shown recently to increase during cell cycle progression at the G_2/M checkpoint (McCrann et al., 2009b; Menon et al., 2003). Additionally, Nox4-dependent ROS production in melanoma cells hyperphosphorylates the protein tyrosine phosphatase cdc25, thereby modulating Cyclin B activation to regulate transition through the G_2/M cell cycle checkpoint (Yamaura et al., 2009). Further investigation into the mechanism suggested that cdc25 hyperphosphorylation promotes an increase in binding of cdc25 to 14-3-3, and thus inhibits cdc25 from dephosphorylating cdc2 (Yamaura et al., 2009); however, whether Nox4 promotes an increase in oxidation of the PTP cdc25, or alters its binding affinity for 14-3-3, was not investigated. Determining the mechanism by which the Nox4/Poldip2 axis functions to regulate transition through the G_2/M cell cycle checkpoint, be it through oxidation of cdc25 or alteration of an additional target protein required for cell cycle control, would further increase our understanding of the involvement of Nox4/Poldip2 in multiple pathologies in which cell proliferation is a key component, such as atherosclerosis and restenosis after angioplasty.

CHAPTER 6

Poldip2 Influences the Proper Trafficking of Nox4 and p22phox to Focal Adhesions

6.1. Introduction

Many proteins localize to their proper subcellular compartments by trafficking either 1) from the ER directly to their subcellular compartment or 2) from the trans-face of the Golgi, after having undergone further post-translational modifications (Marie et al., 2008). Very little is known about the trafficking of Nox proteins. As shown in Figure 4.6, we observed that in the absence of Poldip2, Nox4 and p22phox no longer localize to focal adhesions in VSMCs. Therefore, Poldip2 may function as a co-factor to ensure proper Nox4/p22phox complex localization to the focal adhesion compartment in VSMCs, perhaps through modulation of protein trafficking. If Poldip2 is indeed required for proper trafficking of the Nox4/p22phox complex, one would expect that in cells with low or absent Poldip2 expression, these proteins would no longer traffic in VSMCs. Recent evidence indicates that other Nox homologues, namely Duox1 and Duox2, require the co-expression of a DuoxA2 maturation factor for proper processing and localization, otherwise the Duox protein is retained in the ER and is unable to properly traffic to the plasma membrane (Grasberger et al., 2007; Grasberger and Refetoff, 2006; Zamproni et al., 2008). How individual Noxes traffic to their subcellular compartments and what promotes differential subcellular localization is poorly understood and remains to be investigated.

6.2. Methods

6.2.1. Cell Culture

Rat VSMCs were grown in Dulbecco's Modified Eagle's Media (DMEM; Invitrogen, Carlsbad, CA) as described above in 2.2.1. Cells at passages 6 to 12 were used for all experiments.

6.2.2 Antibodies

p22phox antibody used was kindly provided by Dr. Mark Quinn (Montana State University) and the Nox4 antibody was provided by Dr. David Lambeth (Emory University). The PDGFR-β antibody used was purchased from Santa Cruz (Santa Cruz, CA), the GM130 antibody was from Abcam (Cambridge, MA), and the GRP78 antibody was purchased from Cell Signaling (Danvers, MA).

6.2.3 Immunocytochemistry and Confocal Microscopy

VSMCs were trypsinized and plated at 40-50% confluence on collagen-coated glass coverslips (BD Biosciences, Bedford, MA). After 4-6 h, cells were washed with serum-free OPTI-MEM (Invitrogen, Mountain View, CA), and incubated with siRNA + Oligofectamine complexes for 48 h. Cells were incubated in fresh serum free OPTI-MEM for an additional day. Cells were rinsed, fixed, permeabilized, and quenched as described in 2.2.9. Cells were incubated for 1 h in blocking buffer (3-5% bovine serum albumin in PBS) prior to incubating with primary antibody for 1 hour at room temperature. Cells were then incubated for 1 hour at room temperature with fluorophore-conjugated secondary antibodies purchased from The Jackson Laboratory (Bar Harbor, Maine). Nuclei were labeled with DAPI for 10 minutes at room temperature. Cells on coverslips were mounted onto glass slides in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and image acquisition was performed using a confocal

microscope as described in 2.2.9. The primary antibodies used for immunocytochemistry experiments include: 1) Nox4 at 1:50, 3) p22phox Rb5554 at 1:50, 4) PDGFR-β at 1:50, 5) GM130 at 1:150, 6) GRP78 at 1:50, and 7) DAPI (nuclei) at 1:1000. When comparing cells from different treatment groups, all image threshold settings of the confocal microscope remained constant.

6.2.4 siRNA

For transfection with siRNA, VSMCs were trypsinized and plated at 40-50% confluence on collagen-coated substrate. After 4-6 h, cells were washed with serum-free OPTI-MEM (Invitrogen, Mountain View, CA), and incubated with siRNA + Oligofectamine complexes as described in detail in section 3.2.3.

6.2.5 Immunoblotting

VSMCs were lysed in Hunter's buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 0.1 mmol/L Na-orthovanadate, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, and protease inhibitors). Whole cell lysates were utilized for immunoblotting experiments. For IB analysis, proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with appropriate primary antibodies. The GRP78 antibody was used at a dilution of 1:1000 for immunoblotting. Proteins were detected by ECL (GE Healthcare). Band intensity was quantified by densitometry using ImageJ 1.38 software.

6.2.6. Protein Trafficking

VSMCs were transfected with 0 siRNA, siControl, or siPoldip2 for 3 days prior to performing protein trafficking experiments. Cellular proteins were allowed to accumulate in the ER and Golgi compartments by incubation at 19.5°C for 4 hours total, the last 2 hours of which the cells were treated with cycloheximide (100 μg/mL) to block new protein translation and synthesis, thus allowing a single pool of protein to accumulate in that cellular compartment. Upon release by transferring cells back to 37°C, trafficking of that single pool of protein out of the ER/Golgi compartment was followed over time. Cells were then fixed and labeled following standard immunocytochemistry procedures 90 minutes after release at 37°C.

6.3. Experimental Results

6.3.1. Poldip2 is Required for Proper Nox4 and p22phox Localization and Trafficking

The absence of Nox4 and p22phox from focal adhesions in VSMCs with depleted Poldip2 protein levels, as shown in Figure 4.6, raises the possibility that Poldip2 functions to regulate subcellular targeting of these two proteins. A possible role for Poldip2 in trafficking was tested using a combination of cold (19.5°C) to halt trafficking and cycloheximide (100 μg/mL) to prevent new protein synthesis. Confocal microscopy was then used to visualize the release and trafficking of p22phox and Nox4 from the Golgi or central ER after these treatments. In the steady state (SS, cells maintained at 37°C), Nox4 (Figure 6.1, b) and p22phox (Figure 6.2) are distributed in a punctate pattern spread out from the Golgi. Note that these images were acquired at the Golgi/ER plane of the

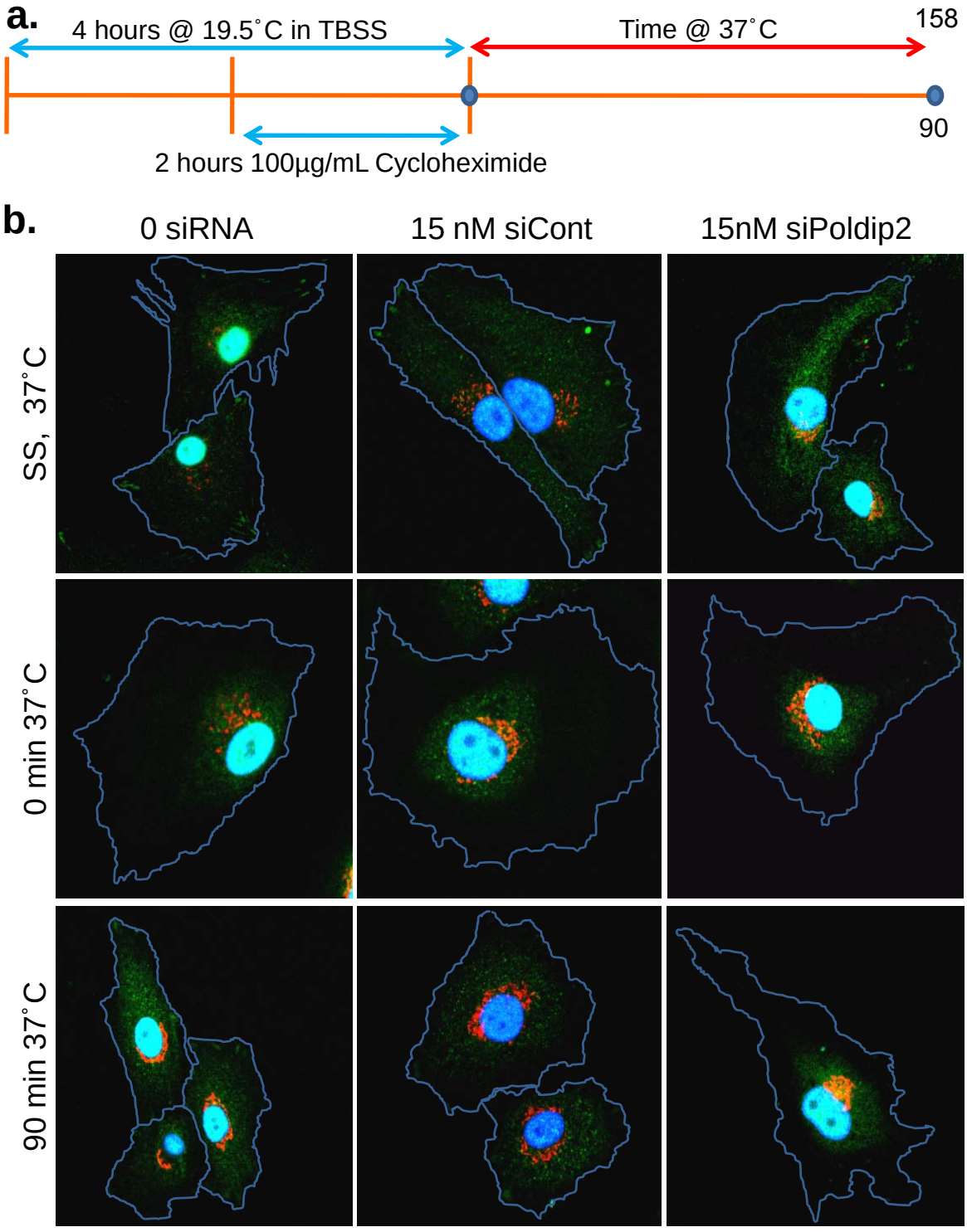


Figure 6.1. siPoldip2 Decreases the Trafficking of Nox4 to Focal Adhesions. Rat VSMCs were transiently transfected with no siRNA (Untreated), control siRNA (siCont), or siRNA against Poldip2 (siPoldip2) prior to transferring to 19.5˚C and treating with cycloheximide or leaving at 37 ˚C (SS). Cells were then released by transfer to 37˚C for 0 or 90 min before fixing and double labeling with GM130, to label the Golgi (red), and Nox4 (green). Nuclei are labeled with DAPI (blue). The outlines of cells are shown in blue.

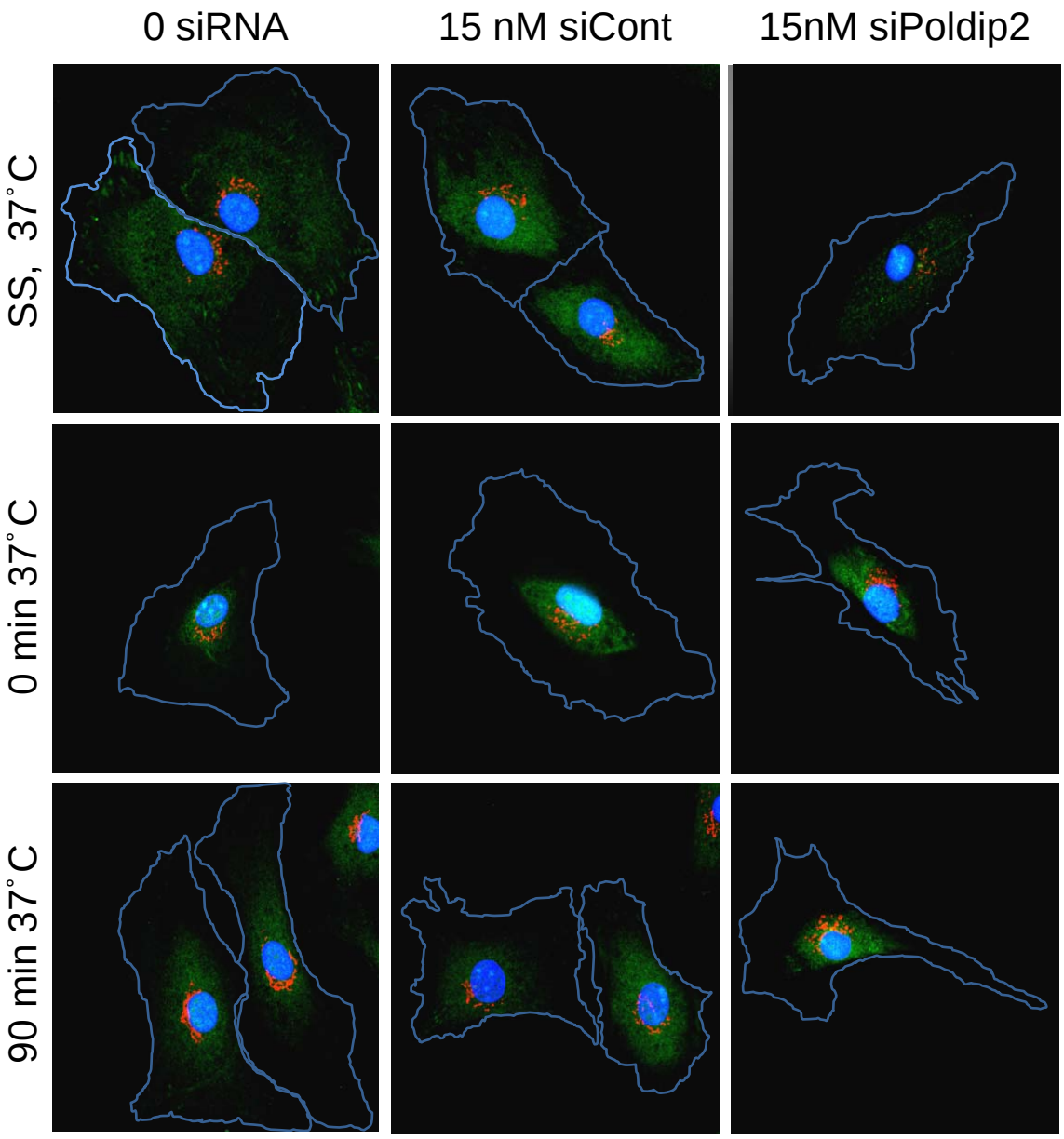


Figure 6.2. siPoldip2 Decreases the Trafficking of p22phox to Focal Adhesions. Rat VSMCs were transiently transfected with no siRNA (Untreated), control siRNA (siCont), or siRNA against Poldip2 (siPoldip2) prior to transferring to 19.5˚C and treating with cycloheximide or leaving at 37 ˚C (SS). Cells were then released by transfer to 37˚C for 0 or 90 min before fixing and double labeling with GM130, to label the Golgi (red), and p22phox (green). Nuclei are labeled with DAPI (blue). The outlines of cells are shown in blue.

cell; therefore, the focal adhesion distribution pattern for both of these proteins is not readily visible. However, after cold + cycloheximide treatment (Figure 6.1, a), these proteins were trapped in or near the Golgi compartment. Reinitiating protein trafficking by rewarming the cells to 37°C for 60-90 minutes induced trafficking out of the Golgi/ER region in untransfected cells (0 siRNA) and cells treated with control siRNA (15 nM siCont), but not in cells treated with siPoldip2. To determine if the knockdown of Poldip2 with siRNA globally blocked protein trafficking in VSMCs or specifically affected focal adhesion proteins, we chose to test if the trafficking of the PDGFR-β was affected. The PDGFR-β was selected because it is a protein that is known to traffic through the Golgi and that localizes, in VSMCs, to a compartment other than focal adhesions, specifically caveolae. Using the same experimental procedure described above, we show that the PDGFR-β in steady state cells is localized in a punctate pattern spread out from the Golgi/ER region, as well as in a caveolar pattern, though this is less clear because the image was acquired at the Golgi plane and not the base or top of the cell (Figure 6.3) to allow for visualization of the caveolae in that specific region. Incubation of cycloheximide-treated cells at 19.5°C retained the PDGFR-β in the Golgi/ER region and upon reinitiation of protein trafficking by rewarming the cell to 37°C for 90 minutes, the PDGFR-β was able to traffic out of the Golgi/ER region in untransfected cells (0 siRNA), cells treated with control siRNA (15 nM siCont), and in cells treated with siPoldip2. These data together suggest that Poldip2 is not a global mediator of protein trafficking and that Poldip2 may

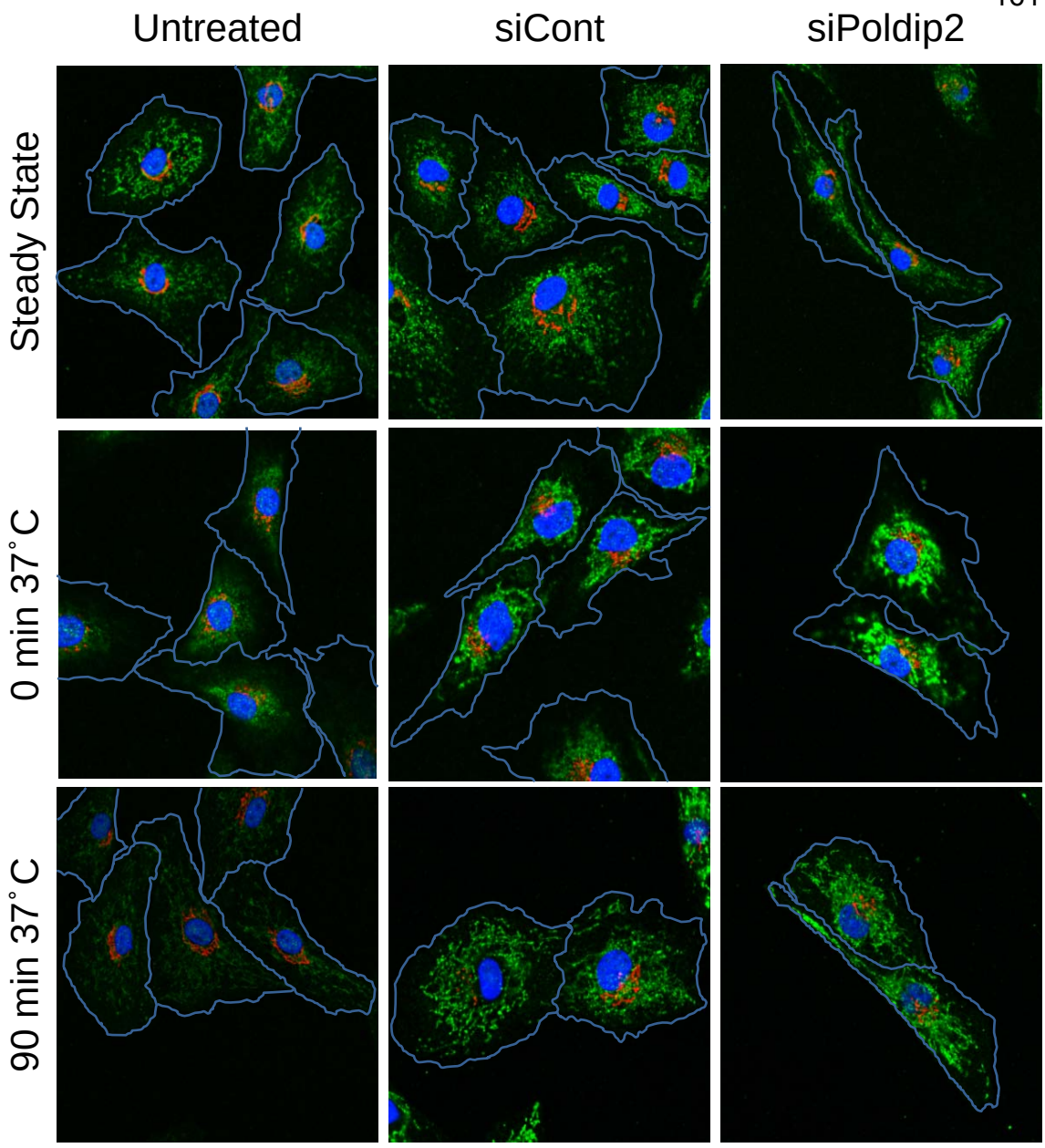


Figure 6.3. siPoldip2 Does Not Affect the Proper Trafficking of the PDGFR-β to Caveolae. Rat VSMCs were transiently transfected with no siRNA (Untreated), control siRNA (siCont), or siRNA against Poldip2 (siPoldip2) prior to transferring to 19.5˚C and treating with cycloheximide or leaving at 37˚C (SS). Cells were then released by transfer to 37˚C for 0 or 90 min before fixing and double labeling with GM130 to label the Golgi (red) and PDGFR-β (green). Nuclei are labeled with DAPI (blue). The outlines of cells are shown in blue.

specifically mediate the trafficking of Nox4 and p22phox, or of focal adhesion proteins in general.

6.3.2. Impaired Trafficking of Nox4 or p22phox Results in Increased Endoplasmic Reticulum Stress in Vascular Smooth Muscle Cells

As mentioned previously, improper protein processing or delayed trafficking of proteins from the ER/Golgi can result in an increase in ER stress and initiation of the unfolded protein response, commonly indicated by an upregulation in the ER chaperone known as GRP78 (Ron and Walter, 2007). The data in Figures 6.1 and 6.2 clearly indicate that in the absence of Poldip2, Nox4 and p22phox no longer properly traffic from the Golgi/ER to the focal adhesion compartment in VSMCs. We therefore used immunocytochemistry and western blot analysis to determine if this lack of proper trafficking of Nox4 and p22phox in siPoldip2 treated cells initiates the ER stress response, as indicated by an upregulation in GRP78. As shown in Figure 6.4a, there is a clear increase in GRP78 expression by immunocytochemistry in siPoldip2 treated cells. Western blot analysis also shows an increase in GRP78 expression in VSMCs treated with siPoldip2 when compared with siControl treated cells (Figure 6.4, b). This increase was greatest after 3-4 days of siPoldip2 treatment. Taken together, these data suggest that Poldip2 is required for proper Nox4 and p22phox trafficking to the focal adhesions and that when proper trafficking of either of these targets is blocked by knockdown of Poldip2, there is an increase in ER stress in VSMCs.

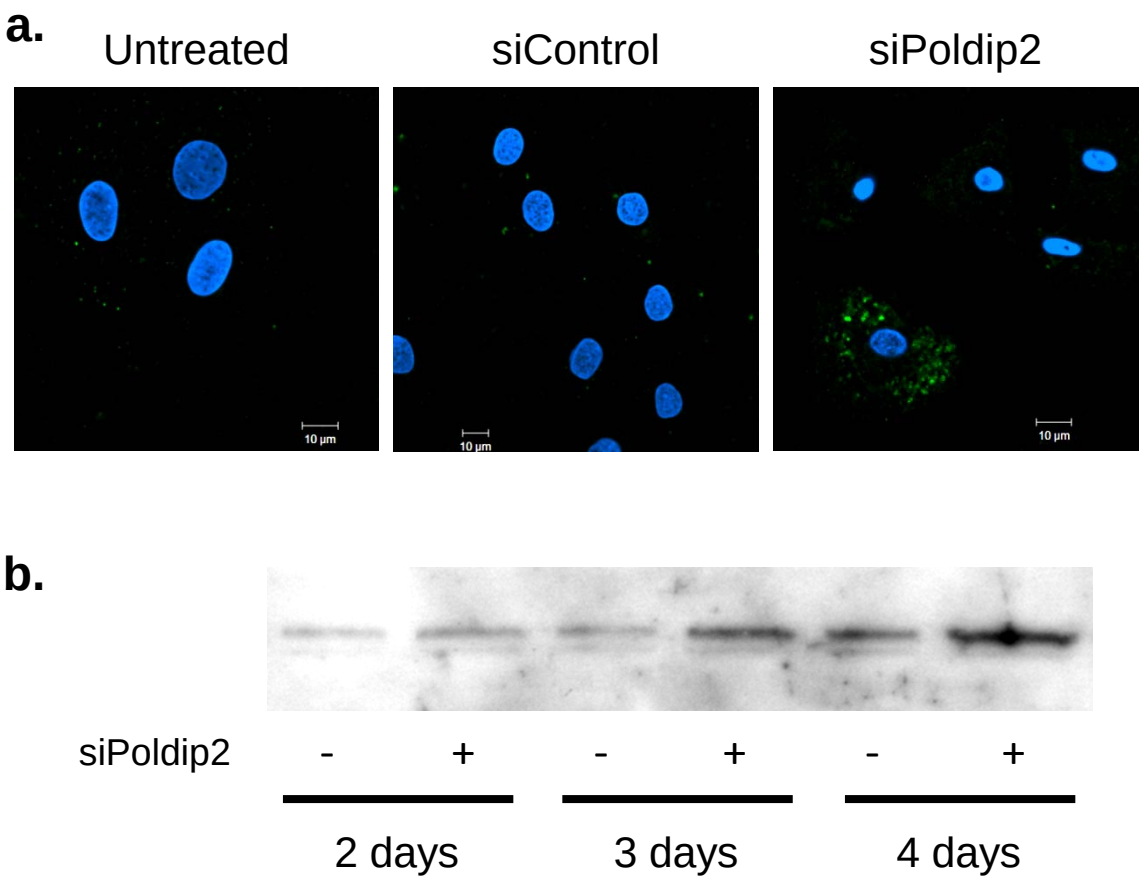


Figure 6.4. siPoldip2 Treatment Increases Expression of the ER Stress Marker GRP78. **a,** Rat VSMCs were transiently transfected with no siRNA (Untreated), control siRNA (siCont), or siRNA against Poldip2 (siPoldip2) for 4 days prior to single labeling with GRP78 (green). Nuclei are labeled with DAPI (blue). Scale bars,10 µm. **b,** Rat VSMCs were transiently transfected with control siRNA (-) or siRNA against Poldip2 (+) for 2, 3, or 4 days prior to use for western analysis to assess GRP78 protein expression.

6.4. Discussion

The complete loss of Nox4 and p22phox localization to focal adhesions in VSMCs with depleted Poldip2 protein levels, as shown in Figure 4.6, suggests that Poldip2 may function to regulate the subcellular targeting of this specific NADPH oxidase complex in VSMCs. In this chapter, we utilized a combination of cold (19.5°C) to halt trafficking and cycloheximide (100 μg/mL) to prevent new protein synthesis, to determine if Poldip2 plays a role in Nox4/p22phox trafficking in VSMCs. As shown in Figures 6.1 and 6.2, the trafficking of Nox4 and p22phox was greatly impaired siPoldip2 treated VSMCs, whereas the trafficking of PDGFR-β to caveolae, the subcellular compartment where Nox1 localizes in VSMCs, was not affected by Poldip2 knockdown (Figure 6.3). While very little is known about the trafficking of Nox proteins, these data support the concept that proper trafficking and subcellular localization are important to proper Nox function.

How individual Noxes traffic to their subcellular compartments and what promotes differential subcellular localization is poorly understood and remains to be investigated. Hilenski, et al. (Hilenski et al., 2004) previously reported that the two Noxes expressed in VSMCs, Nox1 and Nox4, localized to very distinct subcellular compartments, where Nox1 localized to caveolae and Nox4 localized to the nucleus, along stress fibers, and in focal adhesions. The localization of different Noxes seems to be cell type specific. Helmcke, et al. (Helmcke et al., 2008) examined what domains within a particular Nox determines its localization by generating a series of chimeras consisting of differing regions of Nox1 and

Nox4. In reconstituted HEK293 cells, Nox4 localized primarily to the ER and, when the N-terminal region of Nox4 was spliced with the remaining sequence for Nox1, Nox1 no longer localized to the plasma membrane, but was retained just below it in vesicle-like structures. While localization studies such as these help to shed light as to what regions of the Noxes promote specific subcellular localization patterns, most of the studies performed do not examine how these Noxes translocate or traffic within the cell. The specific domain structures that promote differential subcellular distributions may serve as binding sites for protein chaperones that enable trafficking to different compartments within the cell. The findings reported within this chapter clearly demonstrate that Poldip2 is essential for proper trafficking and localization of both Nox4 and p22phox to the focal adhesion compartment and suggest that the differential localization of Noxes may be conferred by the presence of different co-factors that aid in this trafficking process. What specific maturation factors or co-factors are required for proper processing and localization of different Noxes remains to be fully investigated.

The idea that Noxes require maturation factors or co-factors for processing and localization is not new. A recent article reported that other Nox homologues, namely Duox1 and Duox2, require the co-expression of a DuoxA2 maturation factor for proper processing and localization; otherwise, the Duox protein is retained in the ER and is unable to properly traffic to the plasma membrane (Grasberger et al., 2007; Grasberger and Refetoff, 2006; Zamproni et al., 2008). Poldip2 may serve a similar function as that of a co-factor to ensure

proper Nox4/p22phox complex localization to the focal adhesion compartment in VSMCs. When Nox4 is overexpressed in HEK293 or VSMCs, it accumulates within the ER, which could be a consequence of not simultaneously co-expressing Poldip2 to promote maturation or proper translocation from the ER to the focal adhesion compartment; however, this is speculative and remains to be confirmed experimentally. Interestingly, when Poldip2 is knocked down by siRNA, there is an increase in ER stress, suggesting that the improper protein processing or delayed trafficking of Nox4/p22phox from the ER/Golgi results in an increase in ER stress and possibly the initiation of the unfolded protein response (Figure 6.4) (Ron and Walter, 2007).

The roles of different Nox family members are distinct, including the roles of Nox1 and Nox4 in VSMCs, even though they all produce ROS. This is partly due to differential regulation by cytosolic subunits and partly due to differential subcellular localization and compartmentalization of each Nox subtype. Understanding how specific Nox subtypes are processed, trafficked, and regulated could potentially allow one to pharmacologically target, with greater specificity, different Noxes in disease pathologies.

CHAPTER 7

Discussion

7. Discussion

The data presented in Chapters 2 and 3 of this dissertation clearly demonstrate an association between Poldip2 and p22phox in VSMCs. Specifically, we illustrate that Poldip2 functionally associates with the Nox4/p22phox complex in a p22phox-dependent manner and establish Poldip2 as a novel positive modulator of Nox4 enzymatic activity. Furthermore, our functional studies indicate that Poldip2 does not positively influence Nox1 activity, and suggest that Poldip2 may potentially act as a negative modulator of Nox1 in VSMCs. These are the first reports of a protein, in any cell type, that positively regulates Nox4 activity and Nox4-dependent ROS production. Additionally, the data presented in Chapters 4 and 6 of this dissertation demonstrate that Poldip2 regulates the proper localization and trafficking of the Nox4/p22phox complex in VSMCs. Moreover, experimental findings described in Chapters 4 and 5 provide evidence that the Nox4/Poldip2 axis, through production of ROS, alters the cytoskeleton by maintaining focal adhesion integrity and stress fiber formation. Our data also link Nox4/Poldip2 to VSMC polyploidy and implicate this complex in the proper regulation of progression through the G_2/M phase of the cell cycle. Altogether, these data link ROS production by the Nox4/Poldip2 axis to the regulation of cellular functions highly dependent on the tight coordination of cytoskeletal regulation, such as VSMC migration and cell cycle regulation.

7.1. Poldip2, a Novel Binding Partner for p22phox

The association between Poldip2 and p22phox was established and confirmed using multiple experimental methods including yeast two-hybrid, GST-pulldown, co-immunoprecipitation, and co-localization by immunocytochemistry. While the initial interaction between Poldip2 and p22phox was found using a yeast two-hybrid system, additional experiments were performed to confirm the association between these two proteins utilizing tagged versions, as well as transient transfection or adenoviruses to express them. One of the primary concerns associated with the use of tagged proteins and overexpression systems is the possibility that the tagged protein may not undergo proper processing in the cell type being transduced. Additionally, overexpression of proteins can often overwhelm the ability of the cell to properly process and fold the protein, often resulting in protein aggregation in the ER and cellular stress. However, in recognition of the limitations that accompany the use of tagged proteins, transient transfection, and overexpression methods, we generated an antibody against Poldip2 and used it to confirm the association between endogenous Poldip2 and p22phox, as well as the association between endogenous Poldip2 and Nox4 in VSMCs, a cell type known to natively express all of these proteins.

Despite the expression of both Poldip2 and Nox1 in VSMCs, no association between the endogenous proteins was detected, due in large part to the poor quality of available Nox1 antibodies. However, when a tagged version of Nox1 was overexpressed in VSMCs, an association between these two proteins was detected. Additional experiments are needed in the future to verify an interaction between endogenous Nox1 and Poldip2 in VSMCs.

7.1.1. To what specific regions of p22phox does Poldip2 bind?

Poldip2 was first established as a novel p22phox binding partner using the cytosolic C-terminal tail region of p22phox as bait in a yeast two-hybrid screen, thus allowing us to conclude that the p22phox tail region is critical for the association of these two proteins. As discussed in Chapter 1 and Chapter 3, the interaction of cytosolic regulatory subunits with the proline-rich region of p22phox is required for proper activation of Nox1, Nox2, and Nox3; however, the requirement of the cytosolic C-terminus region for proper Nox4 activation was unclear. If one considers the cytosolic C-terminal tail region of p22phox to consist of amino acids 106-195, based on the rat and human sequence numbering, then our findings, in conjunction with recent studies that show amino acids 130-195 of p22phox (which codes for the proline-rich region) are not required for Nox4 activity (von Lohneysen et al., 2008), suggest that the portion of the C-terminal region required for Poldip2 association with p22phox and Nox4 activation consists of amino acids 106-129. This 24 amino acid domain is highly conserved across species and may serve as the site required for Poldip2 association with p22phox. Future studies designed to confirm an association between Poldip2 and this 24 amino acid domain of p22phox in live cells would be useful. To do this, one could generate a p22phox deletion mutant that no longer contains the 106-129 aa region and determine if it is able to bind Poldip2.

At the present time, we do not know what protein regions or domains of Poldip2 mediate its interaction with p22phox. To determine the regions of Poldip2 necessary for association with p22phox, one could utilize protein

modeling, generate Poldip2 deletion mutants, or use peptide blocking in the initial experimental approach. A caveat of utilizing such techniques is that multiple regions of Poldip2 may be involved in the protein interaction with p22phox and, therefore, it may not be possible to pinpoint specific residues that are critical to the association of Poldip2 with p22phox. However, identifying regions of Poldip2 that may be important for association with p22phox, and subsequently important for the regulation of Nox4 and Nox1 activity in VSMCs, could provide insight as to how this protein may differentially regulate these two enzymes despite its association with the same site on p22phox. This type of information would potentially allow one to devise ways to pharmacologically target one enzyme system without interfering with the other, such as the development of a decoy peptide, which would prove quite useful in cellular and physiological studies.

7.1.2. Does Poldip2 Regulate Nox4 and Nox1 Enzymatic Activity?

Multiple methods were used for functional studies to determine how Poldip2 influences Nox enzymatic activity and ROS production, which included lucigenin-enhanced chemiluminescence, DHE-HPLC, ESR, and the Amplex Red Assay. Lucigenin-enhanced chemiluminescence, commonly criticized for the potential to overestimate $O_2^{\bullet}$- due to redox cycling, was the primary method used to measure NADPH oxidase activity. It is well established that when lucigenin is used at a concentration of 5 μmol/L, the concentration used for our experimental measurements, the overestimation caused by redox cycling is insignificant (Dikalov et al., 2007; Li et al., 1998). A number of assays used to measure ROS have limitations, which are discussed and reviewed thoroughly (Dikalov et al.,

2007). To overcome these limitations, avoid any potential artifacts, and confirm our results, we used several techniques to measure ROS generation. For example, to measure $O_2^{\bullet}$-, we used DHE-HPLC. The use of DHE allows for the detection of intracellular $O_2^{\bullet}$- and provides results similar to other $O_2^{\bullet}$- detection techniques without the risk of redox cycling (Dikalov et al., 2007). To measure H_2O_2 production, we used Amplex Red, which is highly specific and sensitive and will detect H_2O_2 levels in the picomole range. In addition, Amplex Red reacts with H_2O_2 with a 1:1 stoichiometry and allows one to detect H_2O_2 in both oxidative and reductive cellular conditions (Dikalov et al., 2007).

Our experimental results clearly demonstrate that Poldip2, when overexpressed, increases basal oxidase activity and ROS production in a Nox4-dependent manner in VSMCs. However, the use of overexpression in these experiments presents the same caveats that were discussed earlier; therefore, to account for these limitations and verify that endogenous Poldip2 is necessary for basal oxidase activity and ROS production, we generated siRNA to knockdown endogenous Poldip2. As evidenced by our results, siPoldip2 significantly impairs basal oxidase activity and ROS generation by Nox4 in VSMCs. Altogether, these results verify that Poldip2 exerts its effects on ROS and Nox activity specifically through Nox4/p22phox.

The functional studies performed in Chapter 3 show that Nox4 requires a novel cytosolic subunit, Poldip2, for proper enzymatic activity and ROS production and suggest that Nox4 may not be a constitutively active enzyme, as previously reported (Martyn et al., 2006; von Lohneysen et al., 2008). However,

it remains to be determined exactly how Poldip2 influences Nox4 activity in VSMCs, since there are multiple possible levels of Nox4 regulation. Our data confirm that altering Poldip2 at the mRNA and/or protein level does not alter the mRNA or protein levels of Nox4 (Figure 3.2 and Figure 3.3 a). Poldip2 could, however, modulate Nox4 through post-translational modification, coordination of appropriate subcellular localization, or regulation of enzymatic activity. While the ability of Poldip2 to increase Nox4 enzyme activity and ROS generation supports the concept that Poldip2 may translocate and activate Nox4 in a fashion similar to that of classical oxidase subunits, these experimental results do not conclusively prove that this is the case. Nor do these data suggest that translocation of Poldip2 to the Nox4/p22phox complex is the only, or even the principal, mechanism by which Poldip2 regulates the Nox4/p22phox complex.

The analysis conducted in Chapter 3 indicates that Poldip2 does not positively influence Nox1 activity and suggests that Poldip2 negatively regulates Nox1 in VSMCs, although the mechanism by which this occurs requires further investigation. One could speculate that Poldip2 inhibits Nox1 activity through its association with aa 106-129 of the cytosolic C-terminal tail region of p22phox. This interaction could potentially result in a conformational change in the cytosolic tail region of p22phox, thus masking the proline rich region and rendering the other the cytosolic subunits necessary for Nox1 activation, p47phox and NoxA1, unable to bind. To determine if Poldip2 negatively impacts the ability of other cytosolic regulatory subunits to bind to the Nox1/p22phox complex, one could utilize a p22phox deletion mutant that no longer contains the

106-129 aa region. If the association of Poldip2 with this region functions to inhibit the binding of p47phox and NoxA1, one would expect that the association between these subunits and the mutant p22phox would increase in response to agonist stimulation, since Poldip2 would no longer be able to associate with the mutant p22phox.

7.1.3. Does Poldip2 function like a classical oxidase regulatory subunit?

While investigating the effects of Poldip2 on Nox activity, the possibility that Poldip2 may potentially act like a classical oxidase regulatory subunit was considered. The known cytosolic regulatory subunits function by translocating to the membrane, where they to bind to the Nox/p22phox cytochrome complex to promote its activation and ROS production. However, none of the experiments conducted in this body of work provide conclusive evidence that this is how Poldip2 functions to regulate the activation of Nox4. Sequence analysis of full-length Poldip2 showed that Poldip2 bears no homology to the classical Nox cytosolic regulatory subunits p47phox and p67phox or their homologues NoxO1 and NoxA1. Further protein analysis predicts that Poldip2 may undergo post-translational modifications, including phosphorylation at serine/threonine residues and/or N-Myristoylation, to name a few. However, whether these post-translational modifications to Poldip2 occur and how/if these modifications influence the ability of Poldip2 to positively regulate Nox4 activity and negatively modulate Nox1 activity requires additional investigation. It is well established that other cytosolic regulatory subunits require phosphorylation (Bedard and Krause, 2007); thus initially it may be most informative to determine if Poldip2 is

also phosphorylated, for example, in response to treatment of VSMCs with TGF-β, an agonist known to activate Nox4. Assessment of protein-protein interactions between the phosphorylated form of Poldip2 and p22phox and Nox4 can be assessed using co-immunoprecipitation, co-localization, and bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) techniques, which enable observation of protein-protein interactions in live cells. If Poldip2 phosphorylation increases in response to agonist stimulation, one could then generate a series of Poldip2 constructs with single or multiple point mutations in the serine and threonine sites predicted to be phosphorylated to establish which sites are necessary for association of Poldip2 with Nox4/p22phox and the subsequent increase in Nox4-dependent ROS production. Alternatively, if phosphorylation is not required for Poldip2 translocation and association with the Nox4/p22phox complex, one could perform additional mutation analysis studies at the predicted site of *N*-myristoylation, which is a co- or post-translational modification described previously to promote weak and reversible protein-membrane and protein-protein interactions, to determine if N-myristoylation is what promotes association of Poldip2 with the Nox4/p22phox complex and subsequent ROS production.

7.1.4. How does Poldip2 regulate the localization and trafficking of Nox4?

A definite role for Poldip2 in the localization and trafficking of Nox4 is suggested by the experimental results presented in Chapter 6; however, how Poldip2 functions to regulate proper Nox4/p22phox localization and trafficking has not been investigated. Poldip2 could potentially mediate proper

Nox4/p22phox localization through regulation of Nox4 post-translational modification and/or coordination of appropriate subcellular localization. Our data show that when Poldip2 is knocked down using siRNA, there is an increase in ER stress and the Nox4/p22phox complex localizes in a peri-nuclear region indicative of the ER/Golgi. Both of these events could be mediated by either improper protein processing or a lack of protein trafficking. Little is known about the post-translational modifications that are made to Nox4. It is known that Nox4 contains 4 predicted N-glycosylation sites (Shiose et al., 2001), which suggests that Nox4 may be glycosylated; however, this has not been confirmed. It is suggested that one potential cause of ER stress is an accumulation of ubiquitinated proteins that fail to be degraded or processed (Kim et al., 2006). It might therefore be of interest to determine if the lack of Poldip2 promotes an increase in the ubiquitination of Nox4 and an increase in Nox4 co-localization with the proteasome, both of which would suggest improper protein processing.

In the absence of Poldip2, there is a clear mislocalization of Nox4/p22phox in VSMCs. The experiments presented in Chapter 6 establish that in the absence of Poldip2, Nox4/p22phox trafficking to the focal adhesions is significantly impaired. Therefore, establishing how Poldip2 functions to regulate the proper trafficking of this complex would be an interesting area for future investigation. To establish if Poldip2 serves as a chaperone to the Nox4/p22phox complex, one could initially determine if Poldip2 associates with and traffics with the complex from the ER/Golgi region to the focal adhesion compartment. One of the limitations of the method utilized in Chapter 6 to

investigate protein trafficking was that the cells had to be fixed at different time intervals after release from cold and cycloheximide. Therefore, the use of live cell imaging would prove a more effective and informative technique for watching proteins traffic and would enable one to do so in real time. While there are limitations associated with the use of BRET or FRET, in this case the use of one of these two methods would be most advantageous and would allow one to visualize both protein-protein interaction and protein trafficking within a single living cell in real time. If Poldip2 functions as a chaperone for the Nox4/p22phox complex, one would expect that these proteins would associate and translocate to focal adhesions together.

While other investigators have studied the localization of Noxes, very few have attempted to determine how Noxes traffic to their proper subcellular compartments. Interestingly, Duoxes require the co-expression of a factor called DuoxA2, an ER resident protein that mediates ER-to-Golgi transition of Duoxes (Grasberger et al., 2007; Grasberger and Refetoff, 2006). Upon co-expression of Duoxes with DuoxA2, the ER retained Duox protein is able to mature and properly traffic to the plasma membrane (Grasberger et al., 2007; Grasberger and Refetoff, 2006; Zamproni et al., 2008). It is possible that Poldip2 serves a similar role for Nox4. To better understand how the Poldip2/Nox4/p22phox complex traffics, establishing if the complex assembles and traffics from the ER or the Golgi would be an interesting place to begin. The method we used to examine trafficking utilized cold temperatures (15°C), which prevents transport of proteins from both the ER and the Golgi compartments (Marie et al., 2008). The

classical view of protein trafficking suggests that a large majority of proteins, after undergoing proper folding in the ER, are transferred to the cis side of the Golgi. As the proteins move through the Golgi stacks, they undergo post-translational modifications, including glycosylation, proteolytic processing, sulfation, and phosphorylation. When the proteins reach the trans-Golgi, they are sorted and assigned to carriers for further transport to various cellular destinations (Marie et al., 2008). However, current studies also indicate that an increasing number of proteins may undergo transport directly after exit from the ER, thereby bypassing Golgi processing altogether (Marie et al., 2008). This type of non-classical protein transport is characterized by its insensitivity to brefeldin A (BFA), which functions to rapidly disassemble the Golgi and thereby block protein Golgi-dependent protein trafficking. Sensitivity to BFA could thus be used to establish if the Poldip2/Nox4/p22phox traffics from the Golgi or directly from the ER by non-classical trafficking methods.

Alternatively, Poldip2 may serve as a chaperone by potentially functioning as an intermediary protein that provides a link between the Nox4/p22phox complex and a functional motor protein that travels along microtubules or stress fibers. In this case, the association of Poldip2 with the Nox4/p22phox complex would suggest that Poldip2, in addition to its role as an activator of Nox4/p22phox, may serve as a scaffolding protein in a preassembled Poldip2/Nox4/p22phox signal transduction cascade known as a ‘transducisome’. Transducisomes can assemble prior to transport to subcellular compartments. Unpublished data from our lab has shown by electron microscopy that Nox4

localizes in vesicle-like structures. It would be interesting to determine if such a transducisome is preassembled prior to transport of the Poldip2/Nox4/p22phox complex to the focal adhesion complex. Some of the previously described experimental approaches would provide an answer to this question; however, it would also be interesting to examine the localization of Poldip2 and Nox4 in more detail to determine if they both localize to vesicular structures in VSMCs using electron microscopy.

Which motor proteins Poldip2 may potentially link to the Nox4/p22phox complex would be determined by whether the complex traffics along microtubules or along stress fibers, which has yet to be established. Because both of these cytoskeletal highways insert into focal adhesions, either is a potential candidate for the trafficking of Nox4/p22phox. To determine if the complex traffics along microtubules, experimentally one could treat with either cytochalasin D, to disrupt the stress fiber network, or nocodozole to disrupt microtubules. Should results indicate that Nox4/p22phox traffics along microtubules, determining how the complex associates with the motor protein kinesin, which functions to carry cargo vesicles from the Golgi/ER region of the cell toward the barbed (+) growing ends of microtubules, would be an interesting avenue of investigation. The motor proteins involved in actin stress fiber trafficking are poorly understood, making it more difficult to determine what protein may be responsible for carrying the Nox4/p22phox complex to focal adhesions in this case.

7.2. Nox4/Poldip2, regulators of cytoskeletal integrity in vascular smooth muscle cells

The functional data provided in Chapter 4 strongly implicate Poldip2/Nox4 in the modulation of cytoskeletal integrity. More specifically, the knockdown of Poldip2 or Nox4 results in the complete loss of two vital cytoskeletal structures, focal adhesions and stress fibers. In contrast, when Poldip2 is overexpressed, both of these structures are strengthened, thus establishing a strong link between Nox4/Poldip2 and cytoskeletal integrity. Additional evidence presented in Chapter 4 shows a strong activation of RhoA by Nox4/Poldip2 and demonstrates that this activation occurs in a ROS-dependent manner. Taken together, these experimental findings suggest that the Nox4/Poldip2 axis functions, through production of ROS and modulation of RhoA, to alter cytoskeletal integrity; however, the precise mechanism by which this regulation occurs is unknown.

7.2.1. How does Nox4/Poldip2 function to regulate RhoA?

The data in Chapters 4 and 6 of this dissertation suggest that Poldip2 may regulate Nox4 by regulating trafficking of the mature protein complex from the Golgi/ER to focal adhesions. As discussed above, proper protein trafficking is dependent on both the actin cytoskeletal network and the maintenance of microtubules. The literature clearly establishes that RhoA is a central regulator of the integrity of both of these cytoskeletal networks, where RhoA regulates microtubules through the activation of mDia2 (Wen et al., 2004) and regulates stress fibers through activation of Rho kinase (ROCK) (Chrzanowska-Wodnicka

and Burridge, 1996; Katoh et al., 2007). Because treatment of VSMCs with siPoldip2 results in disruption of both stress fibers (Figure 4.1) and microtubules (unpublished data from the Griendling lab), this suggests that either of these cytoskeletal networks may contribute to impaired Nox4/p22phox trafficking.

As indicated in Figure 4.7, the activation of Nox4 by Poldip2 results in a pronounced increase in the activation of RhoA, which is ROS-dependent. How the Nox4/Poldip2 axis regulates Rho remains to be determined. There are a number of proteins upstream of Rho activation that could serve as targets of Nox4/Poldip2. This large and sustained activation of Rho could potentially occur either 1) through the inactivation of the Rho GAP, p190RhoGAP, which catalyzes the exchange of GTP to GDP and thus inactivates Rho or 2) through the activation of the Rho guanine nucleotide exchange factor (GEF), p190RhoGEF, which catalyzes the exchange of GDP to GTP and thus activates Rho. While we show that p190RhoGAP protein levels are unchanged by Poldip2 overexpression (Figure 4.9 b), further investigation is needed to determine if Nox4/Poldip2 blocks p190RhoGAP phosphorylation, and thus prevents its activation, or if Nox4/Poldip2 functions by inactivating a PTP upstream of the p190RhoGEF, such as PTP-PEST, thus promoting its hyperphosphorylation and sustained activation, both of which could potentially mediate the increase in RhoA caused by Nox4/Poldip2. An additional target for consideration is the Rho GDP-dissociation inhibitor (GDI), which functions to bind and sequester the inactive Rho-GDP form to prevent the exchange of GDP to GTP and thus blocks Rho activation. Phosphorylation of Rho-GDI by PAK induces its dissociation from

Rho-GDP. PAK is also known to be ROS sensitive. Therefore, Nox4/Poldip2 may function to keep Rho-GDI in a phosphorylated and inactive state by inactivating a PTP upstream of PAK, enabling PAK to remain active and promoting sustained Rho-GDI dissociation from Rho-GDP, which would free Rho to be activated.

7.2.2. How does Nox4/Poldip2 function to regulate stress fibers?

The marked increase in activation of RhoA and the increase in stress fiber formation caused by overexpression of Poldip2, in combination with the data showing that siPoldip2 results in the complete loss of stress fibers in VSMCs, strongly link Nox4/Poldip2 to the regulation of stress fibers. It is well established that stress fiber formation and maintenance by RhoA occurs through activation of Rho kinase (ROCK) (Chrzanowska-Wodnicka and Burridge, 1996; Katoh et al., 2007). While our results in Chapter 4 establish RhoA as a downstream target of the Nox4/p22phox complex, further exploration is needed to determine the signaling involved in Nox4/Poldip2 mediated stress fiber formation and the functional consequences of stress fiber stabilization.

The likelihood of Nox4/Poldip2/p22phox utilizing the stress fiber network as a means to traffic to focal adhesions seems high, since our co-localization experiments indicate that Nox4 and Poldip2 co-localize along stress fiber structures; however, we cannot use this association to rule out the possibility that the complex may traffic along microtubules, since we have seen these proteins localize by immunocytochemistry in distributions indicative of microtubules as well. If the Nox4/p22phox complex actively generates ROS as it transports to

focal adhesions, then the possibility exists that Nox4/Poldip2 may function to increase stress fiber formation by simultaneously activating local pools of RhoA to activate ROCK and oxidizing actin, thus promoting the rapid incorporation of actin into new stress fiber structures in VSMCs (Chrzanowska-Wodnicka and Burridge, 1996; Moldovan et al., 2000). To further delineate the signaling involved in stress fiber formation by Nox4/Poldip2, one could use constitutively active and dominant negative forms of RhoA, to establish its role as a downstream target of Nox4/Poldip2 and as an upstream mediator of ROCK activation. To establish if ROCK is involved in the signaling that mediates the increase in stress fiber formation by Nox4/Poldip2, pharmacological inhibitors and kinase dead mutants of ROCK would be informative tools. Additionally, using the BIAM assay, utilized in Chapter 4 to look at PTP-PEST oxidation, to look at actin oxidation would enable one to establish if oxidation of actin promotes stress fiber formation. Introducing a fluorescently labeled mutant form of actin that cannot be oxidized would also allow one to tease out if the oxidation of actin increases incorporation into actin stress fibers, or prevents it. By fluorescently labeling actin in this way, one can specifically track the mutant actin introduced into the cell using live cell imaging.

7.2.3. How does Nox4/Poldip2 function to regulate microtubule dynamics?

As discussed above, the overexpression of Poldip2 results in a marked increase in activation of RhoA, which is upstream of mDia2 and microtubule stabilization (Palazzo et al., 2001). In contrast, the knockdown of Poldip2 using siRNA results in a loss of microtubules (unpublished data from the Griendling

lab). Preliminary results indicate that when Poldip2 is overexpressed, there is an increase in the formation of stable microtubules, known as Glu-microtubules, known to occur as a result of increased association between mDia2 and microtubules downstream of RhoA activation (Bartolini et al., 2008). While our results establish RhoA as a downstream target of the Nox4/p22phox complex, further exploration is needed to determine if RhoA is involved in maintenance of microtubule integrity and to define the functional consequences of microtubule stabilization by the Nox4/Poldip2 axis.

One question that arises is if Nox4/Poldip2 functions as an active ROS producing complex as it undergoes anterograde transport from the ER/Golgi to the focal adhesion compartment. The ability to determine if ROS is being generated in a specific subcellular compartment, such as a transport vesicle, proves very difficult using the tools presently available; however, one possible way to determine if ROS generation is taking place in Nox4 containing vesicles would be to use Cerium Chloride, a compound that is converted to amorphous electron dense cerium perhydroxide precipitates. The use of Cerium Chloride for transmission electron microscopy would allow one to determine if ROS are being actively generated in vesicle-like structures. Additionally, the use of Cerium Chloride in combination with the use of a Poldip2 or Nox4 antibody in nox1$^{y/-}$ VSMCs, which only express functional Nox4 and would thereby eliminate the possibility that ROS production in vesicles is due to Nox1, would allow us to correlate the localization of Nox4 and Poldip2 with ROS production, thereby establishing if the Nox4/Poldip2 complex is active as it transports or if it is only

active in the focal adhesion compartment of the cell. The use of Cerium Chloride, however, would not allow us to eliminate other non-NAPDH oxidases as sources for this ROS production, thus making it critical to establish a co-localization of Nox4 or Poldip2 to the sites of Cerium Chloride conversion.

A potential mechanism by which Nox4/Poldip2 may function to stabilize microtubules through the activation of RhoA is through the subsequent activation of its downstream effector mDia2, which promotes an increase in Glu-microtuble formation (Bartolini et al., 2008). To further delineate the signaling involved in microtubule stabilization by Nox4/Poldip2, one could use mDia2 mutants, in which the formin homology domain is altered to block association with microtubules, to determine if these effects are mediated by RhoA regulation of mDia2 binding to microtubules to promote stabilization.

7.2.4. What are the functional consequences of improper cytoskeletal regulation?

Ultimately, the consequences of a lack of proper cytoskeletal regulation and a lack of cytoskeletal dynamics are the misregulation of cellular processes that require a dynamic cytoskeleton, for example cell cycle progression, which is required for cell proliferation, as well as cell polarization and migration. As shown in Chapter 5, ROS generated by the Nox4/Poldip2 axis contributes to the regulation of cell cycle progression at the G_2/M checkpoint (McCrann et al., 2009b; Menon et al., 2003), potentially through the inactivation of the PTP cdc25, which would lead to the observed increase in basal phosphorylation at Tyr15. However, further experiments are required to determine if this block at the G_2/M

checkpoint is due to the lack of cytoskeletal dynamics or if it is due to ROS modulation of a signaling pathway in which the inactivation of PTP cdc25 leads to a block in dephosphorylation of cdc2, and ultimately to a block in cyclin B activation. If it is a lack of cytoskeletal dynamics that contributes to the block at G_2/M, this may be reflected in the positioning of the centrioles, which must move to opposite poles of the cell for mitosis to properly progress. This movement of the centrioles requires the destabilization of microtubules and repolymerization of new microtubules (Gomes et al., 2005; Schmoranzer et al., 2009). Failure of centrioles to move to opposite poles of the cell during mitosis would result in cell cycle inhibition. Interestingly, this latter hypothesis involving the lack of cytoskeletal dynamics blocking cell cycle progression may also potentially explain how Nox4/Poldip2 functions to block proper cell migration.

One of the key events required for cell migration is establishment of cell polarity for the cell to properly extend filopodia and lamellipodia in the direction of migration. Inhibiting the continual turnover and rearrangement of either the actin cytoskeleton or microtubules would ultimately function to impair the dynamic processes necessary for both VSMC migration and cell cycle progression. Ultimately, how the Nox4/Poldip2 axis functions to regulate either of these processes is an important area of future investigation.

Additionally, dynamic regulation of focal adhesions is required for cell migration and division. In the case of migration, cells must form new focal adhesions in the front/leading edge to promote cell attachment to the extracellular matrix where transmembrane integrins link the matrix to the actin

cytoskeleton. Cells also require dissolution of focal adhesions in the rear of the cell to allow for forward cell progression. At sites of cell attachment, focal adhesions contain a number of proteins including RhoA and PTP-PEST. The potential mechanisms by which Nox4/Poldip2 may function to regulate RhoA activation is discussed above. Future experiments are needed to investigate how the oxidation of resident focal adhesion proteins, such as PTP-PEST, which we have shown to be oxidized by Nox4/Poldip2 (Figure 4.10), would affect focal adhesion turnover and the migration process. PTP-PEST functions to modulate a number of signaling proteins upstream of RhoA, such as FAK and, therefore, may serve as a potential target for ROS mediated regulation of VSMC migration. One potential pathway that links PTP-PEST to migration involves RhoA. PTP-PEST oxidation and inactivation by Nox4/Poldip2 would lead to hyperphosphorylation of FAK, thus increasing p190RhoGEF and RhoA activation. This activation of RhoA would promote an increase in focal adhesion formation and a decrease in turnover, thus blocking cell migration. The possible involvement of this pathway in mediating Nox4/Poldip2 effects on focal adhesion turnover is supported by the increase in oxidation of PTP-PEST mediated by Nox4/Poldip2, the increase in RhoA activation, as well as by the increase in focal adhesion formation and decrease in cell migration caused by Nox4/Poldip2, but future work is necessary to establish the contribution of each of these molecules.

7.3. Implications for Nox4/Poldip2 in Cardiovascular Disease Pathologies and Future Directions

Together, the data provided in this body of work link ROS production by the Nox4/Poldip2 axis to the regulation of cellular functions highly dependent on the tight coordination of cytoskeletal regulation, such as VSMC migration and cell cycle regulation. Two vascular pathologies in which both VSMC migration and proliferation are clinically important are restenosis after angioplasty and atherosclerotic lesion formation. To better understand if Poldip2 could potentially serve as a pharmacological target for restenosis and atherosclerosis, determining how Poldip2 functions in each of these settings is important. The Griendling lab recently generated a Poldip2 knockout model that can be used for animal studies to shed more light on Poldip2's role in an in vivo setting. Interestingly, the Poldip2 homozygotes appear to be developmentally impaired and, thus, embryonic lethal; however, the Poldip2 heterozygote animals are viable can be used for animal studies. Examples of animal studies that could be conducted to determine the role of Poldip2 in an in vivo animal model of VSMC migration and proliferation include: 1) a femoral artery injury model to examine neointimal formation, which requires both migration and proliferation of VSMCs, and involves Nox4 (Clempus et al., 2007; Szocs et al., 2002), 2) Poldip2 heterozygotes in an ApoE-/- background on a high fat diet to examine atherosclerotic plaque formation, and 3) an ischemic injury model to look at collateral vessel formation, which requires proper migration of both ECs and VSMCs for new vessel formation. Nox4 was previously implicated in EC migration (Datla et al., 2007; Natarajan et al., 2008); therefore, it would be

interesting to investigate how Poldip2 regulates Nox4, cytoskeletal dynamics, and migration in ECs as well.

Despite clinical advances in stent development, restenosis is still an ongoing issue, since a sizeable number of patients still develop this complication after the stent insertion procedure. It is known that after vascular injury, medial VSMCs proliferate and then migrate into the intimal layer and recent evidence shows that ROS, specifically derived from Nox1 and Nox4 in VSMCs, are involved in both the proliferative and migratory responses of VSMCs in the neointimal formation process of restenosis. However, the vast majority of clinical studies utilizing antioxidant therapies in an attempt to block restenosis have had disappointing results. This could be because, despite the fact that the primary product generated by NADPH oxidases is $O_2^{\bullet}$-, it is the more stable and freely diffusible H_2O_2 and other peroxides that are more likely important in disorders where smooth muscle proliferation and migration are the critical components. Therefore, a better approach to preventing restenosis would be to target the direct source of ROS generation in the vascular wall, such as the Nox enzymes, and prevent the production of ROS, rather than trying to scavenge $O_2^{\bullet}$-, H_2O_2, or one of the other myriad of ROS produced. For this reason, it becomes crucial to understand how each of the Noxes found in the vascular wall and, more specifically how the Noxes expressed in VSMCs, are regulated. Therefore, understanding how Nox1 and Nox4 are differentially regulated and how each of these Noxes functions in the proliferation and migration of VSMCs would allow one to specifically target and prevent the activation of one or both Nox subtypes.

The data presented herein concerning the role of Poldip2 in the regulation of Nox4 and Nox1 not only further our understanding about how these Noxes are regulated in VSMCs, but also begin to shed light on a protein, Poldip2, that may serve as a novel pharmacological target for vascular pathologies with a significant VSMC proliferative and migratory component, such as restenosis.

Because numerous cells types express Nox4 and Poldip2, our findings could be expanded to other cells types and disease models that involve cell migration and proliferation. One disease situation in which both of these processes are integral to the disease pathology is cancer. NADPH oxidases and high levels of ROS contribute to cancer by promoting cancer cell proliferation and may promote cancer cell migration (Bhandarkar et al., 2009; Yamaura et al., 2009). Additionally, NADPH oxidases and ROS are linked to the process of angiogenesis (Ushio-Fukai and Nakamura, 2008), which is needed for tumor growth and metastasis. Therefore, it would be interesting to use a skin carcinoma mouse model, which has already implicated Nox4 in endothelial tumor progression (Bhandarkar et al., 2009), to determine the specific role of Poldip2 in cancer cell migration, proliferation, and angiogenesis.

Additionally, Nox-dependent ROS generation in pulmonary endothelial and smooth muscle cells was described previously (Ismail et al., 2009; Mittal et al., 2007). Nox4 is thought to be the predominant isoform expressed in pulmonary smooth muscle and may serve a primary role as an oxygen sensor. Hypoxia is believed to promote Nox4 activation, ROS generation and smooth muscle cell proliferation, which could result in an increase in vascular media

thickness and a decrease in lumen size, ultimately contributing to the process of pulmonary hypertension (Ismail et al., 2009; Nisbet et al., 2009a; Nisbet et al., 2009b). The use of Poldip2 heterozygote mice will allow one to further elucidate the role of Nox4, and more specifically the role of Poldip2, in hypoxia-induced ROS generation and proliferation in pulmonary arteries and how they contribute to pulmonary hypertension.

Another setting in which both Nox4 and Poldip2 are highly expressed is the kidney. A role for Nox-derived ROS in diabetic nephropathy is well established, where the kidney, in this disease state, shows an increase in Nox4 mRNA and protein expression (Gorin et al., 2005). Additionally, treatment of diabetic animals with antisense Nox4 decreases the kidney pathology (Gorin et al., 2005). The role of Nox4 in this particular disease is suggested to be as an inducer of mesangial cell hypertrophy and fibrosis (Block et al., 2008; Gorin et al., 2005). Therefore, it would be interesting to determine if there is a role for Poldip2 in Nox4 regulated hypertrophy, which may be mediated by cell cycle regulation, and fibrosis in diabetic nephophathy.

Additional future studies could also potentially include looking at how Poldip2 is regulated by agonist stimulation. At this point, we know that Poldip2 functions to regulate Nox4 activity and ROS production and that Nox4/Poldip2 regulate cytoskeletal dynamics and cell migration and proliferation. What drives the regulation and activation of Poldip2 itself remains unknown. It is also unknown whether Poldip2 is regulated at the mRNA level, the translational level, or by specific post-translational modifications, and what agonists may promote

the regulation of Poldip2. Conducting a systematic search for potential agonists that may function to modulate Poldip2 would prove an interesting study, where one would initially test agonists known to activate Nox4 in VSMCs.

In summary, the results reported in this dissertation may help in our overall understanding of how NADPH oxidases are differentially regulated and contribute to multiple disease pathologies. Unraveling the mechanisms by which different NADPH oxidase traffic and signal may allow for the identification of novel targets for the development of new and specific pharmacological agents to aid in the treatment of restenosis and atherosclerosis. In a broader context, the results reported in this dissertation contribute to the rapidly expanding field of NADPH oxidases in general, particularly by providing the first reports of a protein, in any cell type, that expressly functions to positively regulate Nox4 activity and Nox4-dependent ROS production. As Noxes are implicated in an increasing number of disease pathologies and it becomes clear that antioxidant therapies may not be the best approach to combating pathological levels of ROS generation, Nox enzymes themselves become more suitable pharmacological targets. Targeting the primary sources of ROS thus becomes a more effective pharmacological therapy than trying to scavenge $O_2^{\bullet}$-, H_2O_2, or one of the other myriad of ROS produced. Therefore, understanding how each Nox is regulated is critical in the pursuit of novel therapeutic development.

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