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Zoila Isabel Fernandez **April 18, 2012**

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Abstract

Nox1 meditated Reactive Oxygen Species Induce Oxidation of 14-3-3 cysteine residues in Vascular Smooth Muscle Cells

By Zoila Isabel Fernandez

Vascular smooth muscle cell (VSMC) migration is important during vascular development and contributes to vascular lesion formation. It plays a role in diseases such as atherosclerosis and postangioplasty restenosis. The mechanism regulating migration of this cell type are therefore of great interest. Recent work in our laboratory has shown that platelet-derived growth factor (PDGF) induces VSMC migration by activating the cofilin phosphatase slingshot (SSH1L) via a reactive oxygen species (ROS) mediated mechanism. The exact mechanism of SSH1L activation, however, is not completely understood. Previous studies have revealed that SSH1L is associated with 14-3-3 proteins in an inhibitory complex that is disrupted by PDGF. In this study, we found that the phosphorylation of serine 834 within 14-3-3 binding motif in SSH1L regulates its binding to 14-3-3 proteins. Interestingly, we describe that the phosphatase responsible for the Serine 834 dephosphorylation and therefore phosphatase activation is SSH1L itself through a mechanism that required Nox1-based NADPH oxidase. In addition, we established that Nox1-mediated 14-3-3 oxidation of cysteine residues leads to an initial SSH1L/14-3-3 complex disruption that allowed SSH1L autodephosphorylation to occur. Because of the key role of 14-3-3 oxidation, we generated cysteine mutants to identify the redox sensitive sites in 14-3-3 that affect its binding capacity.

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Introduction

Cardiovascular Disease

Cardiovascular disease is the leading cause of death among adults in the United States (Miniño, 2008). In fact, one out of three adults has one or more types of cardiovascular disease (Roger et al., 2011). These conditions include hypertension, atherosclerosis, post-angioplasty restenosis, stroke and coronary heart disease. Atherosclerosis, the underlying cause of the majority of cardiovascular events, is caused by plaque build-up in the arteries. Plaque is a substance composed mainly of cholesterol deposits and cellular components that accumulate and narrow the arteries over time. Atherosclerosis by itself accounts for nearly three fourths of all deaths from cardiovascular disease (Gotto, 2009). Moreover, it can result in coronary heart disease, which affects 7% of the U.S population 20 years and older (Roger et al., 2011). Because of its close association with other cardiovascular diseases and its extensive prevalence, understanding the molecular mechanisms that control the development of atherosclerosis has become extremely significant.

The artery consists of three distinct layers or coats called tunicae. The tunica intima is consists of a single layer of flattened endothelial cells with marginal underlying connective tissue. This endothelial layer is in direct contact with blood flow and provides the necessary smooth lining and slippery inner surface of blood vessel walls. The middle layer or tunica media is composed of vascular smooth muscle cells (VSMCs) that constrict to regulate and restrain the diameter of the vessel. In larger arteries, the tunica media is divided from the tunica externa by a significant amount of elastic tissue. The outermost layer or tunica externa follows and is largely made of loosely woven collagen fibers that protect the vessel and stabilize it by anchoring the vessels to nearby structures such as organs (Porth, 2011). The primary cell type of the outermost

layer is the fibroblast, a connective tissue cell that is capable of synthesizing and secreting collagen fibers required for wound repair. Fibroblast cells possess unique histological, biochemical and functional features that allow them to regulate vascular response to injury (Fang et al., 2006).

During diseases such as atherosclerosis, the composition of the tunicae is altered (Fang et al., 2006). Chronic exposure to high plasma lipids causes damage to the endothelial cell layer in the intima which results in the formation of a fatty streak by macrophages (Hansson, 2005). This is followed by fibroblast activation and proliferation which causes increased production of collagen (Fang et al., 2006). The accumulation of connective tissue is what leads to the development of a fibrous cap (Dzau et al., 2002). A key component in fibrous cap growth lies in the accumulation of smooth muscle cells (VSMC) in the atherosclerotic region, due in part to the migration of medial smooth muscle cells into the intima (Schwartz, 1997).

Vascular Smooth Muscle Cells

VSMCs comprise a large portion of vessels and the majority of the tunica media layer. In physiological conditions, they are fully differentiated and contractile, a feature that permits them to regulate blood flow and pressure by properly contracting and relaxing. On the other hand, under pathological circumstances such as the formation of an atherosclerotic lesion, the phenotype of VSMCs is altered. VSMCs lose their contractile proteins and dedifferentiate to a mobile form. Basically, they switch from a contractile, differentiated state to a proliferative, migratory phenotype which enables them to travel from the tunica media to the tunica intima (Kraemer, 2000). The cells then begin to divide and secrete extracellular matrix proteins that lead to the development of a fibrous cap which is a characteristic of advanced atherosclerotic lesions.

Migration

Cellular migration is an important process for normal development and homeostasis. It allows for morphogenesis of the embryo as well as immune response, tissue repair and regeneration. Cell migration begins with polarization, as a cell must make a clear distinction between its front and rear by sensing a concentration, spatial or temporal gradient (Lauffenberger, 1996). After the cell establishes its polarity, it can then extend protrusions of active membranes processes in the direction of movement. These protrusions can be in the form of lamellipodia or filopodia. The formation of both structures is closely coupled with actin polymerization. In fact, it is the reorganization of actin that allows for change of cell shape, polarity and migration. In VSMCs, the generation of lamellipodia is driven by the extension of F-actin-rich fibers (San Martin and Griendling 2010). These actin fibers are polar and possess barbed, fast growing ends that point toward the direction of protrusion (Koestler et al., 2009). The constant assembly and disassembly of actin fibers allows for proper direction and extension of the membrane protrusions formed at the leading edge of migrating cells. The cycles of actin polymerization and depolarization are essential to lamellipodia formation in cell migration (Small et al., 2012).

Cofilin

Cofilin is a small ubiquitous protein that belongs to a family of related proteins, ADF/cofilin (Yamaguchi and Condeelis, 2007). It plays a major role in actin dynamics during cell migration. Cofilin can bind both monomeric and filamentous actin and cut actin filaments to increase the number of barbed ends, creating sites for polymerization or depolymerization to occur (Pfaendtner et al., 2010). Moreover, it continuously produces actin monomers for polymerization and provides a rapid turnover of actin filaments (Lappalainen and Drubin, 1997).

In VSMCs, lamellipodium formation is dependent on the formation of free barbed ends on existing actin filaments (Wear et al, 2000). Activation of cofilin has been demonstrated to maintain and protrude lamellipodia at the leading edge of migrating VSMCs (Bambourg, 1999).

Cofilin is negatively regulated by phosphorylation on a Serine residue at position 3. Upstream kinases such as LIM Kinase and TES kinases, phosphorylate and thus inactive cofilin which prevents it from binding to F-actin (Yamaguchi and Condeelis, 2007). On the other hand, phosphatases belonging to the Slingshot (SSH) and chronophin (CIN) families are known to dephosphorylate cofilin and consequently activate the protein. The phosphorylation and dephosphorylation of cofilin allows for the regulation of actin reorganization.

Slingshot

Slingshot belongs to a family of protein phosphatases which specifically dephosphorylate and activate cofilin (Endo et al., 2003). The name, *slingshot,* comes from the bifurcation phenotype of the bristles and hairs seen in *slingshot* deficient mutant flies (Niwa et al., 2002). Loss of the *slingshot* gene results in increased levels of actin filaments which cause defective cell morphogenesis and the observed phenotype. The *slingshot* gene encodes a phosphatase that is conserved among various animal species. Currently, there are three human and mouse isoforms, SSH1L, SSH2L and SSH3L, each with long and short variants (Soosairajah et al., 2004). Structurally, mammalian slingshots belong to the dual phosphatases family of proteins and thus are potentially capable of dephosphorylating phosphoserine and phosphothreonine residues (Patterson et al. 2009). They contain a conserved protein tyrosine phosphatase (PTP) catalytic domain and binding motifs for 14-3-3 proteins, F-actin and Src homology 3 (Patterson et al., 2009). All isoforms are widely expressed but each isoform has its own unique tissue expression pattern and phosphatase activity. In cultured mammalians cells, the expression of both SSH1L

and SSH2L lead to a decrease in phospho-cofilin, implying that these SSHL phosphatases dephoshrorylate cofilin (Niwa et al., 2002). However, under experimental conditions, the cofilin phosphatase activity of SSH1L is higher than that of SSH2L (Niwa et al., 2002). Our laboratory has demonstrated that in VSMCs, SSH1L is activated and required for platelet derived growth factor (PDGF)-mediated migration (San Martin et al., 2008).

PDGF Signaling

Human PDGF is a dimer composed of disulfide-linked polypeptide chains A and B (Johnsson et al., 1982). It is the major promigratory stimulus for VSMC migration *in vivo* (Jackson et al., 1993; Grotendorst et al., 1981; Jawien et al., 1992) as well as a serum growth factor for fibroblasts and glia cells (Andrae et al., 2008). Studies of PDGF and its receptors have demonstrated that they are crucial during the development of various tissues, such as cranial and cardiac neural crest, the gonads and the skeleton. However, their role in normal physiological processes in adults appears to be not as critical. Nonetheless, PDGF signaling has been associated with a range of diseases where increased PDGF activity has been observed (Andrae et al., 2008). For instance, activation of PDGF signaling has been associated with leukemia, sarcomas and epithelial cancers. Of relevance, PDGF has also been closely linked to the development of atherosclerotic lesions, where chains A and B are expressed at high level in every cell type of an atherosclerotic arterial wall (Raines, 2004). Similarly, PDGF receptors such as PDGF-α and PDGF-β are also highly expressed in the vessel walls. In mormal conditions, there are low levels of PDGF-β in the walls of blood vessels (Heldin and Westermark, 1999). However, during atherosclerosis, PDGF-β receptors are readily synthesized. Presently, the mechanism that results in the increased PDGF and PDGF receptor expression observed during atherosclerosis is not well known.

Regardless of the process, PDGF is an important component of atherosclerosis pathogenesis. PDGF secreted by inflammatory cells at the site of the atherosclerotic plaque attract VSMCs to migrate from the tunica media to tunica intima and induce their subsequent proliferation via the activation of PDGF-β receptors. (Ross and Glomset, 1976) The activation of receptor PDGF-β itself is linked to phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ, which in turn is connected to changes in myoplasmic calcium, hydrolysis of 4,5-biphosphate (PIP_2) and activation of mitogen-activated protein kinases (MAPK) (Gerthoffer et al., 2007). Calcium and PIP_2 regulate several actin-binding proteins such as Wiskott–Aldrich syndrome protein (WASP), WASP-family verprolin- homologous protein (WAVE) and actin-related protein 2/3 complex. These proteins work together to enhance actin polymerization. Meanwhile, depolymerization of actin at the minus end is stimulated by cofilin, which restricts the length of the filaments and produces a rapid turnover of existing monomers thus enabling VSMC cell migration. We know that the activation of cofilin is controlled by SSH1L, which is induced and activated by PDGF in VSMCs (San Martin et al., 2008). Taken together, all of these processes serve to extend the leading edge of the cell.

Control of VSMC Migration by ROS

Reactive oxygen species (ROS) play a significant role in the development of various cardiovascular diseases such as hypertension, atherosclerosis and restenosis after angioplasty (San Martin et al., 2010). ROS encompasses a series of highly reactive small molecules that have unpaired valence shell electrons in the oxygen atom that raise their reactivity. Hydrogen peroxide (H_2O_2), unlike the other ROS, is not a free radical, which increases its stability and makes it a good candidate as a cell signaling molecule. Despite its increased stability, H_2O_2 is a strong oxidizing agent with a high reduction potential. For example, it is responsible for the oxidation

of various thiol-containing proteins (Winterbourn and Hampton, 2008). Thiols possess a low PK_a, which make them an easy target for H_2O_2. Once they are oxidized, they can become sulfenic (SOH), sulfinic (SO_2H), and sulfonic (SO_3H) acids or protein disulfides (PrSSPr).

The generation of ROS occurs through the sequential reduction of atomic oxygen which adds one or two electrons to the oxygen atom, forming superoxide ($O_2^{\bullet-}$). It is largely produced by mitochondria where it is a by-product of the electron transport chain during cell respiration (Murphy, 2009). In VMSCs, ROS are produced by proteins such as xanthine oxidase, lipoxygenases, nitric oxide synthases and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (San Martin and Griendling, 2010). NADPH oxidases are the main source of $O_2^{\bullet-}$and H_2O_2 production within the vessel wall. They can produce ROS both extracellularly and intracellularly within different compartments. Consequently, they are involved in many signaling pathways in a paracrine, autocrine or even intracrine manner. NADPH oxidases consist of multi-subunit enzymes in which the catalytic subunit is composed of a Nox protein. In human VSMCs, Nox1, Nox2, Nox4 and Nox 5 dominate NADPH oxidase activity in different sites of the arteries (Gupte et al., 2009). For instance, VSMCs from large arteries express mainly Nox1 and Nox4. Similarly, the activity of each Nox protein has specific requirements. Unlike Nox4, both Nox1 and Nox2 require cytosolic subunits for activity. Nox 1 NADPH oxidase associates with two cytosolic factors, $p47^{phox}$ and Nox1, as well as the small G protein, Rac. Nox2 is regulated by $p47^{phox}$ and $p67^{phox}$. Despite their differences, all of the oxidases possess low activity in VSMCs, a characteristic which makes them good candidates as signaling molecules.

A landmark study by Sundaresan et al. presented a new possible role for ROS in VSMC migration. Their work demonstrated PDGF-induced migration in VSMCs depended on H_2O_2 (Sundaresan et al., 1995. Later on, VSMC migration was shown to depend on Nox1-based

NADPH oxidase which was stimulated by PDGF (Lee et al., 2009). In correlation to this finding, Nox1 mRNA levels were shown to be elevated in the presence of PGDF (Brown and Griendling 2009). We and others have now discovered that PDGF-induced generation of ROS occurs via the activation of Nox1 (Lee et al., 2009). PDGF activates PDGF receptors which stimulate the activation of phosphatidylinositol 3-Kinase (PI3-K) (Brandes and Kreuzer, 2005). PI3-K then leads to the activation of Rac which interacts with cytosolic regulators of Nox 1 to produce superoxide (Clempus and Griendling, 2006).

PDGF induces SSH1L activation via a Nox1-dependent pathway

Nox1-derived ROS lead to the activation of SSH1L (San Martin et al., 2008). The exact mechanism behind this process is not completely understood. However, we know that VSMC migration induced by PDGF depends on ROS generation and that PDGF stimulates the production of ROS through a Nox1-based NADPH oxidase pathway (Sundaresan et al., 1995; Lee et al., 2009). Moreover, we have recently published that VSMCs derived from $Nox1^{-/y}$ (knockout) animals display defective migration and decreased levels of active cofilin (Lee et al., 2009). This finding correlates with our previous results that demonstrate that SSH1L activity is induced by PDGF in wildtype VSMCs but not in $Nox1^{-/y}$ cells (Maheswaranathan et al., 2011), indicating that SSH1L activation appears to be mediated by Nox1 generated H_2O_2 (Maheswaranathan et al., 2011). However, H_2O_2 does not activate SSH1L directly as typically any oxidative alteration on a phosphatase such as SSH1L would be inhibitory. It has been shown the activity of SSH1L can be regulated by its association with regulatory protein 14-3-3 (Nagata-Ohashi, 2004). Interestingly, studies in our laboratory revealed that 14-3-3 forms an inhibitory complex with SSH1L that is disrupted after PDGF treatment (Maheswaranathan et al., 2011).

This finding reveals a possible role for the SSH1L/14-3-3 complex in Nox1 dependent, PDGF-induced SSH1L activation in VSMCs.

14-3-3 Protein and 14-3-3/SSH1L Complex

14-3-3 proteins belong to a family of proteins that assemble spontaneously as dimers and are ubiquitously expressed in eukaryotes (Bridges et al., 2005). They have multiple binding partners which explain the large number of cellular processes in which they are involved. They are essential to various processes such as cell cycle control, apoptosis and cell growth (Fu et al, 2000). Studies have shown that there several isoforms of the protein, which depend on the organism. For instance, there are seven isoforms of 14-3-3 in mammals while only two exist in yeast (Brides et al., 2005). These isoforms recognize phosphoserines or phosphothreonines in their binding partner (Bridges and Moordhead, 2005). The specific function of 14-3-3 is currently not well understood and thus remains the subject of active investigation. However, we and others have demonstrated that different isoforms of 14-3-3 have been shown to interact with SSH1L and form an inhibitory complex by sequestering SSH1L in the cytosolic fraction (Nagata-Ohashi et al., 2004, Kim et al., 2009 and Maheswaranathan et al., 2011). This association prevents cofilin dephosphorylation and SSH1L translocation to F-actin abundant areas (Huang et al., 2006). As discussed earlier, there is a reduction in the binding of SSH1L and 14-3-3 after administration of PDGF to VSMCs. The release of SSH1L from the SSH1L/14-3-3 complex results in its activation. Thus, the mechanism behind the complex disruption is essential in understanding the cofilin pathway and ultimately VSMC migration.

Hypothesis

As discussed above, VSMC migration misregulation contributes to serious cardiovascular conditions such as atherosclerosis (Schwartz, 1997). Effective migration relies chiefly on proper remodeling and reorganization of the actin cytoskeleton. Actin assembly and disassembly, in turn, depends on the function of cofilin, a protein that belongs to the actin depolarizing factor family (Chen et al., 2000). Thus, the regulation of cofilin is of particular interest. Previous studies in our laboratory identified Slingshot-1L (SSH1L) as the phosphatase responsible for cofilin dephosphorylation and activation in VSMCs after treatment with platelet-derived growth factor (PDGF), the most relevant migratory stimulus for VSMC in vivo and in vitro (San Martin, 2008, Andrae et al., 2008). These findings underline the importance of SSH1L as a major regulator of VSMC migration and a potential therapeutic target for atypical migration.

The activity of SSH1L has been shown to be regulated by its association with regulatory protein, 14-3-3 (Kim et al., 2009). In VSMCs, different isoforms of 14-3-3 bind to SSH1L and form an inhibitory complex (Nagata-Ohashi et al., 2004, Kim et al., 2009 and Maheswaranathan et al., 2011). We have recently published that PDGF activates the SSH1L/cofilin pathway through the generation of reactive oxygen species (ROS) by the Nox1-based NADPH oxidase (Maheswaranathan et al., 2011). However, the mechanism of SSH1L activation by ROS is not completely understood. The purpose of this study is to investigate the role of Nox1 derived ROS in the regulation of SSH1L/14-3-3 complex. *We hypothesize that PDGF-induced Nox1-produced ROS induces the oxidation of cysteine residues within the 14-3-3 protein that ultimately impairs its ability to bind to SSH1L, leading to SSH1L activation.*

Materials and Methods

Cell Culture

Vascular Smooth Muscle Cells (VSMCs) were isolated from wild type (wt) and $Nox1^{-/y}$ mouse aortas by enzymatic disruption (Ohmi et al. 1997). Human Embryonic Kidney (HEK) cells were purchased from Stratagene (AD293 cat. #240085). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics, and used between passages 6 and 12. Cultures were maintained at $37^{o}C$ in a 5% CO_2 atmosphere. Cultures at 70-80% confluence were used for transfection or H_2O_2 treatment in growth media. Cells were made quiescent by incubation in serum-free media for 48 hours prior to treatment with 10ng/mL of platelet-derived growth factor-BB (PDGF-BB, human recombinant, BD Biosciences).

Plasmids

The plasmid containing the hexahistidine tag human 14-3-3γ was constructed by subcloning 14-3-3 cDNA into vector, pDEST^{TM} 26 (Clontech) and was a gift from Dr. Haian Fu, Emory University. The plasmid coding for the (CFP)-tagged human SSH1L (SSH1L-CFP) was generated using the pECFP-C1 (Clontech) vector and was donated by Dr. Kenzaku Mizuno from Tohoku University. The SSH1L mutants, SSH1L S834A-CFP or SSH1L S834D-CFP, were constructed in our laboratory (Maheswaranathan et al., 2011).

Total Cell Lysate Preparation

Media from the cell culture dishes was aspirated while on ice. The dishes were then washed 3 times with 5 mL of cold Phosphate Buffered Saline (PBS). Residual PBS was removed. Lysis buffer was added to each plate and cells were scraped from the dishes and set to

shake for 30 minutes at 4^{o}C. Next, cells were centrifuged at 14,000 RPM for 10 minutes at 4^{o}C to pellet cellular debris. The supernatant was then transferred to a microcentrifuge tube.

To measure the concentration of protein in the samples, a BCA protein assay was performed using bovine serum albumin (BSA) as the standard (Thermo Scientific). Samples were read using a µQuant spectrophotometer microplate reader and KC4 software. Cell lysates were normalized for the total amount of protein using lysis buffer.

Transfection

Cells were transfected by electroporation using the Amaxa Cell Line Nucleofector Kit V. Media was removed from cultured HEK293 cells and cells were washed with 2.5mg/mL Trypsin. Once cells were detached, the trypsinization reaction was neutralized with culture medium. Next, 2 x 10^{6} cells were counted for each reaction sample using Septer 2.0 Handheld Automated Cell Counter (Millipore). The cells were then collected by centrifugation at 0.5xg for 5 minutes at room temperature. The cell pellet was resuspended with 100µL of Cell Line Nucleofector Solution V. After mixing, the cell suspension was combined with 4µg of mutant SS1L-SD DNA and 1.5µg of His-tag wildtype or mutant 14-3-3. The entire suspension was transferred to a cuvette which was inserted into the Nucleofector cuvette holder and electroporated using the A23 program. The contents of the cuvette were then mixed with culture medium and plated.

Western Blot

Samples were loaded into a SDS-PAGE gel (NuPAGE, Invitrogen) and separated by size through electrophoresis. Next, they were transferred to PVDF membranes for 1 hour at 100 volts. The membranes were then blocked for 1 hour with 5% milk and washed 3 times with 0.1% TBS-

Tween for 5 minutes each time. After washing, membranes were incubated over night 14-3-3 primary antibody (Santa Cruz Biotechnology 1657) or anti-GFP antibody with CFP cross-reactivity (Abcam 6662) for 1 hour in a 1:1000 concentration.

The membranes were then washed again 3 times with 0.1% TBS-Tween and incubated for 1 hour with either anti-mouse secondary antibody (Abcam 97023) or an anti-goat secondary antibody (Santa Cruz Biotechnology 2020) containing horseradish peroxidase (HRP). Luminescence in the membrane was assessed after incubation with the HRP substrate (GE Healthcare RPN 2132) for 5 minutes using an image station (KODAK image station 4000 MM Pro series). The images were analyzed by densitometry using ImageJ software (National Institutes of Health).

Co-Immunoprecipitation (IP)

To test for SSH1L/14-3-3 interactions, HEK 293 cells were transfected by electroporation with mutant SS1L-S834D-CFP DNA and either His-tag 14-3-3 wildtype protein, His-tag 14-3-3 mutant C97/98A or His-tag 14-3-3 mutant C192A. Cells were incubated for 24 hours prior to immunoprecipitation. They were then lysed with a Tris pH 7.5 buffer with 0.5% Nonidet P-40 and harvested as described earlier. After that, cell lysates were transferred to a microcentrifuge containing 30µL of His-Tag isolation magnetic beads (dynabeads, Invitrogen) and incubated for 30 minutes at 4°C. Immunoprecipitates were then collected using a magnet to gather the protein complex attached to the magnetic beads. The supernatant was aspirated and discarded. The samples were washed 3 times with lysis buffer and prepared for western blotting with primary antibody against GFP (Abcam 6662) to evaluate the amount of CFP-tagged SSH1L co-precipitated with His-tagged 14-3-3.

Phosphatase Activity Assay

To determine the ability of active SSH1L to dephosphorylate endogenous SSH1L, SSH1L-S834A-CFP and PP2A were collected through immunoprecipitation (Abcam b291 and 32141) and mixed with endogenous SSH1L, retrieved from untransfected VSMCs (ab76943). Phosphate release was assessed by a colorimetric reaction with malachite green using color intensity measurements acquired from a μQuant spectrophotometer microplate reader at 600 nm. The quantity of free phosphate was based on a standard curve established using inorganic phosphate

For the SSH1L phosphatase activity assay *in vivo*, VSMCs from wildtype animals were used to immunoprecipitate total SSH1L, using a specific antibody (Abcam 291). The amount of phosphorylated SSH1L was assessed using an antibody that recognized the phospho-serine 14-3-3 binding motif (Cell signaling 9606). Total SSH1L was measured in the same membrane after reblotting.

Site-Directed Mutagenesis

Primers were created to introduce mutations in the 14-3-3 protein using the QuikChange XL site-directed mutagenesis kit from Stratagene. Four primers were constructed for this. Primer 97/98SA+ and primer 97/98SA- contain a mutation highlighted bellow for cysteines at positions 97 and 98 (TGT → GCT). Primer 192SA+ and primer 192SA- carry were used to mutate the cysteine at position 192 (TGC → GCC). DNA from different clones was subjected to Sanger-based automated DNA sequencing by Agencourt Bioscience using custom-made primers and commercially available primers, Dest +1 and Universal T7.

97/98SA-: 5’ – TTTGTCCAGTACATCCAGAATGTCAGCAGCGATTAACTTTAGCTCAGTCTCAACC – 3’

97/98SA+: 5’ – GGTTGAGACTGACCTAAAGTTAATCGCTGCTGACATTCTGGATGTACTGGACAAA – 3’

192SA+: 5’ – CCCCTGACCGTGCCGCCAGGTTGGCAAAAG – 3’

192SA-: 5’ – CTTTTGCCAACCTGGCGGCACGGTCAGGGG – 3’

Labeling with 5-Iodoacetamido Fluorescein (5-IAF)

To determine the amount of oxidation of cysteine residues, a 5-IAF label was used as previously described (Wu et al., 1998). First, VSMCs from wild type and $Nox1^{-/y}$ animals were stimulated with PDGF (10ng/mL) for 15 minutes and lysed with MES buffer pH 6.5 that was bubbled with argon for 60 minutes before the experiment. The 5-IAF solution was added to the cell lysate to a final concentration of 10 µM. The reaction was then incubated for 1 hour in the dark at 4°C. After the incubation period, β-mercaptoethanol was added to a final concentration of 20 µM to stop the reaction. Cell lysates were then mixed with 5 µg of fluorescein antibody (Abcam 6213) for overnight immunoprecipitation. The reactions were then incubated with 40µL of an anti-mouse antibody (Santa Cruz Biotechnology 2336) bound to L-Agarose beads. The binding reaction was performed at 4°C for 1 hour. Immunoprecipitates were centrifuged at 4,000 rpm for 5 minutes and washed with lysis buffer. This step was repeated 3 times before the samples were ready for western blotting. The amount of 14-3-3 pulled down was measured by blotting with a 14-3-3 specific antibody (Santa Cruz Biotechnology 1657).

Statistical Analysis

Results are expressed as means ± SEM (standard error of the mean). Differences among groups were analyzed using t-test or 1-way ANOVA, with post hoc contrasts adjusted to the Duncan or Bonferroni correction using SPSS 14.0 for Windows. A value of $p < 0.05$ was considered to be statistically significant.

Results

Phosphorylation of Serine 834 site controls SSH1L binding to 14-3-3

Previous results in our laboratory demonstrated that PDGF stimulates SSH1L activity by serine 834 dephosphorylation in wild type VSMCs but not in $Nox1^{-/y}$ cells (Maheswaranathan et al., 2011). Interestingly, Serine 834 is found in a 14-3-3 consensus binding motif, raising the possibility that 14-3-3 regulates SSH1L activity by formation of an inhibitory complex.

In order to confirm that serine 834 regulates SSH1L/14-3-3 complex formation, the San Martín laboratory created two mutants using site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene) (Maheswaranathan et al., 2011). One mutation generated a phospho-mimetic mutant (S834D) by mutating the serine 834 residue to aspartic acid while the other produced a phospho-deficient mutant (S834A) by mutating the serine to alanine. Due to similarities in their chemical structures, the aspartic acid mutation mimics a constant phosphorylated serine residue and the alanine mutation mimics a dephosphorylated serine.

Studies of enzymatic activity demonstrated that the phosphodeficient mutant SSH1L-S834A behaves as a constitutively active phosphatase presumably because of the inability to bind to its inhibitory partner 14-3-3 (Maheswaranathan et al., 2011). In order to evaluate how the phosphorylation of serine 834 affects SSH1L binding to 14-3-3 and regulation of enzymatic activity, HEK cells were co-transfected with a 14-3-3-His tag protein and either SSH1L-S834D-CFP or SSH1L-S834A-CFP. His-tagged proteins were immunoprecipitated and the amount of SSH1L bound was measured using an antibody against CFP (Abcam 6662). The results of this experiment demonstrate that there is a higher amount of the phosphomimetic mutant bound to 14-3-3 than the phosphodeficient mutant (Figure 1). As expected, the phosphorylation at position

834, results in greater binding capacity for 14-3-3 while dephosphorylation at this site decreases its binding. These results indicate that the phosphorylation at serine 834 keeps SSH1L bound to 14-3-3 while dephosphorylation reduces its binding.

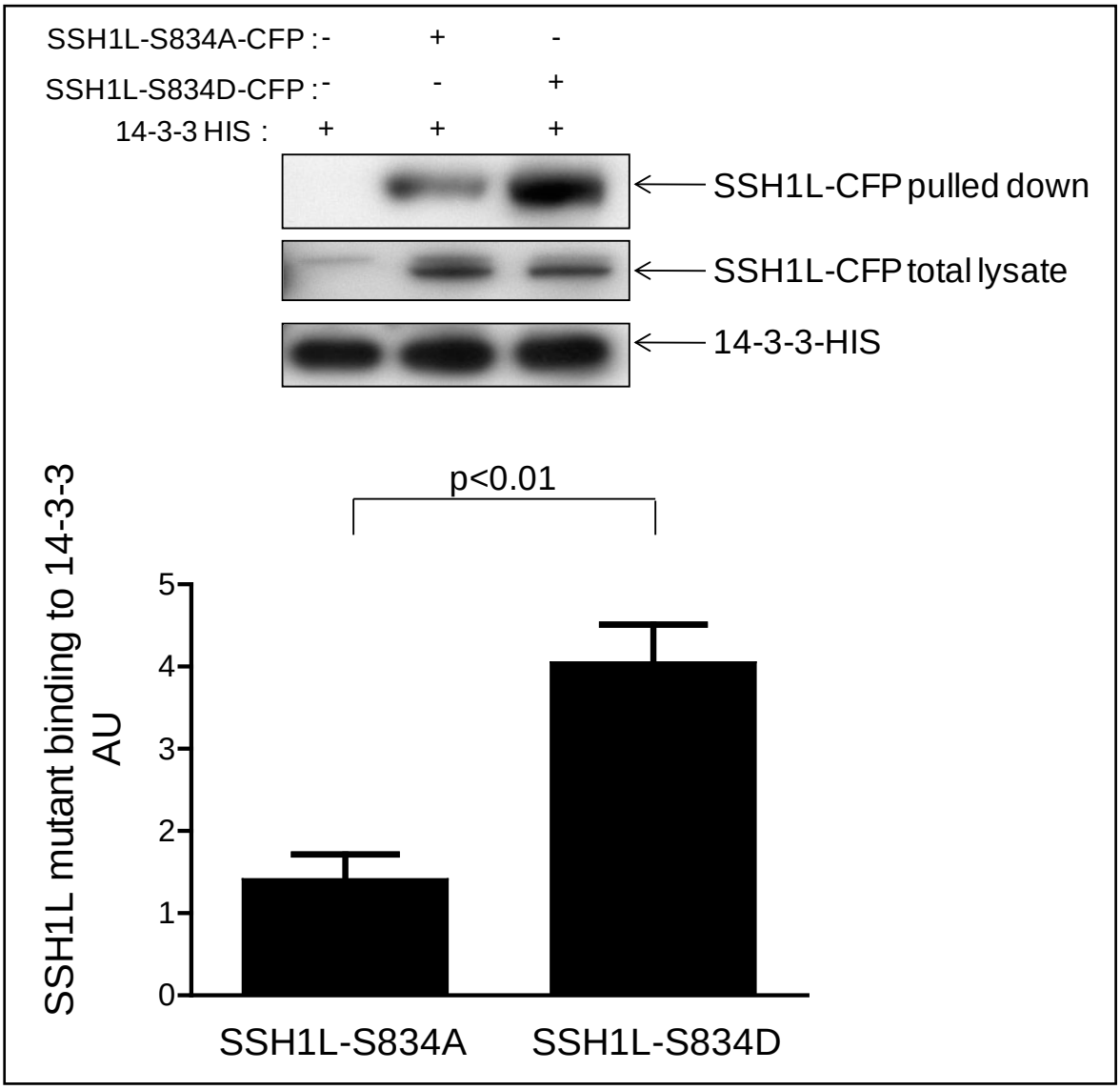

Figure 1. P**hosphorylation of serine 834 regulates SSH1L binding to 14-3-3.** HEK cells were co-transfected with His-tagged 14-3-3 and either SSH1L S834A-CFP or SSH1L S834D-CFP by electroporation. After 24 hours, His-tagged 14-3-3 proteins were immunoprecipitated using dynabeads and bound SSH1L was quantified using an antibody against CFP. Bars indicate mean ± S.E. Average of four independent experiments. [Figure obtained courtesy of *Journal of Biological Chemistry*, Maheswaranathan et al., 2011]

SSH1L is autodephosphorylated

As discussed previously, serine 834 regulates 14-3-3 binding and SSH1L activity induced by PDGF in VSMCs. Consequently, the next goal was to find the phosphatase responsible for dephosphorylation. Previous studies showed that vanadate, an inhibitor against tyrosine phosphatases, reduce SSH1L activity (Lee et al., 2006). Since the dephosphorylation occurs on a serine residue (Figure 1), our laboratory reasoned that the phosphatase involved must be a dual phosphatase that is inhibited by vanadate. Interestingly, there are very few dual phosphatases known in VSMCs and only one activated by PDGF, SSH1L. Therefore, it was hypothesized that SSH1L could be acting on itself. To validate the theory, the San Martín laboratory assessed the ability of SSH1L to dephosphorylate endogenous SSH1L using a constitutively active form of SSH1L, the phospho-deficient mutant S834A (Maheswaranathan et al., 2011). The amount of free phosphatase released was measured using a colorimetric reaction with malachite green (Maheswaranathan et al., 2011). Figure 2A shows there is a significant increase of phosphatase released by endogenous SSH1L in the presence of SSH1L-S834A, which is not observed by other phosphatases such as protein phosphatase 2 (PP2A). This result implies that endogenous SSH1L is dephosphorylated by SSH1L-S834A.

Next, the quantity of phospho-SSH1L was also evaluated using specific antibody against the phospho-serine in the 14-3-3 binding motif. In the presence of SSH1L-S834A, no amount of phosphorylated SSH1L remains within the endogenous SSH1L protein, suggesting that all SSH1L is dephosphorylated (Figure 2B) by the SSH1L-S834A mutant. Collectively, these experiments demonstrate that SSH1L is autodephosphorylated.

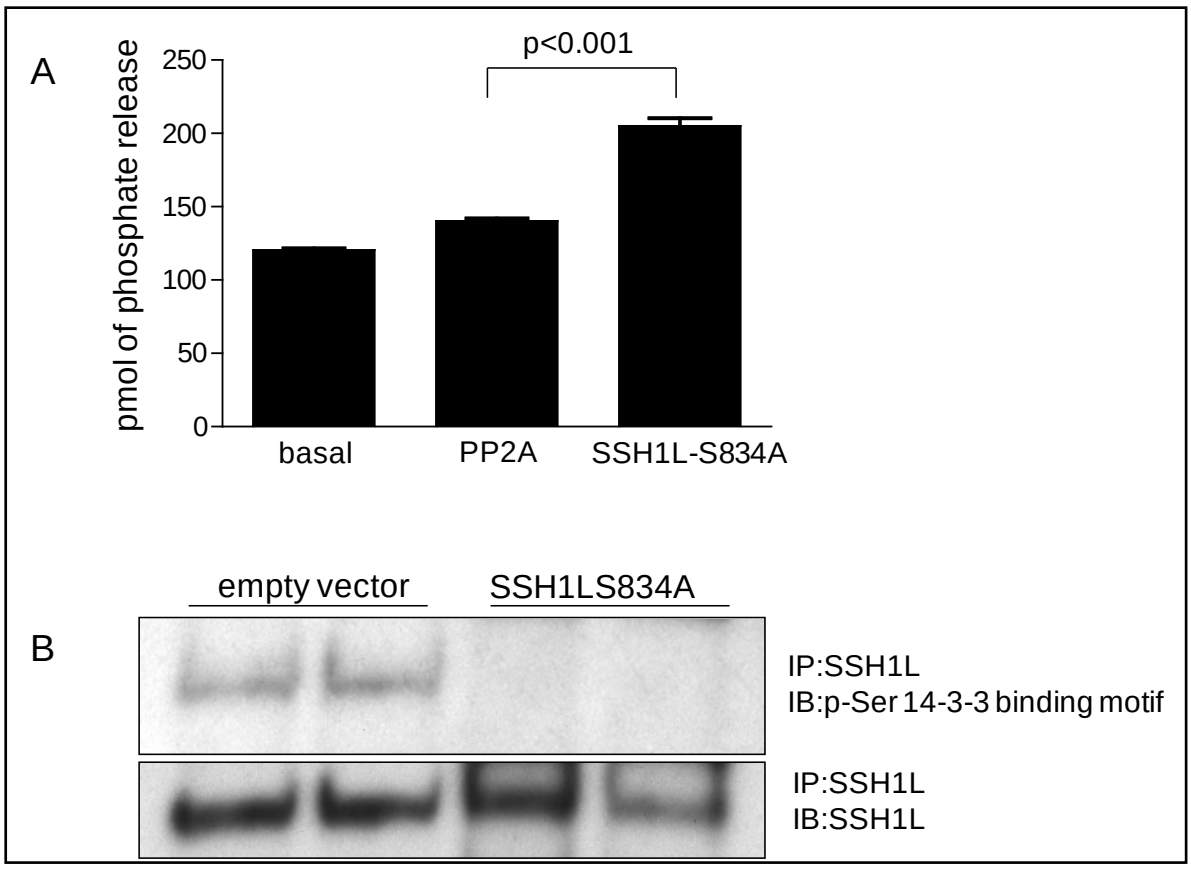

Figure 2. SSH1L autodephosphorylation. HEK cells were transfected with empty vector or SSH1L-S834A-CFP. *A,* Amount of released endogenous SSH1L using PP2A or SSH1L-S834A. S834-GFP and PP2A were retrieved by immunoprecipitation and reacted with endogenous SSH1L immunoprecipitated from untransfected cells. Phosphatase released was measured using a colorimetric reaction with malachite green. *B,* Dephosphorylation of the serine residue in 14-3-3 binding motif by S834A. Endogenous SSH1L was immunoprecipitated and the amount of phospho-SSH1L was quantified using specific antibodies against phospho-Ser-14-3-3 binding motif. To evaluate that same amount of SSH1L protein, the same membrane was reblotted. Western blot represents four independent experiments. [Figure obtained courtesy of *Journal of Biological Chemistry*, Maheswaranathan et al., 2011]

14-3-3 cysteine residues are oxidized after PDGF treatment which reduces 14-3-3 binding to SSH1L

It was evident from these experiments that SSH1L activation depends on the dephosphorylation of serine 834 and the disruption of its complex with 14-3-3. However, in basal conditions, SSH1L is constitutively phosphorylated in VSMCs and has no detectable activity (San Martín et al., 2008). Moreover, the dephosphorylation of SSH1L by PDGF requires Nox1. These observations suggest that there is an additional step that does not require phosphatase activity for the activation of SSH1L to occur. We hypothesized that this initial step is Nox1-mediated oxidation of cysteine residues within 14-3-3 that disrupt the SSH1L/14-3-3 complex formation and therefore induce SSH1L activation. To confirm the PDGF-induced oxidation of 14-3-3, we used 5-Iodoacetamido Fluorescein (5-IAF) to label cysteine residues in 14-3-3. First, we confirmed that cysteine groups in 14-3-3 contained redox sensitive cysteines that are oxidizable by H_2O_2 (Figure 3A). Next, we studied the effect of PDGF treatment, which is a known activator of the Nox1 NADPH oxidase. Figure 3B shows that there is a decrease in the amount of 14-3-3 cysteine groups in wildtype cells after PDGF treatment, implying that oxidation occurs in these residues. On other hand, the amount of cysteine groups remain relatively the same for $Nox1^{-/y}$ cells, suggesting that 14-3-3 oxidation does not occur in $Nox1^{-/y}$ cells. Thus, PDGF induces the oxidation of cysteine groups within 14-3-3 in VSMCs via a mechanism that requires Nox1.

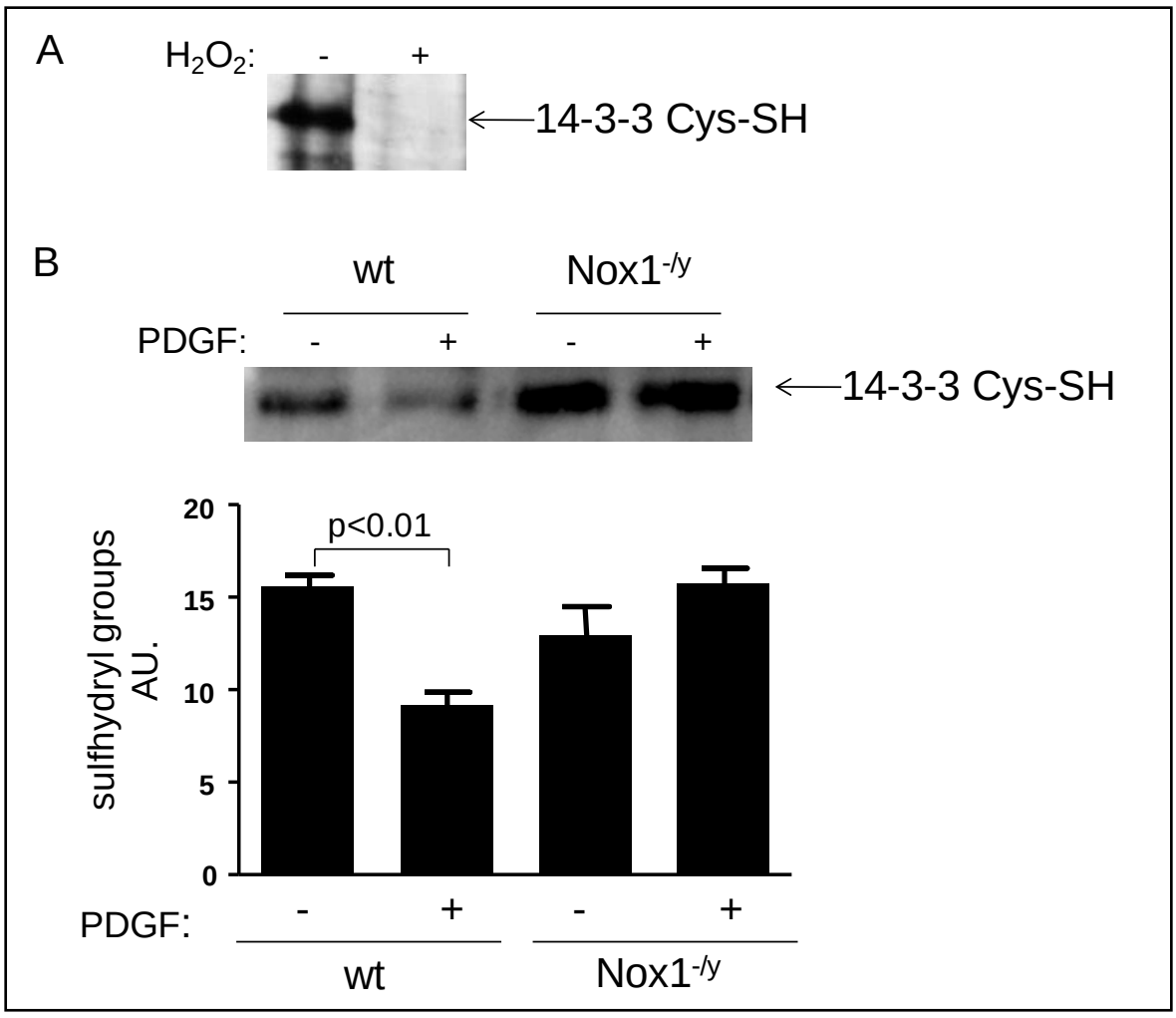

Figure 3. PDGF stimulates the oxidation of 14-3-3 cysteine groups in VMSCs but not Nox1$^{-/y}$cells. *A,* 14-3-3 cysteine residues are oxidizable. His-tagged 14-3-3 proteins were immunoprecipitated after 15 minutes of H_2O_2 treatment or control conditions. Cysteine groups were then detected using 5-IAF in anaerobic conditions. *B*, Nox1 is required for 14-3-3 oxidation by PDGF. Cells from wild type and Nox1$^{-/y}$ animals were stimulated with PDGF (for 15 min, and cysteine oxidation was measured as described in "Materials and Methods"). Bars represent mean ± S.E. average from four independent experiments. [*Journal of Biological Chemistry*, Maheswaranathan et al., 2011]

To connect this important finding to the increase in SSH1L activity previously observed after PDGF treatment, we investigated the effect of 14-3-3 oxidation on the SSH1lL/14-3-3 complex. To do this, we measured the binding of 14-3-3 to a constitutively phosphorylated SSH1L, the phospho-mimetic mutant S834D, before and after H_2O_2 treatment. The data from this experiment demonstrates that the addition of H_2O_2 decreases the amount of complexed 14-3-3 with the mutant SSH1L-S834D mutant (Figure 4). Therefore, 14-3-3 oxidation appears to reduce binding to SSH1L even in the presence of a phosphorylated 14-3-3 binding motif in SSH1L. Considering that Nox1 is required for the oxidation of 14-3-3 and that 14-3-3 oxidation decreases SSH1L binding (Figure 3B), it is logical that Nox1 derived H_2O_2 leads to the initial disruption of the SSH1L/14-3-3 complex that ultimately leads to the activation of SSH1L.

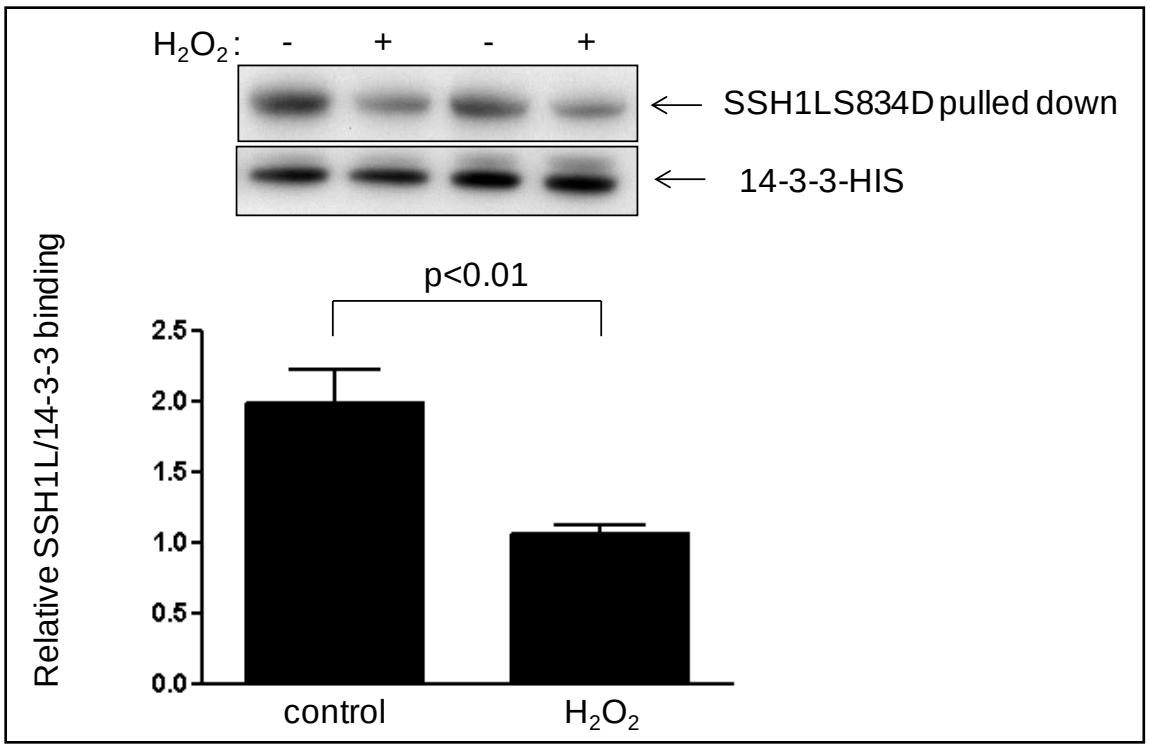

Figure 4. 14-3-3 oxidation decreases binding to SSH1L.14-3-3 binding to SSH1L before and after H_2O_2 treatment. HEK cells were transfected with 14-3-3 His and SSH1L-S834D-CFP. His-tagged proteins were immunoprecipitated after H_2O_2 treatment or control conditions. A western blot using an antibody against 14-3-3 was used to identify 14-3-3. SSH1L/14-3-3 binding was calculated using an antibody that recognized the CFP label. [Figure obtained courtesy of *Journal of Biological Chemistry*, Maheswaranathan et al., 2011]

14-3-3 Structure and Generation of 97/98 and 192 Mutants

Considering that the oxidation of the 14-3-3 protein is a crucial step for SSH1L phosphatase activation, we decided to investigate the site of oxidation within the 14-3-3 protein. For this, we examined the human epsilon 14-3-3 structure using published data (Yang et al., 2006). Analysis of the 14-3-3 structure shows three exposed, low pKa cysteine residues, which could potentially be involved in the redox reaction due to the presence of sulfyhydryl (SH) groups.

Next, we acquired the amino acid sequence of the protein from a gene database (NCBI). Two of the cysteine residues were found in positions 97 and 98, and the other cysteine was located at position 192. Since the 192 cysteine group is contiguous to an arginine residue, it is more acidic and reactive than the other two and thus more likely to be oxidized. Because of this, we hypothesized that this is the site of oxidation. Nevertheless, we studied all three cysteines as possible sites of 14-3-3 oxidation and generated two 14-3-3 mutants using site-directed mutagenesis (QuikChange Site-directed Mutagenesis Kit, Stratagene). One mutant modified the 97 and 98 cysteine groups to alanine residues and the other changed the 192 cysteine group into to an alanine as well. The alanine group was chosen because of its non-reactivity and its insensitivity to oxidation.

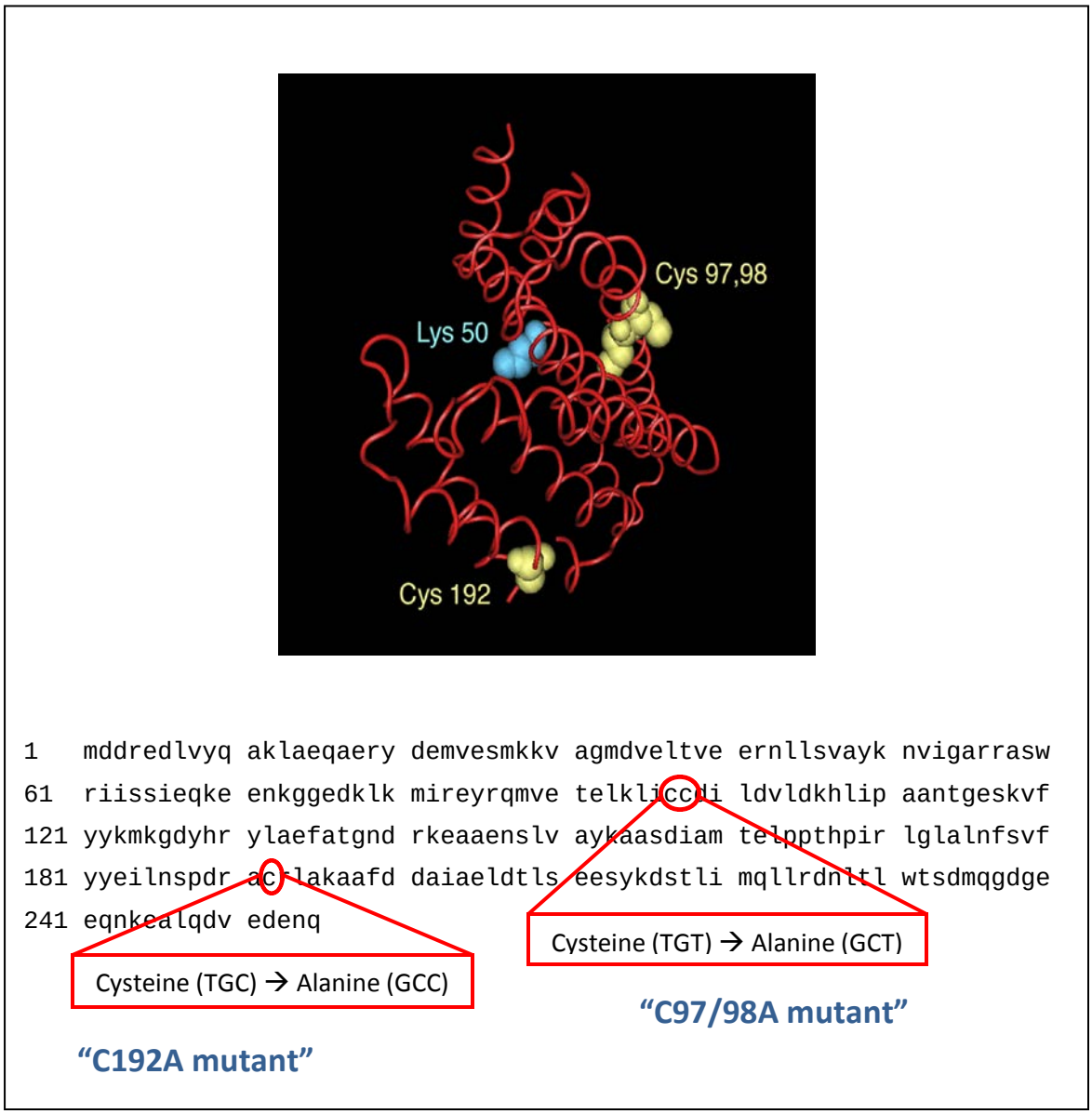

Figure 5. 14-3-3 protein structure and sequence. Crystal structure of the human epsilon 14-3-3 protein. Cysteine groups at positions 97, 98 and 192 are circled in red. Two mutants, "C192A" and "C97/98A" were generated using site-directed mutagenesis.

To verify that there were no DNA insertions in the desired mutants, we collected DNA from the 14-3-3 mutant clones and performed PCR using universal T7 primer and custom-made primers, Dest +1 and Primer +3 (see map in Figure 6). The PCR yielded two different fragments of sizes 386 and 987 base pairs. We identified the mutant clones with the correct size of PCR product using agarose gel electrophoresis. Mutants 97/98#2, 9798#3, 192#1, 192#2, and 192#3, 192#7 and 192#9 contained the right length of PCR fragments, suggesting there were no random insertions (Figure 6). These were chosen for whole gene sequencing (Beckman Coulter Genomics) to verify the desired mutations with no additional changes in the gene sequence.

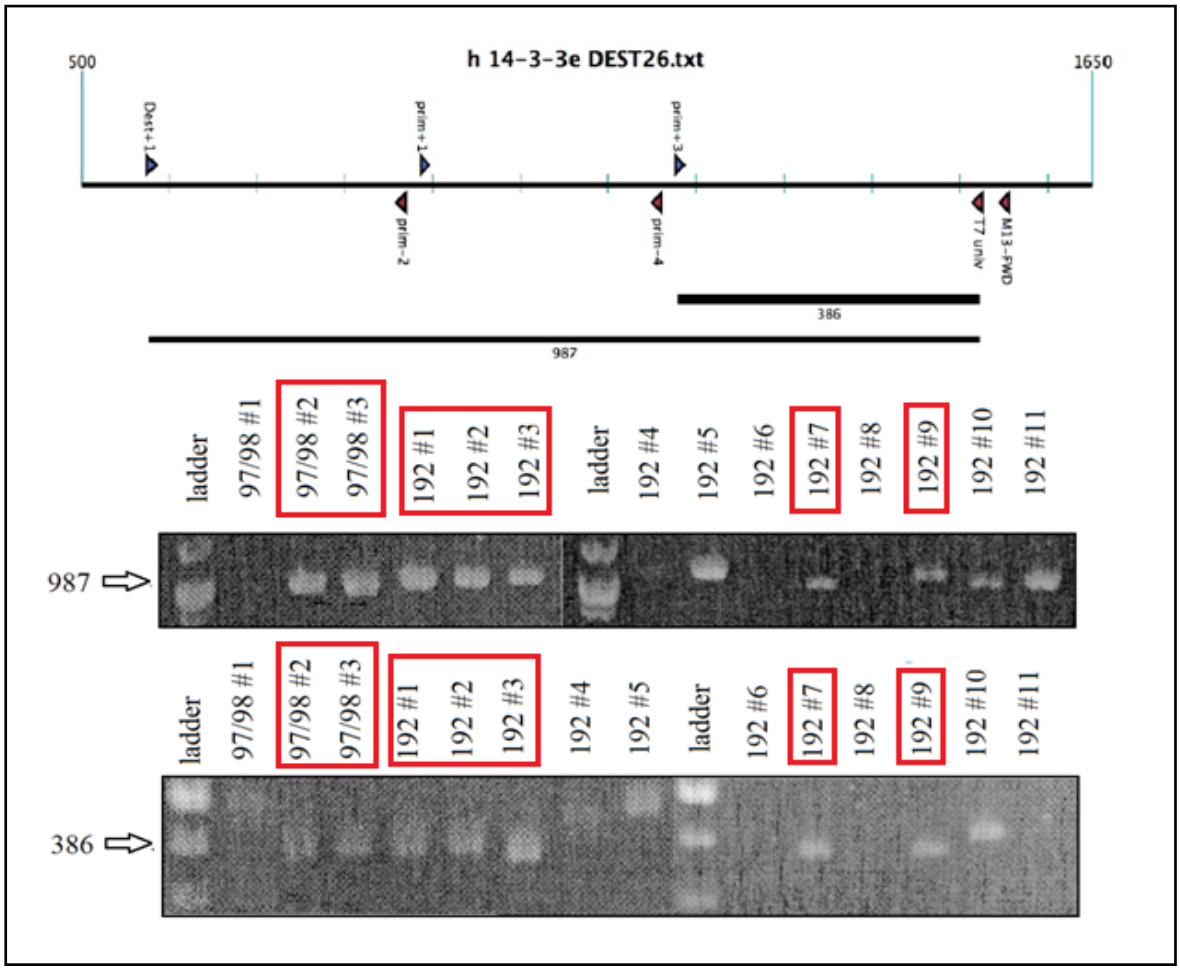

Figure 6. PCR results of 14-3-3 mutants using two primers Dest +1, Primer +3 and Universal T 7. Mutants 97#2, 97#3, 192#1, 192#2, and 192#3, 192#7 and 192#9 possess mutants of interest.

14-3-3 mutant binding capacity to SSH1L in basal and H_2O_2-treated conditions (Preliminary)

Based on the fact that H_2O_2 treatment reduces the binding of 14-3-3 to a phosphomimetic mutant of SSH1L, we hypothesize that the oxidation of SH groups within 14-3-3 will change its ability to bind SSH1L and that the mutation of these redox sensitive cysteine residues will produce a redox insensitive 14-3-3, unable to release SSH1L. In order to determine the cysteine responsible for the H_2O_2-induced disruption of 14-3-3/SSH1L complex, we transfected HEK cells with SSH1L-S834D and either wildtype 14-3-3, C97/98A or C192A mutants. As expected, all three mutants are able to interact with SSH1L-S834D (Figure 7). In addition, the 14-3-3/SSH1L complex is disrupted by H_2O_2 as demonstrated by the decrease in 14-3-3 wild type binding to SSH1L after H_2O_2 treatment. On the other hand, mutants C97/98A and C192A show very little difference in SSH1L binding capacity. However, there is also significantly less amount of 14-3-3 His-tagged protein. Normalization of transfected protein in the future will hopefully reduce any source of error and allow us to clearly determine the effect of the cysteine mutation found in this experiment.

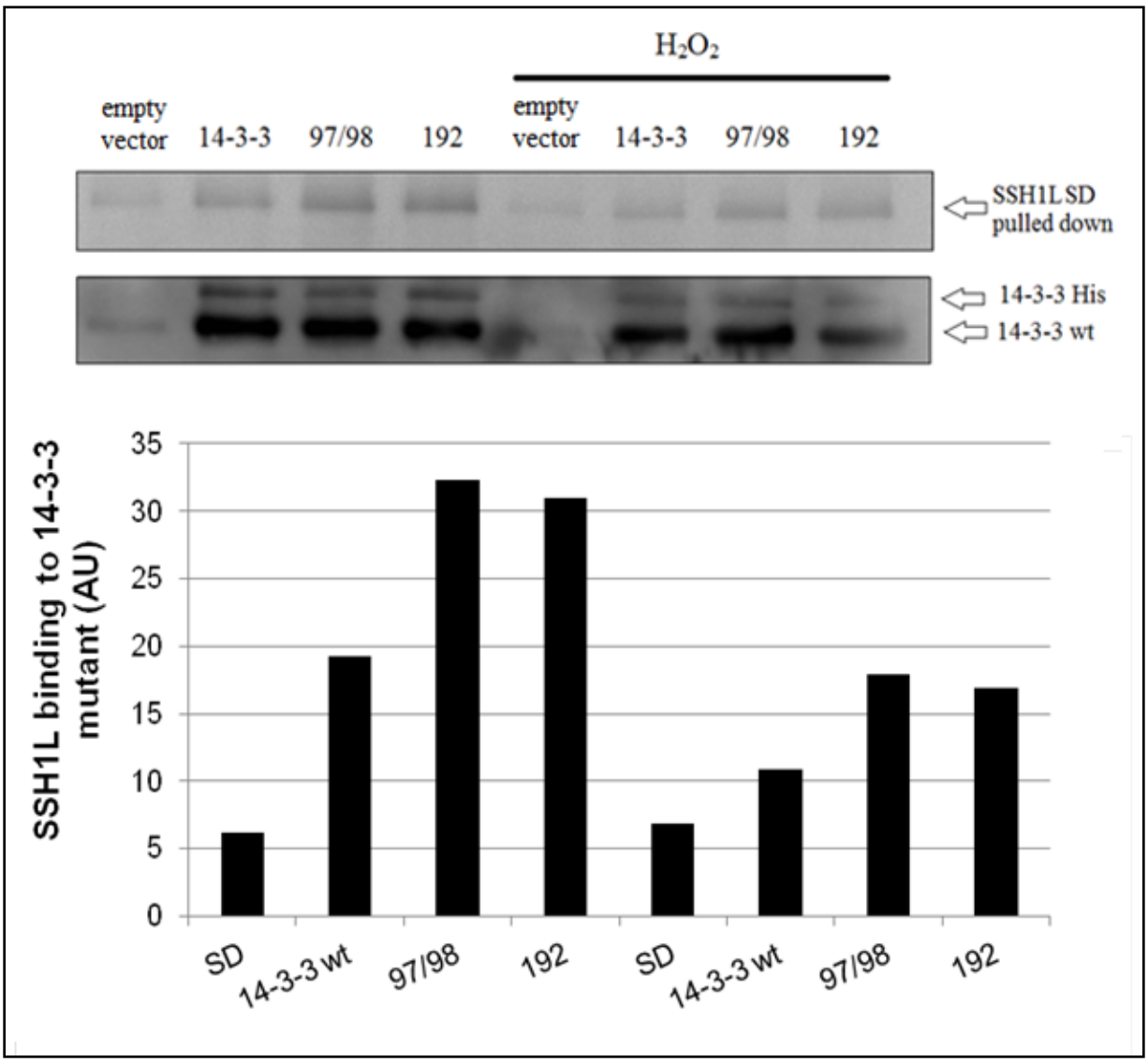

Figure 7. 14-3-3 mutant binding capacity to SSH1L before and after H_2O_2 treatment. HEK cells were transfected with SSH1L-S834D-CFP and either 14-3-3 His, C97/98A or C192A mutant. His-tagged proteins were immunoprecipitated after 30 minutes treatment with H_2O_2 or control conditions. A western blot using an antibody against 14-3-3 was used to identify 14-3-3. Bound SSH1L was detected using an antibody that recognized the CFP tag.

Discussion

Our results present an interesting model for SSH1L activation by PDGF in VSMC. Previous work in our laboratory indicated that Nox1 derived ROS are required for SSH1L dephosphorylation and consequently phosphatase activation. In this study, we found that the dephosphorylation of serine 834 within the 14-3-3 binding motif governs SSH1L binding to 14-3-3, as demonstrated by the decreased binding of phospho-deficient SSH1L to 14-3-3 (Figure 1), and ultimately SSH1L activation (Maheswaranathan et al., 2011). Further experiments showed that the phosphatase responsible for this dephosphorylation was the SSH1L itself (Figure2). However, SSH1L is constitutively phosphorylated in VSMCs and possesses no activity in basal conditions. Thus, the question of initial SSH1L activation after agonist stimulation still remained unanswered. Considering that SSH1L dephosphorylation by PDGF depends on Nox1, we then concluded that there must be an additional step to the mechanism that did not use the phosphatase activity. Since the mechanism of activation is dependent on Nox1 activation, we hypothesized that the first step of SSH1L/14-3-3 complex disruption and therefore SSH1L activation is the oxidation of 14-3-3 by Nox-1 derived ROS and that this oxidation changes 14-3-3 binding activity facilitating SSH1L/14-3-3 complex disruption.

In order to determine if PDGF stimulation leads to the oxidation of 14-3-3, we used a label against reduced cysteine residues within 14-3-3. Our data demonstrate that cysteine residues are oxidized in wild type cells treated with PDGF, as shown by the overall decrease of SH groups (Figures 3B). On the other hand, PDGF does not induce the oxidation of 14-3-3 in cells derived from $Nox1^{-/y}$ (Knockout) animals. These results imply that the mechanism of cysteine oxidation by PDGF depends on Nox1, as we saw no significant SH groups after PDGF treatment in the cells lacking Nox1 protein.

We have demonstrated that the SSH1L/14-3-3 complex is disrupted by dephosphorylation of the Serine 834. Therefore, to evaluate the effect of 14-3-3 oxidation on its binding to SSH1L, we studied the effect of H_2O_2 treatment on 14-3-3 binding to the phospho-mimetic SSH1L mutant (SSH1L-S834D) in which the increase in phosphatase activity should not have an effect. We confirmed that H_2O_2 treatment results in decreased 14-3-3 binding to the SSH1L-S834D mutant (Figure 4). This experiment confirmed our hypothesis that Nox1-mediated oxidation of 14-3-3 by PDGF causes the initial SSH1L/14-3-3 complex disruption. This initial separation is what leads to the SSH1L autodephosphorylation cascade that results in SSH1L activation. The proposed mechanism is shown in Figure 8.

Given that ROS-mediated signaling by PDGF is significant to SSH1L activation and that 14-3-3 proteins play a key role in this mechanism and other important biological processes, we decided to analyze further the oxidation of 14-3-3. Analysis of the structure showed three possible sites of oxidation, cysteine residues in positions 97, 98 (likely to produce an S-S bridge) and 192 (Figure 5). To pinpoint the cysteine residue responsible for 14-3-3 oxidation, we generated two mutants and analyzed their binding to the phosphomimetic mutant SSH1L-S834D (Figure 7). We hypothesized that the ROS insensitive mutant would show no change in binding before and after H_2O_2 treatment. Our results for this experiment, however, were inconsistent (Figure 7). While H_2O_2 appears to reduce wild type 14-3-3 binding to SSH1L-SD, it also decreases the binding of both C97/98A and C192A mutants. This observation, however, could be due in part to the unequal amount of 14-3-3 protein transfected across the samples, resulting in different amounts of 14-3-3 pulled down and therefore SSH1L binding. It can also be attributed to the fact that all three cysteines are participating in the redox regulation of 14-3-3 binding capacity. We hope to address this issue in the future by repeating the experiment and careful

measurement of protein concentration and to repeat this experiment with a mutant that has mutations in all three cysteine residues. Nevertheless, we believe that by studying each cysteine residue in 14-3-3, we can better understand the formation/disruption of the 14-3-3 complex with SSH1L and, on a broader scope, with other protein partners.

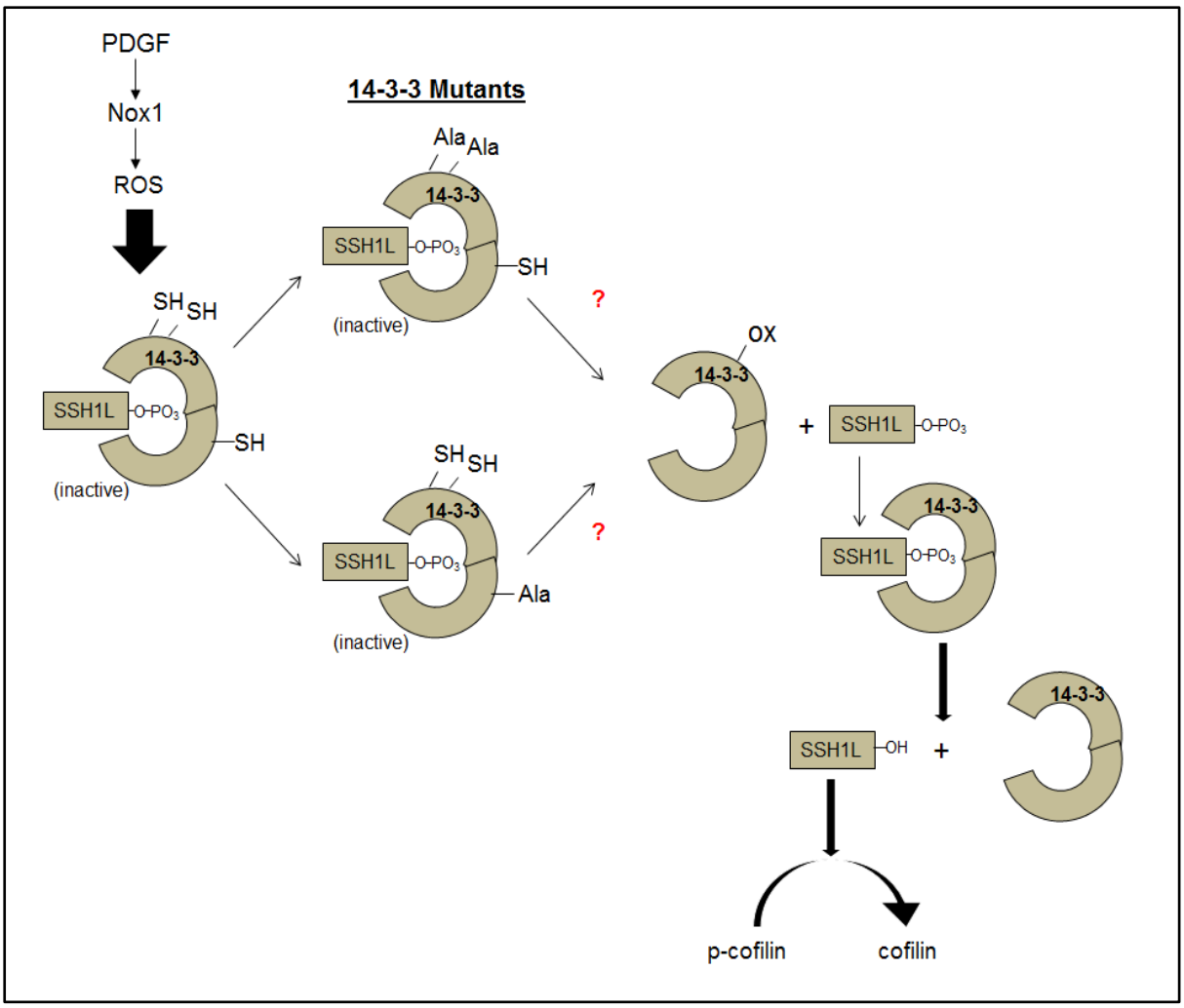

Figure 8. Model of SSH1L activation by PDGF in VSMCs. This diagram demonstrates our model for the mechanism of SSH1L activation by PDGF that results in VSMC migration. PDGF activates Nox1 to produce ROS. ROS act on cysteine residues within 14-3-3 to oxidate the protein. We created mutants to identify the site of oxidation. 14-3-3 oxidation leads to disruption of the SSH1L/14-3-3 complex. This step allows for the SSH1L autodephosphorylation to occur which ultimately leads to the dephosphorylation of cofilin, actin reorganization and ultimately migration.

Limitations and Future Directions

As we discussed previously, 14-3-3 proteins exist in dimers, creating the possibility of different protein configurations for 14-3-3 in our experiments: endogenous 14-3-3 dimer, endogenous 14-3-3 and 14-3-3 mutant dimer and 14-3-3 mutant dimer. Our experiment isolates only His-tagged 14-3-3 mutants. However, because of dimerization of 14-3-3 proteins, His-tagged 14-3-3 mutants may be coupled to another 14-3-3 mutant or a wildtype 14-3-3 protein. Now, if the dimer is composed of only the mutant, oxidation will not occur in the redox insensitive mutant. On the other hand, if the dimer is composed of a 14-3-3 mutant and wildtype, it is possible that oxidation of the wildtype might disrupt the SSH1L/14-3-3 complex. If that occurs, the effect on 14-3-3 mutant binding to SSH1L will be reduced even if the mutant is redox insensitive. Nevertheless, based in previous experiments, we expect that 14-3-3 mutant homodimers due to protein overexpression will be able to override the heterodimers.

We plan to continue the analysis of the binding capacity of each 14-3-3 mutant in H_2O_2 and basal conditions. We will also repeat this experiment in PDGF-treated conditions. Afterwards, we will determine the sensitivity of each mutant to oxidation by transfecting VSMCs with the mutants and using our cysteine residue probe, 5-IAF. For this experiment, transfected VSMCs will be treated with H_2O_2, PDGF and vehicle prior to immunoprecipitation and labeling as mentioned earlier. If the SSH1L/14-3-3 complex is disrupted in both mutant cells, we will perform a triple mutation using C97/98A as template for site-directed mutagenesis, as mentioned earlier. This will allow us to determine whether all cysteine residues are involved in the oxidation of 14-3-3. Additionally, we want to study the possible modifications of the cysteine residues and in the future, the 14-3-3 oxidized structure. We hope that by fully understanding the oxidation of 14-3-3, we can not only shed light on SSH1L activation and cell migration, but also

the general function of 14-3-3 as regulatory proteins and their association with other binding partner proteins.

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