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Daniel Kikuchi March 16, 2017

Investigating the mechanism by which Poldip2 activates Ect2 and the downstream consequences of this activation in vascular smooth muscle cells

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Abstract

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During atherogenesis, cell migration, proliferation, and extracellular matrix secretion are mis-regulated in vascular smooth muscle cells (VSMCs). Polymerase δ interacting protein 2 (Poldip2) is a critical regulator of these processes and many functions of Poldilp2 are mediated by its interactions with NADPH oxidases (Noxes) and RhoA. In the vasculature, Noxes serve as important enzymatic sources of reactive oxygen species (ROS) and at physiological levels ROS derived from Noxes function as signaling molecules. While the mechanism remains unclear, one consequence of Poldip2-generated ROS is activation of RhoA. The Rho family of GTPases are critical regulators of VSMC function; however, under pathophysiological conditions, their downstream effectors promote cardiovascular pathologies. In this study, we investigated the mechanism by which Poldip2 activates RhoA in VSMCs.

Canonically, Rho GTPases are activated by Rho guanine nucleotide exchange factors (GEFs). Thus, we first performed RhoA(17A) pulldowns for active GEFs in cells overexpressing Poldip2 or vector control. Analyzing these pulldowns by mass spectrometry, and confirming this by immunoblotting, we identified Ect2 as a GEF activated by Poldip2 and began to investigate the mechanism for this activation. After finding Poldip2 does not regulate Ect2 expression or subcellular localization, we hypothesized that ROS-derived from Poldip2/Nox signaling may be involved in activating Ect2. We began by testing the effect of ROS on Ect2 and found that exogenous H_2O_2 activates Ect2. Next, using VSMCs with genetic deletion of Nox1 or Nox4, we found that Nox4 is not required for Poldip2 to activate Ect2, but instead Nox1 is likely required. The effect of Poldip2 on Nox1 activity remains unreported; however, our data suggest that Poldip2 acts as a positive regulator of Nox1 activity. Finally, while considering the downstream consequences of this signaling, we used siRNA to probe the role of Polidp2 and Ect2 in VSMC proliferation, and showed that both of these molecules promote VSMC proliferation. Interestingly, Poldip2 and Ect2 may promote VSMC proliferation by inhibiting senescence and we are currently investigating the effect of Poldip2 and Ect2 knockdown on VSMC senescence. Thus, we propose Poldip2 signals through Nox1 to activate Ect2 in a ROS-dependent manner and enhances VSMC proliferation by inhibiting senescence.

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ABBREVIATIONS

Ab: Antibody

ApoB-100: Apolipoprotein B100

β-galactosidase: β-gal

BRCT: BRACA1 C Terminus

DH: Dbl-Homology

DMEM: Dulbecco's Modified Eagle's Media

DSB: Double-Strand Break

ECT2: Epithelial Cell Transforming 2

GEF: Guanine Nucleotide Exchange Factor

H_2O_2: Hydrogen Peroxide

LDL: Low-Density Lipoprotein

MASM: Mouse Aortic Smooth Muscle

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NOX: NADPH Oxidase

$O_2^{\cdot -}$: Superoxide

PCNA: Proliferating Cell Nuclear Antigen

PH: Pleckstrin Homology

PHGDH: Phosphoglycerate Dehydrogenase

PKC: Protein Kinase C

POLDIP2: Polymerase δ Interacting Protein 2

RASM: Rat Aortic Smooth Muscle

ROS: Reactive Oxygen Species

SEM: Standard Error of the Mean

SMC: Smooth Muscle Cell

VSMC: Vascular Smooth Muscle Cell

BACKGROUND

Atherosclerotic Plaque Formation

One-third of all deaths worldwide are caused by cardiovascular diseases.[1] Moreover, coronary heart disease arising from atherosclerosis is the most common type of cardiovascular disease.[1] Atherosclerosis is a chronic disorder characterized by plaque formation and luminal narrowing (stenosis) of large and medium-sized arteries.[2] The process is initiated in the endothelial-lined lumen of the intima (the innermost layer of an artery).[3] Endothelial dysfunction and structural damage expose negatively charged extracellular matrix proteoglycans[2, 4], which lie just beneath the lumen.[3] These proteoglycans promote the accumulation of low-density lipoprotein (LDL) in the intima.[2, 4] LDL transports cholesterol in the blood and is composed of lipids and proteins stabilized by Apolipoprotein B100 (ApoB-100).[5] ApoB-100 ionically interacts with exposed proteoglycans, trapping LDL particles in the intima.[2, 4] In the intima, LDL is prone to oxidative modification by reactive oxygen species (ROS) and enzymatic attack, which initiates an inflammatory response.[2]

Oxidation of LDL particles activates endothelial cells and triggers the production of adhesion molecules and chemokines.[2] Subsequently, monocytes, dendritic cells, and T cells are recruited into the intima.[2] Cytokines secreted from activated endothelial cells promote the differentiation of monocytes to macrophages.[2] These monocyte-derived macrophages take up oxidized LDL, which turns them into foam cells.[2] Early lesions, called fatty streaks are composed of lipid filled foam cells and activated T cells.[2, 4] As the disease progresses, cell debris and cholesterol crystals accumulate in fatty streaks to form a necrotic core.[2, 4] Smooth muscle cells (SMCs) begin to proliferate and migrate

into the plaque, which is eventually covered by a fibrous cap composed of collagen and SMCs.[2, 4] Plaque growth can cause stenosis and ischemia in the surrounding tissues. At its most advanced stage, atherosclerosis commonly manifests as acute coronary syndrome, myocardial infarction, or stroke when a plaque ruptures and causes thrombosis.[4]

Atherosclerosis and Vascular Smooth Muscle Cells

As described above, the development and progression of atherosclerosis involves a series of complex interactions between endothelial cells, lymphocytes, macrophages, and SMCs. Of particular interest is the contribution of SMCs in this process. Most SMCs reside in the media layer (immediately beneath the intima) of vessels; however, upon injury, a significant number migrate into the intima itself.[3] These two populations of SMCs exhibit distinct phenotypes. SMCs in the media layer predominantly express contractile proteins, which confer a contractile phenotype.[6] SMCs in the intima, however, express lower levels of these proteins.[6] Instead, they are highly proliferative and have increased synthesis of extracellular matrix and cytokines.[6] This synthetic phenotype can be induced by various stimuli including extracellular matrix, cytokines, ROS, and lipids.[6]

A number of studies have begun to characterize the role synthetic SMCs play in atherogenesis. Briefly, SMCs are a major source of extracellular matrix.[6] In healthy vessels, SMCs produce type I and type III fibrillary collagen, while in atherosclerotic lesions they also produce proteoglycans.[6] These proteoglycans interact with ApoB-100 to promote LDL accumulation in the intima.[2, 4] Oxidation of LDL particles stimulates

SMCs to secrete more proteoglycans, accelerating lesion progression.[6] Additionally, synthetic SMCs have increased LDL receptors that enhance lipid uptake and promote foam cell formation.[6] These foam cells, in addition to the macrophage-derived foam cells, comprise the initial fatty streak from which atherosclerotic plaques develop.[2, 4, 6] Finally, synthetic SMCs migrate into the fatty streak where they produce cytokines that activate leukocytes, induce SMC proliferation, and stimulate production of extracellular matrix, promoting atherosclerotic plaque growth.[7] Further study is required to characterize the unique mechanisms by which SMCs contribute to atherogenesis.

Vascular Smooth Muscle Cells and Reactive Oxygen Species

Reactive oxygen species include a number of free radicals, such as superoxide ($O_2^{\cdot-}$), and other highly reactive small molecules, such as hydrogen peroxide (H_2O_2).[8] In vascular smooth muscle cells (VSMCs), ROS are produced by NADPH oxidases (Noxes), xanthine oxidase, the mitochondrial electron transport chain, and nitric oxide synthase.[9] Under physiological conditions, moderate levels of ROS act as intercellular and intracellular second messengers to regulate normal VSMC function.[10] ROS modulate VSMC growth and migration through regulation of lamellipodia formation, focal adhesion turnover, actin cytoskeleton remodeling, and contraction of the cell body.[9] Chronic or acute increases in ROS that overwhelm antioxidant defenses, however, cause oxidative stress and cardiovascular disease.[10] Specifically, one way that ROS may promote cardiovascular disease is through activation of RhoA.[11, 12] Rho-kinase, a downstream effector of RhoA, promotes inflammation of the arterial wall and VSMC proliferation.[13] ROS-mediated damage to macromolecules, such as DNA, is also

believed to play a causal role in cardiovascular disease.[14] Interestingly, when DNA damage produces double stranded breaks (DSB), γH2AX, a phosphorylation variant of the histone H2AX, is observed.[15] Importantly, many risk factors for cardiovascular disease including, hypercholesterolemia, diabetes, smoking, aging, and hypertension increase production of ROS.[16] Therefore oxidative stress represents an overarching mechanism for cardiovascular disease risk factors. One area of particularly intense focus has been enzymatic sources of free radicals in the vasculature.

NADPH Oxidases in Vascular Smooth Muscle Cells

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Noxes) are the only known enzymes whose dedicated function is the production of ROS.[17] In VSMCs, ROS derived from Noxes mediate cellular functions and signaling pathways, such as apoptosis, differentiation, fibrosis, proliferation, growth, and cytoskeletal dynamics.[18] Under pathophysiological conditions, however, Noxes are involved in promoting the development of cardiovascular disease, including atherosclerosis.[18] The Nox family of enzymes is comprised of seven homologues, four of which are found in the vasculature (Nox1, Nox2, Nox4, and Nox5).[19] These enzymes, which consist of two-transmembrane subunits (p22phox and a Nox homologue) and cytosolic regulatory subunits (except for Nox5, which has no binding partners and is regulated by calcium), possess distinct regulatory mechanisms, downstream targets, functions, and patterns of expression.[19] All Noxes, however, generate $O_2^{\cdot -}$ from molecular oxygen using NADPH as an electron donor.[19] Depending on Nox subcellular localization, this ROS is released inside organelles or extracellulary.[18] Superoxide tends to act locally due to its short half-

life and relative inability to cross membranes.[19] Moreover, superoxide dismutase rapidly converts $O_2^{\cdot-}$ to H_2O_2, which more readily crosses membranes and is longer-lasting.[19] Therefore, while some effects of Noxes are directly mediated by $O_2^{\cdot-}$, most are attributed to protein oxidation by H_2O_2.[19] To study the functions of NADPH oxidases in VSMCs, the Lee et al.[20] and Carnesecchi et al.[21] has generated Nox1 and Nox4 knockout lines of mouse aortic smooth muscle cells (MASMs), respectively. Because Nox5 is not found in the murine vasculature and Nox2 is only found in small vessels, I will subsequently only focus on Nox1 and Nox4.

Nox1 generates $O_2^{\cdot-}$ and is found in the plasma membrane, caveolae, and endosomes of VSMCs.[18] Different agonists activate Nox1 at specific subcellular locations; extracellularly by thrombin, in endosomes by IL-Iβ and TNFα, and globally by AngII.[19] In vitro studies have shown Nox1 overexpression increases serum-stimulated proliferation and PDGF-induced migration in VSMCs.[20] Consistent with these proatherosclerotic functions, knockout of Nox1 reduces atherosclerosis, especially in diabetic animal models.[10] Nox4 also generates $O_2^{\cdot-}$; however, it contains an extracytosolic E-loop, which results in spontaneous dismutation of $O_2^{\cdot-}$ to H_2O_2.[22] Unlike Nox1, Nox4, is present in VSMC nuclei, endoplasmic reticulum, focal adhesions, as well as stress fibers and confers protective effects against atherosclerosis.[17] Nox4-derived H_2O_2 inhibits proliferation of VSMCs and prevents vascular inflammation and remodeling.[18] Nevertheless, detrimental effects of Nox4 have been reported in murine models of ischemic stroke, diabetic cardiomyopathy, and cardiac hypertrophy.[10]

Nox1 requires p22phox, the Nox organizers p47phox or Noxo1, the Nox activators p67phox or NOXA1, and Rac1 for activation.[10] In contrast, Nox4 only requires

p22phox and is believed to be constitutively active.[10, 23] Significantly, our lab has reported polymerase δ interacting protein 2 (Poldip2) binds Nox4 in a p22phox dependent manner and enhances its activity. Poldip2 was also found to bind Nox1 in a p22phox dependent manner, however, the effects of this interaction remain to be reported. Thus, novel regulators of Noxes, such as Poldip2, require further investigation.

Polymerase δ Interacting Protein 2

Poldip2 is a ubiquitously expressed 42-kDa multifunctional protein that has been observed in nuclear and cytoplasmic compartments.[24, 25] It is comprised of an N-terminal mitochondrial targeting sequence, which is cleaved in some tissues, including VSMCs, to form a functional 37-kDa variant and two functional domains: ApaG/F box A domain and a hemimethylated DNA binding domain.[26] Outside of the vasculature, Poldip2 has been implicated in DNA repair[27, 28] and regulation of the cell cycle.[25] Poldip2 was originally identified as a binding partner of the sliding DNA clamp proliferating cell nuclear antigen (PCNA).[26] In addition to PCNA, Poldip2 is known to directly bind Rev1, Rev7, and Polη, which are involved in a cell's response to DNA damage.[27] While little has been reported on the effect of these interactions, there is some evidence to suggest Poldip2 functions in the DNA damage response. In fibroblasts, depletion of Poldip2 increases Polη foci and decreases survival after UV-treatment.[28] Interestingly, Poldip2 has also been observed in mitotic spindle fibers and loss of function results in cytokinesis failure in some cell types.[25] Consistent with Poldip2 functioning in the cell cycle, our lab has previously reported that knockdown of Poldip2 in mouse embryonic fibroblasts reduces growth.[26]

In VSMCs, Poldip2 regulates aortic stiffness[24], focal adhesion turnover[29], and collagen accumulation.[30] Moreover, many of these effects are thought to be mediated by Poldip2-Nox4 derived ROS. To study the effect of Poldip2 in VSMCs, Sutliff et al.[26] generated Poldip2 heterozygous (HET) mice; homozygous deletion of Poldip2 induces embryonic lethality. While Poldip2 HET cells show no change in p22phox, Nox1, or Nox4 mRNA levels, they do have decreased NADPH oxidase activity.[24] Interestingly, Poldip2 HET MASMs exhibit fractured elastic lamellae and excess collagen production, resulting in reduced smooth muscle contraction and vascular compliance.[24] Our lab has also reported Poldip2 activates RhoA in a ROS-dependent manner.[11] The mechanism for this activation remains to be reported. Thus, while some functions of Poldip2 in the vasculature have been characterized, further study is required.

RhoA and Epithelial Cell Transforming 2

The Rho family of GTPases form part of the Ras superfamily and consist of 22 mammalian members, including RhoA.[31] In the vasculature, RhoA regulates normal functions including contraction, motility, proliferation, and apoptosis. Excessive RhoA activity, however, promotes cardiovascular disease.[13] In general, Rho GTPases function as bi-molecular switches, cycling between active GTP-bound and inactive GDP-bound conformations.[31] Canonically, Rho GTPases are activated by guanine nucleotide exchange factors (GEFs), which promote nucleotide dissociation, allowing the more prevalent GTP to bind.[31] Interestingly, however, RhoA possesses redox-sensitive cysteine residues and can be directly activated by ROS.[12]

The Dbl-family of GEFs is comprised of over 60 mammalian members and is the largest group of Rho GTPase activators.[31] This family of GEFs is characterized by tandem Dbl-homology (DH) and pleckstrin homology (PH) domains.[32] The DH domain binds Rho GTPases and catalyzes the release of nucleotides.[33] The PH domain, while not required for GEF activity, is important for Dbl-family GEF localization.[33] In cells, DH domain interactions are tightly regulated and modulate GEF activity. In epithelial cell transforming 2 (Ect2), a Dbl-family GEF, for example, tandem BRCA1 C-terminus (BRCT) domains interact with and sterically block the DH domain, resulting in autoinhibition.[32] Phosphorylation of key residues or binding of proteins to the BRCT domains releases autoinhibition and activates Ect2.[32] Interestingly, these BRCT domains are conserved across many proteins involved in the DNA damage response; however, these functions have not yet been attributed to Ect2.[34] Ect2 has been implicated in cytokinesis: Pebble, the *Drosophila* homologue, is required for cytokinesis.[35] Additionally, Ect2 activation of RhoA is required for cleavage furrow formation and Ect2 is released from the midbody during the final steps of cytokinesis.[36]

Hypothesis

Our lab has previously reported Poldip2 regulates Nox activity and activates RhoA.[11] While Rho and Nox signaling are important for the development of cardiovascular disease, it remains unclear if and how Poldip2 unites these pathways. I hypothesize that ***ROS generated by Poldip2/Nox signaling enhances RhoA activity, to promote atherosclerosis.***

MATERIALS AND METHODS

Tissue Culture

Rat aortic smooth muscle cells (RASMs, passages 6-13) were cultured in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% calf serum, 4.5 g/l glucose, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Similarly, MASMs (passages 6-13), including $Nox1^{-/-}$, $Nox4^{-/-}$, and Poldip2 HET MASMs, were grown in the same media as RASMs but supplemented with 10% fetal bovine serum instead of calf serum. Cells were passaged approximately every five days using 0.25% trypsin and fed every three to four days.

H_2O_2 Treatment

Samples were treated with 100 µM or 500 µM H_2O_2 in serum-free DMEM for 10 or 30 minutes at 37°C and 5% CO_2. Samples were then rinsed in serum-supplemented media (same as described above) and allowed to recover at 37°C or rinsed in PBS before use in immunoblotting or immunofluorescence microscopy.

Adenovirus Infection

Infection was performed in serum-free DMEM for two hours at 37°C and 5% CO_2. Infection efficiency ranged from 80-90% and was visualized by GFP. Following adenovirus infection, cells were serum deprived for 48 hours before being used in experiments.

Immunofluorescence Staining and Microscopy

VSMCs were seeded and grown to 50-60% confluence on collagen-coated glass coverslips before being immediately fixed, infected with adenovirus (as described above), or subjected to H_2O_2 treatment (as described above). All samples were fixed in 4% paraformaldehyde. Samples were subsequently permeabilized with 0.1% Triton X-100 and incubated with anti-Ect2 polyclonal rabbit antibody (Ab, Millipore; 1:100 dilution), anti-myc monoclonal mouse Ab (Cell Signaling; 1:500), and/or anti-γH2AX monoclonal rabbit (Cell Signaling; 1:400) overnight. Samples were then blocked in 5% BSA for 30 minutes and incubated with secondary antibodies and/or Alexa Fluor568-conjugated phalloidin (ThermoFisher Scientific; 1:100) for one hour at room temperature. Secondary antibodies used were rabbit Alexa Fluor 478 and mouse Alexa Fluor 568 (both from ThermoFisher Scientific; 1:100). Finally, cells were mounted with Vectashield and imaged using a Zeiss LSM 510 META Laser Scanning Confocal Microscope System.

Small Interfering RNA

Transfection was performed in Opti-MEM reduced serum media. Cells were incubated in a solution containing 25 nM of siRNA against the protein of interest and Lipofectamine RNAiMAX Reagent (5 μL in 200 μL; Invitrogen) for five hours. A stealth siRNA (Invitrogen) against Poldip2 (siPoldip2; 5'-GCCCACAUAUAUCUCAGAGAUCUCA-3') and matching stealth control siRNA sequence (siControl-1; Invitrogen) were used. Two sequences of siRNA (both from Sigma) against Ect2 (Ect2-A and Ect2-B; 5'-CUGACUUACAUGGUACUUU-3' and 5'-GUAACACUAACCAACAGUU-3', respectively) and matching control (Qiagen) siRNA

sequence (siControl-2; 5'- GGGUAUCGACGAUUACAAAUU-3') were also used. Cells were rinsed in supplemented DMEM (same as described above) and experiments were preformed 72 hours later.

Growth Curves

Prior to the start of the experiment, an equal number of VSMCs were plated in triplicate. Cells were transfected 24 hours later (Time 0) with stealth siControl-1, stealth siPoldip2, two unique sequences of siRNA against Ect2 (siEct2-A and siEct2B), or siControl-2, as described above. Cells were subsequently counted every 24 hours using a Scepter Cell Counter (Milipore) for a total of three days.

Immunoblotting

VSMCs were lysed in a buffer containing 20 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 1 M DTT, and protease inhibitors (1 mg/ml aprotinin, 0.5 mg/ml leupeptin, 1 mM PMSF). Samples were sonicated and Precision Red Advanced Protein Reagent (Cytoskeleton) was used to assess protein concentrations. Equal amounts of protein were aliquoted and samples were brought to equal volume with H_2O and SDS sample buffer. After boiling for 10 minutes at 100^{o}C, samples were resolved in 7.5-12% acrylamide gels using 25 mAmps/gel and transferred to PVDF membranes overnight at 30 V. PVDF membranes were blocked in 5% BSA for at least 30 minutes before incubation with primary antibodies overnight. Primary antibodies used are as follows: anti-Poldip2 monoclonal rabbit Ab (Abcam; 1:1000), anti-Ect2 polyclonal rabbit Ab (Millipore; 1:100), anti-myc monoclonal mouse Ab (Cell Signaling; 1:1000), anti-p115

polyclonal rabbit Ab (Santa Cruz; 1:1000), anti-tubulin monoclonal mouse Ab (Sigma; 1:10,000), and anti-phospho-p38 Thr180/Tyr182 polyclonal rabbit Ab (Cell Signaling; 1:1000). Next, membranes were rinsed in TBST for 30 minutes before incubation with secondary antibodies for 1 hour. Secondary antibodies were used at twice the dilution of primary antibodies and are as follows: anti-mouse (Amersham) and anti-rabbit (Cell Signaling) horseradish peroxidase conjugated antibodies. PVDF membranes were treated with enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; ThermoFisher Scientific) and blots were developed in a darkroom.

Preparation of RhoA(17A) Beads

As previously described,[37] nucleotide-free RhoA [RhoA(17A)] was transformed into bacteria and cultured in LB overnight at 37^{o}C. Once an optical density of 0.5-0.6 was achieved, expression of RhoA(17A) was induced with 1 mM IPTG. Next, RhoA(17A) was incubated with glutathione (GST) agarose beads for 1 hour at 4^{o}C as previously described.[38] Just prior to use, GST-RhoA(17A) beads were blocked in 5% BSA for 30 minutes. Critically, finished beads were stored at 4^{o}C and used within 24 hours.

GST-RhoA(17A) Pulldown Assay

GST-RhoA(17A) pulldowns were performed as described previously.[38] Briefly, VSMCs were lysed using a buffer containing 20 mM HEPES, 150 mM NaCL, 5 mM $MgCl_2$, 1% Triton X-100, 1 M DTT, and protease inhibitors (1 mg/ml aprotinin, 0.5 mg/ml leupeptin, 1 mM PMSF). Using Precision Red Advanced Protein Reagent (Cytoskeleton), an equal amount of protein for each condition was combined with an equal volume of GST-

RhoA(17A) beads and rocked for 45 minutes at 4^{o}C. Rho(17A) pulldowns were examined by western blot. Total cell lysate, which was aliquoted after cells were lysed, was also examined by western blot to confirm equal loading of pulldown lysate.

β-galactosidase Senescence Assay

VSMCs were seeded and grown to 70-80% confluence before being fixed in 0.2% glutaraldehyde for 5 minutes at room temperature. Cells were then rinsed twice in PBS before being stained with a solution containing $MgCl_2$ (2 mM), potassium ferrocyanide (5 mM), potassium ferricyanide (5 mM), PBS pH 6.0, and X-gal (30 mg/mL DMSO) for 24 hours at 37^{o}C and 5% CO_2. Using bright field, cells were imaged at 4x and 10x magnification and scored blindly.

RESULTS

Poldip2 activates the RhoGEF Ect2

Our lab has previously shown Poldip2 activates RhoA, a member of the Rho family of GTPases, in VSMCs.[11] The mechanism for this activation has yet to be described. Canonical activation of Rho GTPases, however, is mediated by RhoGEFs.[31] Furthermore, the Dbl-family of RhoGEFs comprise the largest family of direct activators of Rho GTPases.[31] Thus, we hypothesized Poldip2 increases RhoA activity via regulation of a Dbl-family RhoGEF. Nucleotide-free RhoA (Rho17A) binds the DH domain in GEFs free of autoinhibition and thus can be used to specifically pull down active GEFs.[38] The GST-RhoA(17A) pulldown products from cells infected with Poldip2 adenovirus (AdPoldip2) or adenovirus containing empty vector (AdCMV) were analyzed using mass spectrometry to identify any RhoGEFs that Poldip2 activates. Although no RhoGEFs came down specifically in the Poldip2 overexpressing group, phosphoglycerate dehydrogenase (PHGDH) was found to bind Rho17A five time more in Poldip2 overexpressing cells compared to the control group. Interestingly, PHGDH has been shown to bind the BRCT domains of the RhoGEF Ect2.[39] Other proteins that bind Ect2 BRCT domains[40] as well as mutation of these domains[41] have been found to activate Ect2. Based on these findings, we sought to determine if Poldip2 activates Ect2. GST-RhoA(17A) pulldowns for active GEFs were performed on VSMCs infected with AdCMV or AdPoldip2-Myc, and immunoblotted for Ect2 (Figure 1a). Using p115-RhoGEF as a negative control,[42] we discovered that Poldip2 specifically activates the RhoGEF Ect2 ($n=5$, $p<0.001$; Figure 1b). Subsequent experiments, were focused on elucidating the mechanism by which Poldip2 activates Ect2.

Poldip2 does not increase Ect2 expression

One possible mechanism for the increased amount of active Ect2 upon Poldip2 overexpression is regulation of Ect2 expression by Poldip2. To test this possibility, VSMCs were infected with AdCMV or AdPoldip2 in triplicate. Cells were lysed and immunoblotted for Ect2 (Figure 2a). Densitometry analysis revealed no difference in Ect2 expression between the AdCMV and AdPoldip2 groups (Figure 2b). Therefore, we concluded that Poldip2 does not increase Ect2 expression.

Poldip2 does not regulate Ect2 subcellular localization

After finding Poldip2 does not regulate Ect2 expression, we investigated the effect of Poldip2 on Ect2 subcellular localization. Ect2 is autoinhibited by BRCT domains and inhibition is released by different kinases/proteins at various subcellular localizations.[43] Thus, we hypothesized Poldip2 increases Ect2 activity by regulating Ect2 subcellular localization. To test this possibility, we first validated the Ect2 antibody for immunofluorescence. VSMCs transfected with siControl or siEct2 were fixed and stained for Ect2 (Figure 3a). In cells transfected with siControl, Ect2 was observed in the nucleus and this signal was diminished in cells transfected with siEct2. Knockdown of Ect2 was confirmed by western blot (Figure 3b). Using the validated Ect2 antibody, the localization of Ect2 was compared in cells expressing AdCMV or AdPoldip2 using immunofluorescence (Figure 3c). The CMV backbone of the Poldip2 plasmid contains the green fluorescent protein (GFP) gene under the control of its own promoter; thus, GFP was used as a marker for infection by AdPoldip2 or AdCMV. In cells expressing empty vector, Ect2 was localized in the nucleus and its localization did not change with

Poldip2 overexpression. Thus, we concluded Poldip2 does not regulate Ect2 subcellular localization at basal conditions.

Hydrogen peroxide activates Ect2

After ruling out expression and localization as mechanisms for activation of Ect2 by Poldip2, we tested a ROS-driven model. Our lab has previously reported that Poldip2 increases H_2O_2 levels in VSMCs.[11] Moreover, Ect2 possesses several cysteines, which are capable of being directly oxidized.[44] Ect2 is also known to be regulated by kinases and phosphatases, which can also be oxidized.[43] To determine if ROS is capable of activating Ect2, we began by testing the effect of ROS on Ect2. GST-RhoA(17A) pulldowns were performed on three groups of VSMCs (control, polyethylene glycol [PEG], and PEG-catalase) that were treated with 100 μM H_2O_2 for 10 minutes (Figure 4). Phospho-p38 has been shown to be redox sensitive[45] and was used as a positive control for H_2O_2 treatment. Without PEG-catalase, which catalyzes the reduction of H_2O_2 to H_2O,[46] H_2O_2 treated cells had increased levels of active Ect2 compared to VSMCs without H_2O_2 treatment. This increase in active Ect2 upon H_2O_2 treatment was attenuated by PEG-catalase; suggesting activation of Ect2 was specifically due to H_2O_2 treatment. Interestingly, the PEG-catalase group without H_2O_2 treatment had the highest levels of active Ect2. This result suggests an optimal level of ROS is required for Ect2 activation; deviation from basal levels (above or below) appears to activate Ect2. Thus, we determined exogenous H_2O_2 can activate Ect2; however, it remains unclear if this activation is due to direct or indirect oxidation.

Nox4 is not required for Poldip2 activation of Ect2

Nox4 is expressed throughout the cardiovascular system and preferentially generates H_2O_2.[19] Moreover, our lab has previously shown that Poldip2 enhances Nox4 activity (H_2O_2 production) in VSMCs.[11] After showing exogenous H_2O_2 is capable of activating Ect2, we hypothesized that Poldip2 activates Ect2 by increasing ROS through its interaction with Nox4. GST-RhoA(17A) pulldowns for active Ect2 were performed in $Nox4^{-/-}$ MASMs infected with AdCMV or AdPoldip2 (Figure 5). Overexpression of Poldip2 increased Ect2 activity in both WT and $Nox4^{-/-}$ MASMs. Because Poldip2 could still activate Ect2 in cells without Nox4, we concluded Nox4 is not required for activation of Ect2 by Poldip2.

Nox1 may be required for Poldip2 activation of Ect2

Nox1 is the other predominant Nox homologue in VSMCs and generates superoxide,[19] which is rapidly converted to hydrogen peroxide by superoxide dismutase.[46] Although the effect of Poldip2 on Nox1 has not been previously described, our lab has reported Poldip2 binds Nox1 in a p22phox dependent manner, and overexpression of Poldip2 in VSMCs with Nox4 knockdown produces a modest increase in superoxide,[11] suggesting that Poldip2 may also activate Nox1. After showing Nox4 is not required for Ect2 activation by Poldip2, we tested the hypothesis that Nox1 is required. GST-RhoA(17A) pulldowns for active Ect2 were performed in $Nox1^{-/-}$ and WT MASMs infected with AdCMV or AdPoldip2 (Figure 6). Overexpression of Poldip2 in WT cells increased the amount of active Ect2; however, this increase was not observed in $Nox1^{-/-}$ cells. This result suggests that Nox1 is required for Poldip2 to activate Ect2. $Nox1^{-/-}$ cells expressing AdCMV, however, had high levels of active Ect2, possibly obscuring any

increase in active Ect2 by Poldip2. Thus, we concluded that Nox1 may be required for activation of Ect2 by Poldip2.

Different WT and Nox1$^{-/-}$ cell lines have variable levels of active Ect2

After observing a difference in the levels of active Ect2 in Nox1$^{-/-}$ and WT cells expressing AdCMV, we sought to determine if this trend held true across multiple WT and Nox1$^{-/-}$ lines. GST-RhoA(17A) pulldowns for active Ect2 were performed in three WT lines (31016, 814, 7913 with two different passages) and three Nox1$^{-/-}$ lines (2212, 311, and 294 with two different passages) (Figure 7). Within WT and Nox1$^{-/-}$ groups the level of active and total Ect2 were heterogeneous. Nox1$^{-/-}$ lines 249, 2212, and 311 and WT line 31016 had similarly low levels of active Ect2. Nox1$^{-/-}$ line 61813 and WT line 7913 had the highest levels of active Ect2. WT line 814 had more active Ect2 than Nox1$^{-/-}$ lines 249, 2212, and 311, but less than Nox1$^{-/-}$ line 61813. Due to the heterogeneous levels of active Ect2, no conclusion regarding the effect of Nox1 on Ect2 could be made. Moreover, we suspect that differences in the isolation and preparation of the cell lines are largely responsible for the heterogeneous levels of active and total Ect2 within WT and Nox1$^{-/-}$ lines.

Ect2 and Poldip2 regulate VSMC proliferation

As we investigated the mechanism by which Poldip2 activates Ect2, we began to consider the downstream consequences of this activation. Because Nox1 and Poldip2 promote VSMC proliferation[20] and mouse embryonic fibroblast growth, respectively, we first hypothesized Ect2 and Poldip2 regulate VSMC proliferation. To test this hypothesis,

growth curves were performed using MASMs transfected with siEct2 or siPoldip2 and controls (Figure 8). Twenty-four hours after transfection, no significant difference in the number of cells was observed between the siPoldip2 or siEct2 and control groups. After 48 and 72 hours, significantly fewer cells were observed in the siPoldip2 and siEct2 transfected groups compared to the control groups ($n=4$, $p<0.001$). Thus, we concluded Ect2 and Poldip2 enhance VSMC proliferation. Subsequent experiments focused on understanding the mechanism by which Ect2 and Poldip2 promote VSMC proliferation.

Ect2, but not Poldip2, regulates cytokinesis in VSMCs

Outside of the vasculature, Ect2 is known to regulate cytokinesis by recruiting and activating RhoA during the early stages of cytokinesis.[36] Moreover, knockdown of Ect2 in fibroblasts produces an increase in the number of multinucleated cells[47] (an indication of cytokinesis failure).[48] Thus, we hypothesized that Poldip2 and Ect2 regulate cytokinesis to promote VSMC proliferation. To test this hypothesis, immunofluorescence microscopy was used to compare the number of multinucleated cells in MASMs transfected with siPoldip2 or siEct2 and control (Figure 9a). Two unique sequences of siRNA against Ect2 (siEct2A and siEct2B) were tested. To better visualize multinucleated cells, siRNA treated and control groups were stained with phalloidin to label F-actin[49] and DAPI to mark nuclei.[50] Prior to imaging, the identity of the coverslips was concealed and all images were scored blindly. Consistent with what has been previously reported in other cell types, knockdown of Ect2 (using either sequence of siRNA) significantly increased the percentage of multinucleated cells ($n=4$, $p<0.0001$; Figure 9b). Poldip2 knockdown, however, did not significantly increase the percentage

of multinucleated VSMCs. Because Ect2, but not Poldip2, regulates cytokinesis in VSMCs, we continued to test other mechanisms by which Poldip2 and Ect2 promote VSMC proliferation.

Poldip2 does not enhance DNA damage repair

After finding Poldip2 does not regulate cytokinesis in VSMCs, we hypothesized Poldip2 and Ect2 inhibit DNA damage to permit proliferation. Poldip2 is known to bind Rev1, Rev7, Polη, and PCNA, which are involved in a cell's response to DNA damage.[27] Moreover, Ect2 is auto-inhibited by BRCT domains, which are commonly found in proteins involved in the DNA damage response.[34] To investigate the effect of Poldip2 and Ect2 on DNA damage repair, we began by comparing γH2AX[15], a marker of DSB, in WT and Poldip2 HET MASMs after H_2O_2 treatment using immunofluorescence microscopy. WT and HET MASMs were fixed and stained for γH2AX 0, 1, 3, 6, and 12 hrs post-treatment with 500 μM H_2O_2 (Figure 10a). The high dose of 500 μM H_2O_2 (physiologically relevant doses range between 0.20-100 μM)[51] was chosen to overwhelm the effect of Poldip2-Nox4 derived ROS, thus enabling us to isolate any reparative functions of Poldip2. As cells responded to DNA damage induced by H_2O_2 treatment, a decrease in γH2AX staining was observed starting at 1 hour post-treatment. No difference in amount of DNA damage, however, was observed between the WT and Poldip2 HET groups at any time point (Figure 10b). Thus, we concluded Poldip2 does not enhance DNA damage repair. Because Poldip2 was not involved in DNA damage repair, we did not investigate the role of Ect2 in the DNA damage response.

Nox1$^{-/-}$ cells are more senescent than WT

We next hypothesized that Poldip2 and Ect2 inhibit senescence to permit proliferation. Based on our proposed pathway (Figure 12), in which Poldip2 activates Ect2 via Nox1, we expected Nox1 to also inhibit senescence. β-galactosidase (β-gal) staining can be used to detect senescence in live cell cultures.[52] Because Nox1$^{-/-}$ MASMs were readily available, a preliminary experiment, comparing the percentage of β-gal positive WT and Nox1$^{-/-}$ cells was performed. Two WT lines (814, 7913) and three Nox1$^{-/-}$ lines (2212, 311, and 294) were stained (Figure 11a) and scored blindly. The Nox1$^{-/-}$ group had a significantly greater proportion of β-gal positive cells than the WT group ($n= 4$, $p<0.001$; Figure 11b). Thus, we concluded that a lack of Nox1 leads to senescence.

DISCUSSION

In VSMCs, RhoA is an important regulator of the cytoskeleton; however, under pathophysiological conditions, one of its downstream effectors, Rho-kinase, promotes cardiovascular disease.[13] As a result, the regulation of Rho GTPases has been extensively studied and several groups have reported that ROS can directly activate RhoA.[12, 53] Our study is the first, however, to show that oxidation can also modulate the activity of a RhoGEF. Specifically, we found Poldip2 activates the RhoGEF Ect2, possibly in a Nox1-dependent manner, to enhance VSMC proliferation.

After finding Poldip2 does not regulate Ect2 expression or subcellular localization, we proposed a ROS-driven model for the activation of Ect2 by Poldip2. Our lab has previously reported that overexpression of Poldip2 increases NADPH oxidase activity and levels of ROS in VSMCs.[11, 24] Furthermore, many proteins, including RhoA, possess redox sensitive cysteine resides that modulate their activity.[12] Ect2 also possesses cysteine residues (GenBank accession number AY376439) and we hypothesized exogenous H_2O_2 activates Ect2. This hypothesis was confirmed and our findings suggest an optimal level of ROS is required for Ect2 activation. It remains undermined, however, if this activation is due to direct or indirect oxidation. Ect2 is known to be regulated by serine/threonine kinases, including protein kinase C (PKCι) and phosphatases.[34] Interestingly, ROS, which have been found to primarily activate tyrosine kinases and phosphatases, also activate the serine/threonine kinase PKC.[54] The activation of PKC by ROS, however, appears to be mediated by increases in calcium [54] and PKCι, an atypical PKC member, does not require Ca^{2+} for activation.[55]

We next sought to determine the origin of ROS used by Poldip2 to activate Ect2. Our lab has previously reported that Poldip2 binds Nox4 to enhance its activity and RhoA is believed to be a downstream target of this interaction.[11] Thus, we proposed that ROS derived from the interaction between Poldip2 and Nox4 activates Ect2. Surprisingly, we found Nox4 was not required for activation of Ect2 by Poldip2, and we next considered Nox1 as a possible source of ROS.

Poldip2 also binds Nox1 via p22phox,[11] however, the effect of this interaction remains unclear. Several indirect and conflicting pieces of evidence exist. In support of Poldip2 functioning as positive regulator of Nox1, Poldip2 HET cells have lower levels of superoxide[24] and overexpression of Poldip2 in VSMCs with Nox4 knockdown show a modest increase in superoxide levels.[11] In opposition, Poldip2 overexpression in VSMCs with Nox4 knockdown show decreased levels of H_2O_2.[11] Although not definitive, our result showing that Nox1 may be required for Poldip2 to activate Ect2 is an important novel finding, as it suggests Poldip2 regulates Nox1 in a physiologically relevant manner. Further study is needed to definitively characterize the effect of Poldip2 on Nox1.

While investigating the mechanism by which Poldip2 activates Ect2, we also began to consider the downstream consequences of this activation. We first looked at the effect of Poldip2 and Ect2 on VSMC proliferation. Knockdown of Ect2[40, 56] and Poldip2[26] in various cell types and mouse embryonic fibroblasts, respectively, has been shown to inhibit proliferation. Thus, our result showing Poldip2 and Ect2 enhance VSMC proliferation is consistent with what has been previously reported in other cell types. Moreover, overexpression of Nox1 has also been reported to enhance VSMC

proliferation.[20] Thus, our result is also consistent with the idea that Poldip2 activates Nox1. Next, we tested several cellular processes that Ect2 and Poldip2 could possibly regulate to promote VSMC proliferation.

Outside of the vasculature, Ect2 functions throughout cytokinesis, and knockdown increases the number of multinucleated cells.[35, 36] Similarly, Poldip2 has been implicated in mitosis and loss of function in cancer cells increases the percentage of multinucleated cells.[25] Thus, we hypothesized, Poldip2 and Ect2 regulate cytokinesis in VSMCs. Interestingly, we found Ect2, but not Poldip2, regulates cytokinesis in VSMCs. Although, knockdown of Poldip2 was found to increase the number of multinucleated cells in four independent cancer cell lines, the same report also found Poldip2 knockdown had no effect on the percentage of multinucleated cells in a fifth cancer cell line.[25] Thus, specific cell types may be able to compensate for the loss of Poldip2 to complete cytokinesis. Alternatively, Ect2 is known to be activated by proliferation.[40] Thus, activation of Ect2 by Poldip2 may be a downstream consequence of the ability of Poldip2 to promote VSMC proliferation. This pathway would account for the observation that both Poldip2 and Ect2 promote VSMC proliferation, but only Ect2 regulates cytokinesis. Further work is needed to rule out this possibility.

We next considered DNA damage repair as a mechanism for Poldip2 and Ect2 to promote proliferation. Ect2 possesses BRCT domains, which are conserved across many proteins involved in the DNA damage response; however, these functions have not yet been attributed to Ect2.[34] Moreover, Poldip2 is known to bind Rev1, Rev7, Polη, and PCNA,[27] but the effect of these interactions remain unclear. Our result that Poldip2 does not enhance DNA damage repair after ROS treatment is a novel finding. Although,

Poldip2 has been reported to enhance cell survival after UV treatment,[28] UV and ROS trigger different DNA damage repair pathways. Thus, it is not surprising that Poldip2 may mediate repair of UV-induced damage but not ROS-induced damage.

Finally, we proposed that Poldip2 and Ect2 inhibit senescence to promote proliferation. We began by looking at senescence in Nox1 knockout MASMs. Previously, our lab has reported that Nox1 knockout inhibits proliferation.[20] Thus, our result that Nox1 regulates senescence is consistent with previous findings. Moreover, if Poldip2 activates Nox1, this finding is consistent with Poldip2 and Ect2 promoting proliferation by inhibiting senescence. We next plan to look at senescence markers in VSMCs transfected with siRNA against Poldip2 and Ect2. Thus, our working model involves Poldip2 signaling through Nox1 to activate Ect2 and promoting proliferation by inhibiting senescence (Figure 12). In conclusion, the regulation of the RhoGEF, Ect2 in the vasculature by Poldip2 is physiologically relevant and represents a novel mechanism for GEF activation.

WORK CITED

1. Wong ND. Epidemiological studies of CHD and the evolution of preventive cardiology. *Nat Rev Cardiol*. 2014;11:276-289.

2. Hansson GK and Hermansson A. The immune system in atherosclerosis. *Nature immunology*. 2011;12:204-12.

3. Stary HC, Blankenhorn DH, Chandler AB, Glagov S, Insull W, Jr., Richardson M, Rosenfeld ME, Schaffer SA, Schwartz CJ, Wagner WD and et al. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*. 1992;85:391-405.

4. Weber C and Noels H. Atherosclerosis: current pathogenesis and therapeutic options. *Nature medicine*. 2011;17:1410-22.

5. Segrest JP, Jones MK, De Loof H and Dashti N. Structure of apolipoprotein B-100 in low density lipoproteins. *Journal of lipid research*. 2001;42:1346-67.

6. Doran AC, Meller N and McNamara CA. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:812-9.

7. Louis SF and Zahradka P. Vascular smooth muscle cell motility: From migration to invasion. *Experimental & Clinical Cardiology*. 2010;15:e75-e85.

8. Singh U and Jialal I. Oxidative stress and atherosclerosis. *Pathophysiology : the official journal of the International Society for Pathophysiology / ISP*. 2006;13:129-42.

9. San Martín A and Griendling KK. Redox Control of Vascular Smooth Muscle Migration. *Antioxidants & redox signaling*. 2010;12:625-640.

10. Förstermann U, Xia N and Li H. Roles of Vascular Oxidative Stress and Nitric Oxide in the Pathogenesis of Atherosclerosis. *Circulation research*. 2017;120:713-735.

11. Lyle AN, Deshpande NN, Taniyama Y, Seidel-Rogol B, Pounkova L, Du P, Papaharalambus C, Lassegue B and Griendling KK. Poldip2, a novel regulator of Nox4 and cytoskeletal integrity in vascular smooth muscle cells. *Circulation research*. 2009;105:249-59.

12. Aghajanian A, Wittchen ES, Campbell SL and Burridge K. Direct activation of RhoA by reactive oxygen species requires a redox-sensitive motif. *PloS one*. 2009;4:e8045.

13. Shimokawa H, Sunamura S and Satoh K. RhoA/Rho-Kinase in the Cardiovascular System. *Circulation research*. 2016;118:352-366.

14. Borghini A, Cervelli T, Galli A and Andreassi MG. DNA modifications in atherosclerosis: from the past to the future. *Atherosclerosis*. 2013;230:202-9.

15. Kuo LJ and Yang LX. Gamma-H2AX - a novel biomarker for DNA double-strand breaks. *In vivo (Athens, Greece)*. 2008;22:305-9.

16. Harrison D, Griendling KK, Landmesser U, Hornig B and Drexler H. Role of oxidative stress in atherosclerosis. *The American Journal of Cardiology*. 2003;91:7-11.

17. Gray SP, Di Marco E, Kennedy K, Chew P, Okabe J, El-Osta A, Calkin AC, Biessen EAL, Touyz RM, Cooper ME, Schmidt HHHW and Jandeleit-Dahm KAM. Reactive Oxygen Species Can Provide Atheroprotection via NOX4-Dependent Inhibition of Inflammation and Vascular Remodeling. *Arteriosclerosis, thrombosis, and vascular biology*. 2016;36:295-307.

18. Lassegue B and Griendling KK. NADPH oxidases: functions and pathologies in the vasculature. *Arteriosclerosis, thrombosis, and vascular biology*. 2010;30:653-61.

19. Lassegue B, San Martin A and Griendling KK. Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circulation research*. 2012;110:1364-90.

20. Lee MY, Martin AS, Mehta PK, Dikalova AE, Garrido AM, Datla SR, Lyons E, Krause K-H, Banfi B, Lambeth JD, Lassègue B and Griendling KK. Mechanisms of Vascular Smooth Muscle NADPH Oxidase 1 (Nox1) Contribution to Injury-Induced Neointimal Formation. *Arteriosclerosis, thrombosis, and vascular biology*. 2009;29:480.

21. Carnesecchi S, Deffert C, Donati Y, Basset O, Hinz B, Preynat-Seauve O, Guichard C, Arbiser JL, Banfi B, Pache JC, Barazzone-Argiroffo C and Krause KH. A key role for NOX4 in epithelial cell death during development of lung fibrosis. *Antioxidants & redox signaling*. 2011;15:607-19.

22. Takac I, Schroder K, Zhang L, Lardy B, Anilkumar N, Lambeth JD, Shah AM, Morel F and Brandes RP. The E-loop is involved in hydrogen peroxide formation by the NADPH oxidase Nox4. *The Journal of biological chemistry*. 2011;286:13304-13.

23. Serrander L, Cartier L, Bedard K, Banfi B, Lardy B, Plastre O, Sienkiewicz A, Fórró L, Schlegel W and Krause K-H. NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. *Biochemical Journal*. 2007;406:105-114.

24. Sutliff RL, Hilenski LL, Amanso AM, Parastatidis I, Dikalova AE, Hansen L, Datla SR, Long JS, El-Ali AM, Joseph G, Gleason RL, Taylor WR, Hart CM, Griendling KK and Lassègue B. Polymerase Delta Interacting Protein 2 Sustains Vascular Structure and Function. *Arteriosclerosis, thrombosis, and vascular biology*. 2013;33:2154-2161.

25. Klaile E, Kukalev A, Obrink B and Muller MM. PDIP38 is a novel mitotic spindle-associated protein that affects spindle organization and chromosome segregation. *Cell cycle (Georgetown, Tex)*. 2008;7:3180-6.

26. Brown DI, Lassègue B, Lee M, Zafari R, Long JS, Saavedra HI and Griendling KK. Poldip2 Knockout Results in Perinatal Lethality, Reduced Cellular Growth and Increased Autophagy of Mouse Embryonic Fibroblasts. *PloS one*. 2014;9:e96657.

27. Tissier A, Janel-Bintz R, Coulon S, Klaile E, Kannouche P, Fuchs RP and Cordonnier AM. Crosstalk between replicative and translesional DNA polymerases: PDIP38 interacts directly with Poleta. *DNA repair*. 2010;9:922-8.

28. Wong A, Zhang S, Mordue D, Wu JM, Zhang Z, Darzynkiewicz Z, Lee EY and Lee MY. PDIP38 is translocated to the spliceosomes/nuclear speckles in response to UV-induced DNA damage and is required for UV-induced alternative splicing of MDM2. *Cell cycle (Georgetown, Tex)*. 2013;12:3184-93.

29. Datla SR, McGrail DJ, Vukelic S, Huff LP, Lyle AN, Pounkova L, Lee M, Seidel-Rogol B, Khalil MK, Hilenski LL, Terada LS, Dawson MR, Lassegue B and Griendling KK. Poldip2 controls vascular smooth muscle cell migration by regulating focal adhesion turnover and force polarization. *American journal of physiology Heart and circulatory physiology*. 2014;307:H945-57.

30. Fujii M, Amanso A, Abrahão TB, Lassègue B and Griendling KK. Polymerase delta-interacting protein 2 regulates collagen accumulation via activation of the Akt/mTOR pathway in vascular smooth muscle cells. *Journal of molecular and cellular cardiology*. 2016;92:21-29.

31. Rossman KL, Der CJ and Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol*. 2005;6:167-180.

32. Kim JE, Billadeau DD and Chen J. The tandem BRCT domains of Ect2 are required for both negative and positive regulation of Ect2 in cytokinesis. *The Journal of biological chemistry*. 2005;280:5733-9.

33. Hoffman GR and Cerione RA. Signaling to the Rho GTPases: networking with the DH domain. *FEBS letters*. 2002;513:85-91.

34. Callebaut I and Mornon JP. From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. *FEBS letters*. 1997;400:25-30.

35. Lehner CF. The pebble gene is required for cytokinesis in Drosophila. *Journal of cell science*. 1992;103 (Pt 4):1021-30.

36. Nishimura Y and Yonemura S. Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis. *Journal of cell science*. 2006;119:104-14.

37. Huff LP, Decristo MJ, Trembath D, Kuan PF, Yim M, Liu J, Cook DR, Miller CR, Der CJ and Cox AD. The Role of Ect2 Nuclear RhoGEF Activity in Ovarian Cancer Cell Transformation. *Genes Cancer*. 2013;4:460-75.

38. Garcia-Mata R, Wennerberg K, Arthur WT, Noren NK, Ellerbroek SM and Burridge K. Analysis of activated GAPs and GEFs in cell lysates. *Methods in enzymology*. 2006;406:425-37.

39. Woods NT, Mesquita RD, Sweet M, Carvalho MA, Li X, Liu Y, Nguyen H, Thomas CE, Iversen ES, Jr., Marsillac S, Karchin R, Koomen J and Monteiro AN. Charting the landscape of tandem BRCT domain-mediated protein interactions. *Science signaling*. 2012;5:rs6.

40. Yüce Ö, Piekny A and Glotzer M. An ECT2–centralspindlin complex regulates the localization and function of RhoA. *The Journal of Cell Biology*. 2005;170:571-582.

41. Saito Si, Liu X-F, Kamijo K, Raziuddin R, Tatsumoto T, Okamoto I, Chen X, Lee C-C, Lorenzi MV, Ohara N and Miki T. Deregulation and Mislocalization of the Cytokinesis Regulator ECT2 Activate the Rho Signaling Pathways Leading to Malignant Transformation. *Journal of Biological Chemistry*. 2004;279:7169-7179.

42. Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G and Sternweis PC. p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science*. 1998;280:2109-11.

43. Hara T, Abe M, Inoue H, Yu LR, Veenstra TD, Kang YH, Lee KS and Miki T. Cytokinesis regulator ECT2 changes its conformation through phosphorylation at Thr-341 in G2//M phase. *Oncogene*. 2005;25:566-578.

44. Chung HS, Wang S-B, Venkatraman V, Murray CI and Van Eyk JE. Cysteine Oxidative Posttranslational Modifications. *Emerging Regulation in the Cardiovascular System*. 2013;112:382-392.

45. Gutierrez-Uzquiza A, Arechederra M, Bragado P, Aguirre-Ghiso JA and Porras A. p38[alpha]mediates cell survival in response to oxidative stress via induction of antioxidant genes. effect on the p70S6K pathway. *Journal of Biological Chemistry*. 2011.

46. Beckman JS, Minor RL, Jr., White CW, Repine JE, Rosen GM and Freeman BA. Superoxide dismutase and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance. *The Journal of biological chemistry*. 1988;263:6884-92.

47. Cook DR, Solski PA, Bultman SJ, Kauselmann G, Schoor M, Kuehn R, Friedman LS, Cowley DO, Van Dyke T, Yeh JJ, Johnson L and Der CJ. The Ect2 Rho Guanine Nucleotide Exchange Factor Is Essential for Early Mouse Development and Normal Cell Cytokinesis and Migration. *Genes & Cancer*. 2011;2:932-942.

48. Normand G and King RW. Understanding Cytokinesis Failure. *Advances in experimental medicine and biology*. 2010;676:27-55.

49. Chazotte B. Labeling cytoskeletal F-actin with rhodamine phalloidin or fluorescein phalloidin for imaging. *Cold Spring Harbor protocols*. 2010;2010:pdb.prot4947.
50. Chazotte B. Labeling nuclear DNA using DAPI. *Cold Spring Harbor protocols*. 2011;2011:pdb.prot5556.

51. Stone JR and Yang S. Hydrogen peroxide: a signaling messenger. *Antioxidants & redox signaling*. 2006;8:243-70.

52. Debacq-Chainiaux F, Erusalimsky JD, Campisi J and Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. *Nature protocols*. 2009;4:1798-806.

53. Heo J, Raines KW, Mocanu V and Campbell SL. Redox Regulation of RhoA. *Biochemistry*. 2006;45:14481-14489.

54. Xu Q, Huff LP, Fujii M and Griendling KK. Redox regulation of the actin cytoskeleton and its role in the vascular system. *Free Radical Biology and Medicine*.

55. Baldwin RM, Parolin DAE and Lorimer IAJ. Regulation of glioblastoma cell invasion by PKC[iota] and RhoB. *Oncogene*. 2008;27:3587-3595.

56. Tan H, Wang X, Yang X, Li H, Liu B and Pan P. Oncogenic role of epithelial cell transforming sequence 2 in lung adenocarcinoma cells. *Experimental and Therapeutic Medicine*. 2016;12:2088-2094.

FIGURES

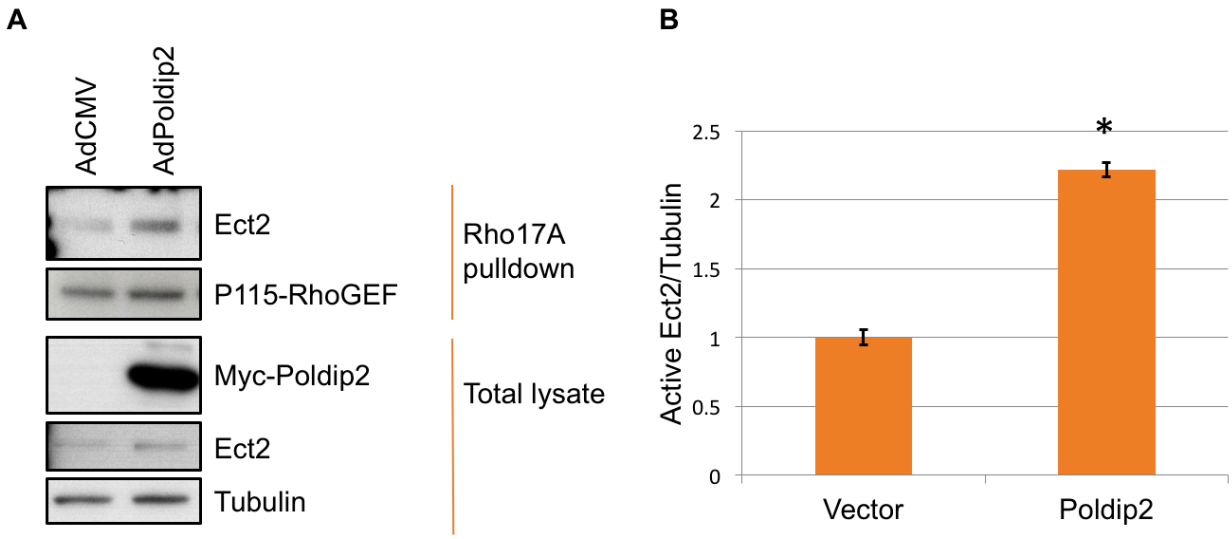

Figure 1. Poldip2 specifically activates the Dbl-family RhoGEF Ect2. a, VSMCs were infected with vector control (AdCMV) or Myc-Poldip2 (AdPoldip2) and used in a GST-RhoA(17A) pull-down assay. GST-RhoA(17A) lysates (top) were immunoblotted for Ect2 and p115 and total cell lysates (bottom) were blotted for myc, Ect2, and tubulin. b, Densitometry analysis was performed and mean levels of active Ect2 in AdCMV and AdPoldip2 groups were graphed. Bars are means ± standard error of the mean (SEM) of five independent experiments. *P<0.0001

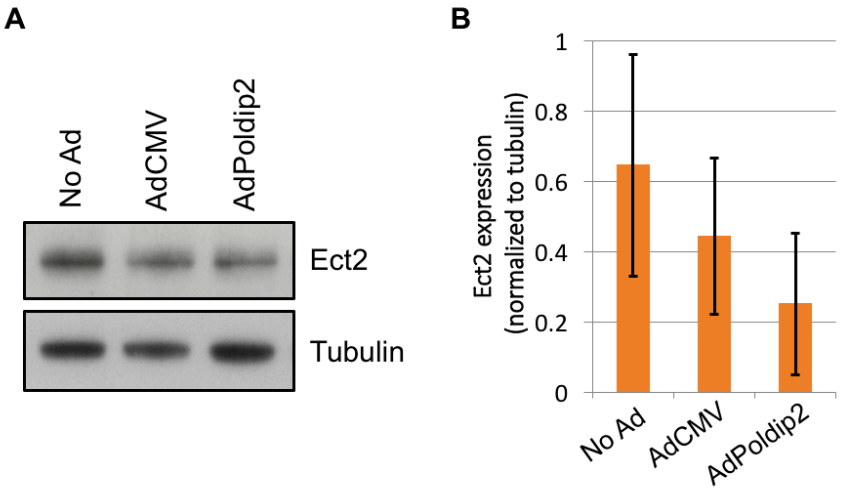

Figure 2. Poldip2 does not increase Ect2 expression. a, VSMCs were infected with vector control (AdCMV) or Myc-Poldip2 (AdPoldip2) in triplicate and immunoblotted for Ect2 and tubulin. b, Densitometry analysis was performed and mean Ect2 expression in VSMC control, AdCMV, and AdPoldip2 groups were graphed. Bars are means ±SEM of one independent experiment done in triplicate.

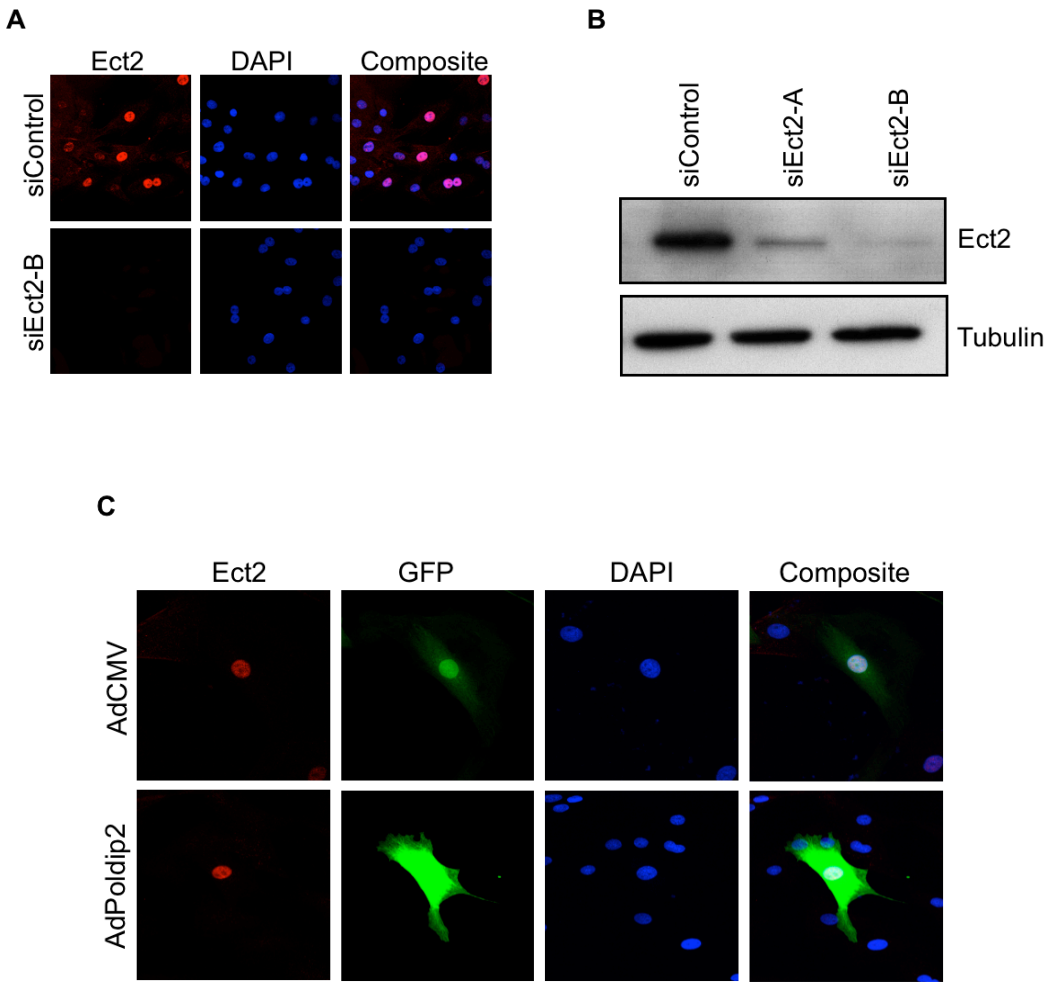

Figure 3. Poldip2 does not regulate Ect2 subcellular localization. a, Confocal images of VSMCs transfected with siControl or siEct2-B. Nuclei were stained with DAPI (blue) and Ect2 was labeled with anti-Ect2 (red) antibody. b, VSMCs were transfected with siControl or two unique sequences of siRNA against Ect2 (Ect2-A and Ect2-B) and immunoblotted for Ect2 and tubulin. c, Confocal images of VSMCs infected with vector control (AdCMV) or GFP-Poldip2 (AdPoldip2). Ect2 was labeled with anti-Ect2 (red) antibody and nuclei were stained with DAPI (blue). GFP (green) indicates infection by AdCMV or AdPoldip2.

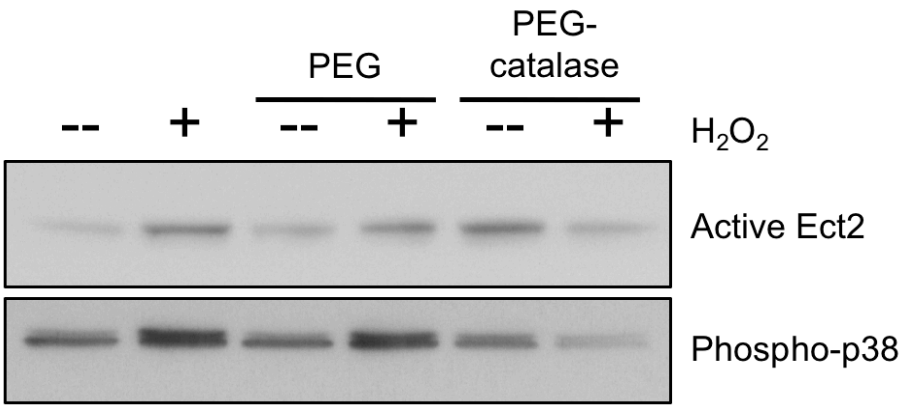

Figure 4. Exogenous H_2O_2 activates Ect2. VSMCs were either left untreated or pretreated with PEG or 200 units/ml PEG-catalase for four hours. Subsequently, all groups were treated with 100 μM H_2O_2 for 10 minutes at 37^{o}C. GST-RhoA(17A) pulldowns were immunoblotted for Ect2 and total lysates were blotted for phospho-p38.

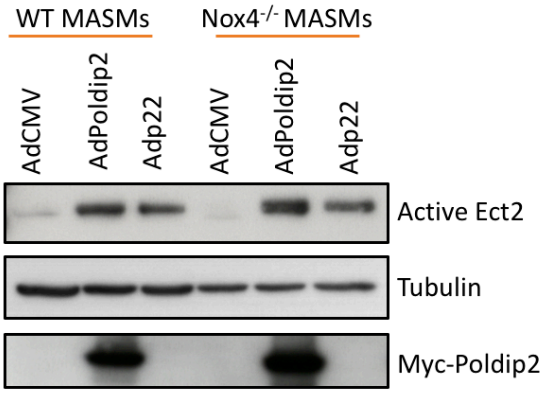

Figure 5. Nox4 is not required for activation of Ect2 by Poldip2. WT MASMs and Nox4$^{-/-}$ MASMs were infected with vector control (AdCMV), Myc-Poldip2 (AdPoldip2), or p22phox (Adp22) and used in a GST-RhoA(17A) pulldown assay. GST-RhoA(17A) pulldowns were immunoblotted for Ect2 and total lysates were blotted for tubulin and myc.

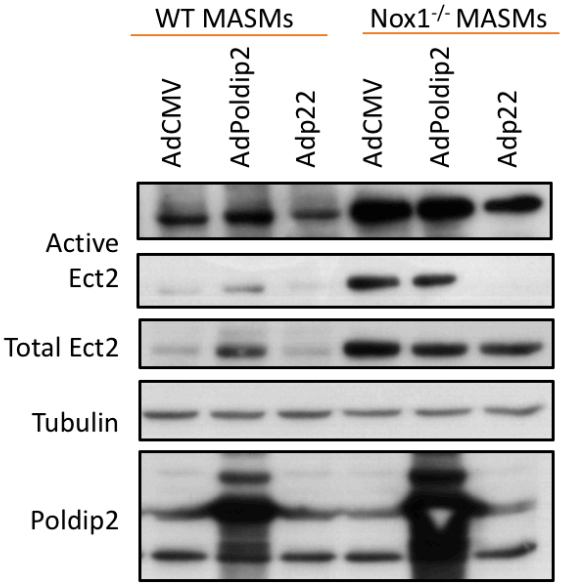

Figure 6. Nox1 may be required for activation of Ect2 by Poldip2. WT MASMs and Nox1$^{-/-}$ MASMs were infected with vector control (AdCMV), Myc-Poldip2 (AdPoldip2), or p22phox (Adp22) and used in a GST-RhoA(17A) pulldown assay. GST-RhoA(17A) pulldowns were immunoblotted for Ect2 and total lysates were blotted for Ect2, tubulin, and Poldip2.

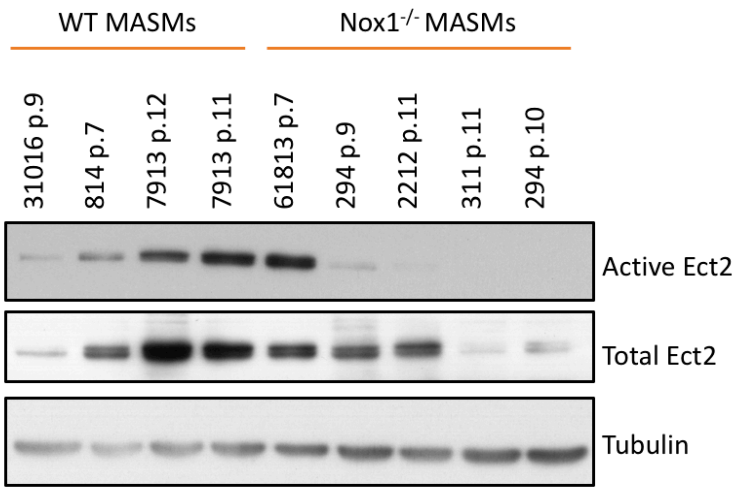

Figure 7. Different lines of Nox1$^{-/-}$ and wildtype MASMs have variable levels of active and total Ect2. Three lines of wildtype MASMs (31016 p.9, 814 p.7, 7913 p.11 and 7913 p.12) and four lines of Nox1$^{-/-}$ MASMs (61813 p.7, 294 p.9, 2212 p.11, 311 p.11, and 294 p.10), where “p” represents the passage number, were used in a GST-RhoA(17A) pulldown assay. GST-RhoA(17A) lysates were immunoblotted for Ect2 and total lysates were blotted for Ect2 and tubulin.

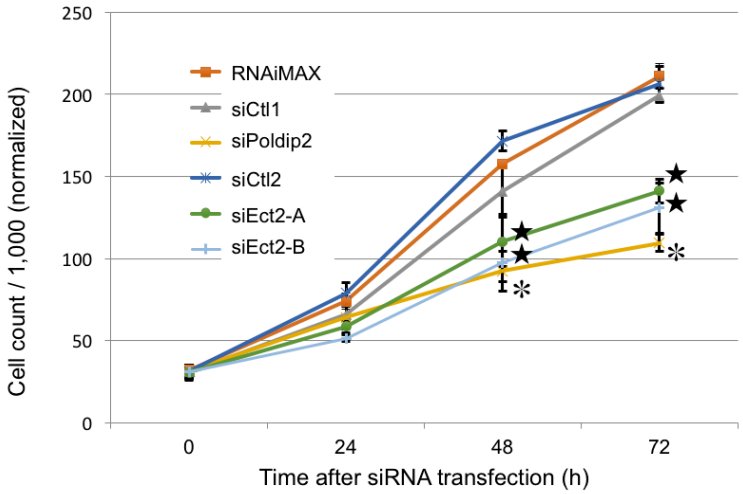

Figure 8. Ect2 and Poldip2 enhance VSMC proliferation. At Time 0, VSMCs were transfected with stealth siControl-1, stealth siPoldip2, two unique sequences of siRNA against Ect2 (siEct2-A and siEct2-B), or siControl-2. Subsequently, cells were counted every 24 hours for a total of three days. Graph depicts the mean number cells for each group. Data points are means ±SEM of four independent experiments. *P<0.001, ★P<0.001.

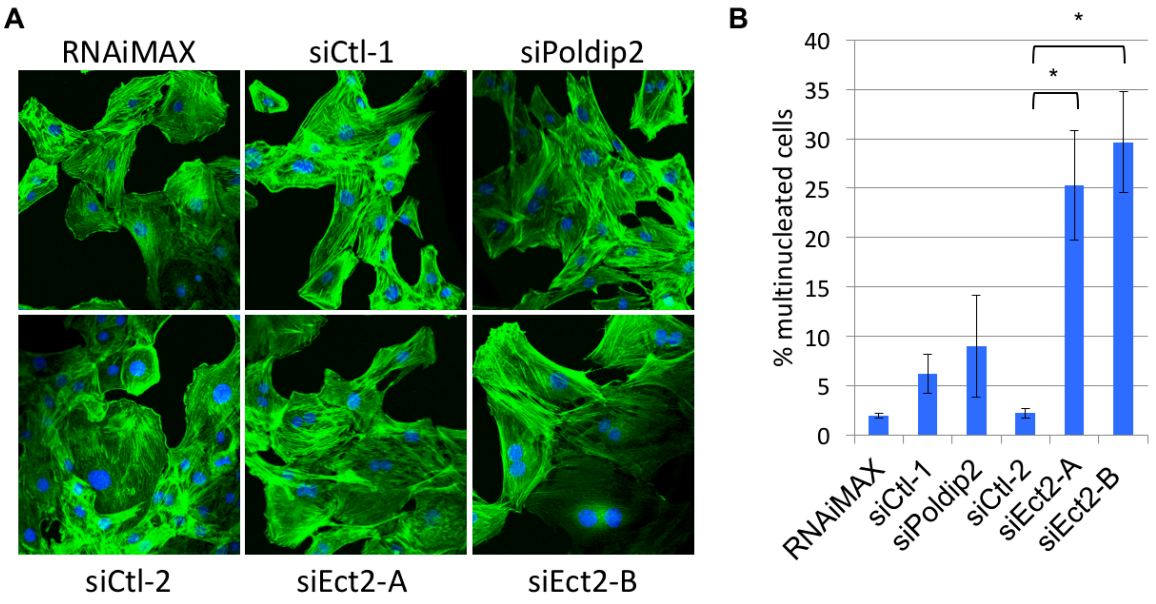

Figure 9. Ect2, but not Poldip2, regulates cytokinesis. a, VSMCs transfected with RNAiMAX, stealth siControl-1, stealth siPoldip2, two unique sequences of siRNA against Ect2 (siEct2-A and siEct2-B), or siControl-2. Nuclei (blue) were stained with DAPI and phalloidin was labeled (green). b, Mean number of multinucleated cells for each treatment was graphed. Bars are means ±SEM of four independent experiments. *P<0.0001.

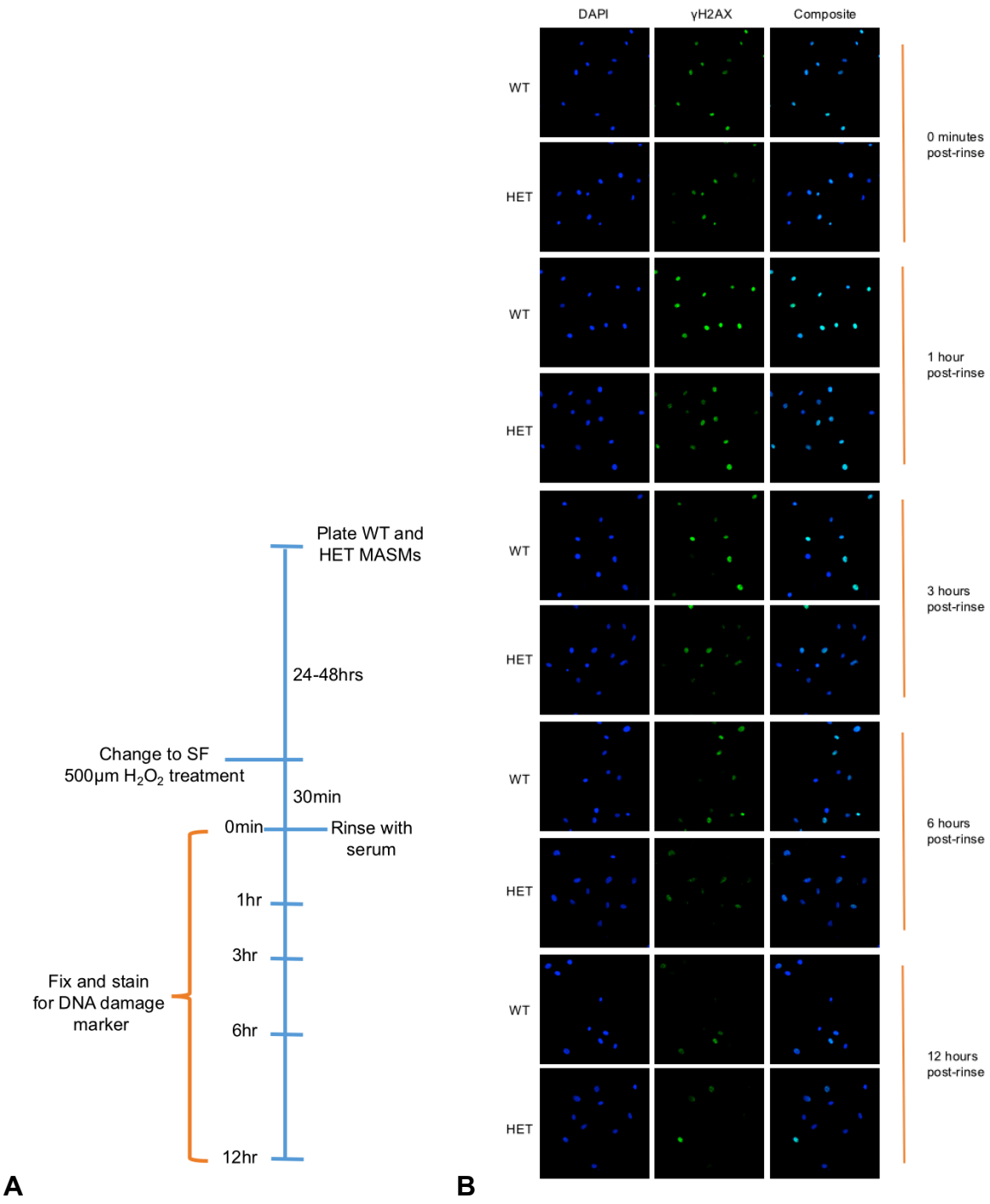

Figure 10. Poldip2 does not enhance DNA damage repair. a, Schematic depicting experimental design. b, Confocal images of WT and Poldip2 HET MASMs treated with 500 µM H_2O_2 for 30 minutes. Nuclei were stained with DAPI (blue) and γH2AX was labeled with anti-γH2AX (green) antibody.

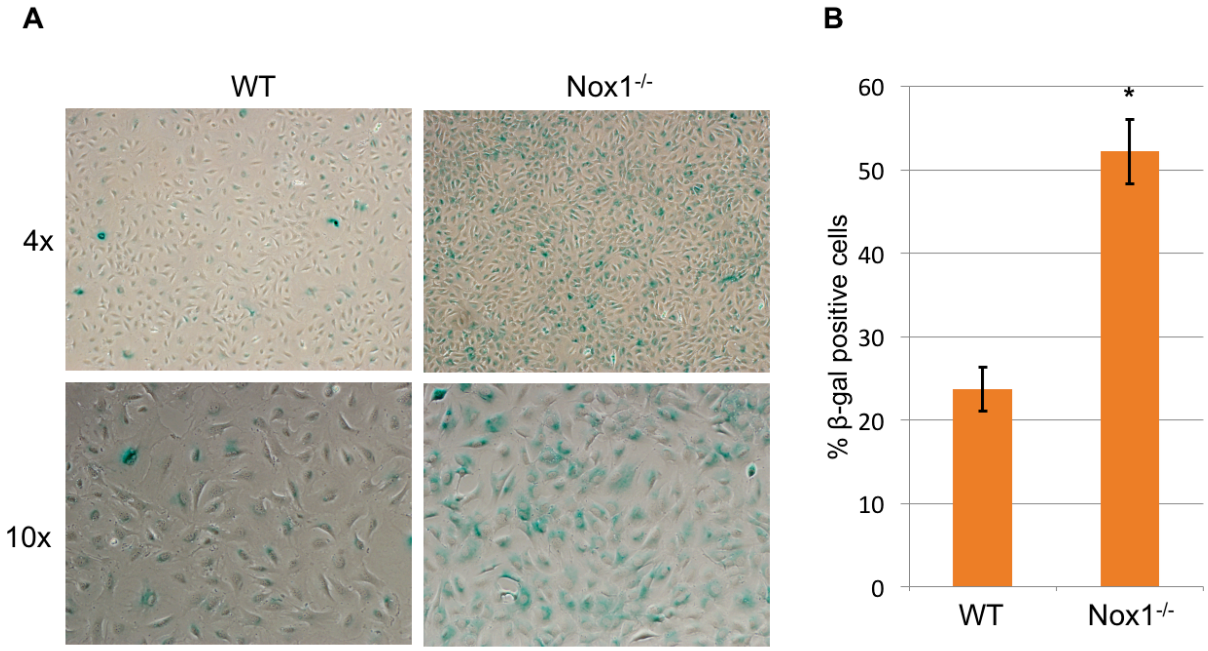

Figure 11. Nox1$^{-/-}$ MASMs are more senescent than WT MASMs. a, WT and Nox1$^{-/-}$ MASMs were treated overnight with β-galactosidase and visualized by bright field at 4x and 10x magnification. b, Mean percentage of β-galactosidase positive cells for WT and Nox1$^{-/-}$ MASMs was graphed. Bars are means ±SEM of four cell batches. *P<0.001.

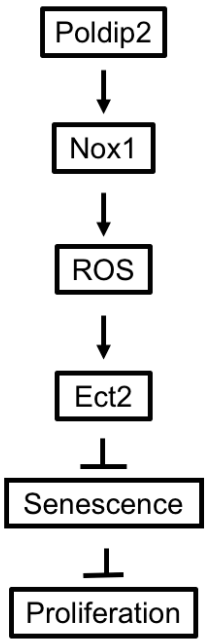

Figure 12. Proposed pathway for the activation of Ect2 by Poldip2 and the downstream consequences of this signaling.